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Aluminium

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Sublethal aluminium phosphide poisoning: using combined vitamins C and D supplementation to modulate pro-inflammatory cytokines release in lungs and kidneys of Wistar rats

Vitamin C and D Mitigate AlP-poisoning

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Abstract
Objective: We investigated the effects of Aluminium phosphide on key intracellular antioxidant enzymes, and the modulatory role of combined vitamins C and D supplementation on interleukin-6 (IL-6) and tumour-necrosis factor-alpha in the lungs and kidneys of Aluminium-phosphide-induced toxicity in Wistar rats.
Methods: Twenty-five male rats (150 -210g) were randomised (n = 5) into five groups (A-E): A. Unexposed control; B. AlP-exposed; C. AlP-exposed+vitamin C (100mg); D. AlP-exposed+vitamin D (10 mg); and E. AlP-exposed+vitamins C & D (100 mg+10 mg) groups. Rats were exposed to AlP (2 mg/kg, w/v) daily using oral gavage; groups C, D and E were treated accordingly, 45 minutes afterwards orally for 28 days. Superoxide dismutase (SOD), glutathione peroxidase, IL-6 and TNF-α levels in kidneys and lungs were assessed using standard methods. Data were analysed using SPSS version 20; $p < 0.05$ was considered statistically significant.
Results: AlP exposure resulted in significant reductions in SOD and GPx activities ($p = 0.000$), increases in IL-6 and TNF-α levels, lymphocytic infiltration and inflammatory lesions in the lungs and kidneys. Vitamin C reduced TNF-α ($p<0.05$) levels in the lungs. In contrast, combined administration of vitamins C and D markedly decreased IL-6 and TNF-α levels, and alleviated lymphocytic infiltration and inflammation in both organs compared with control.
Conclusion: Prolonged exposure to sublethal doses of AlP caused lung and kidney damage. However, continuous and sufficient supplementation with combined vitamins C and D down-regulated pro-inflammatory cytokines and attenuated AlP-induced respiratory and renal damage and could potentially serve as an antidote in occupationally exposed subjects.

Keywords: Aluminium phosphide, Aluminium phosphide poisoning, Cytokine storm, Pro-inflammatory cytokines, Vitamin supplementation

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Plain English summary
Aluminium phosphide (AlP) is a substance used by farmers to preserve grains. However, it is a very deadly substance when it is exposed to air or swallowed. AlP poisoning has no antidote or effective treatment, a major reason for high mortality. Nowadays, reported cases of AlP poisoning are increasing. Therefore, there is a need to continue the search for an antidote and/or improved supportive treatment aiming at reducing the mortality rate due to AlP poisoning. This present study used experimental Wistar rats exposed to a low dose of AlP to investigate the role of combined vitamins C and D supplementation on the lungs and kidneys of the AlP-exposed animals. Results showed that a small amount (2 mg/kg body weight) of AlP did not cause death to the experimental animals within 28 days; however, they showed signs of injury in their lungs and kidneys. Combined vitamins C and D supplementation for 28 days significantly reduced signs of injury in the lungs and kidneys. Research findings are relevant since they can be translated to human beings who are frequently exposed to this deadly substance. Results suggest that, combined vitamins C and D, if taken in adequate amounts, especially by people whose occupations frequently expose them to AlP, may prevent lung and kidney diseases, and reduce death rates.

Background
Aluminium phosphide (AlP) is a potent solid pesticide (56% AlP and 44% carbamate); mainly formulated as dark grey or dark yellow pellets, tablets, or granules (1). Its wide use by farmers has brought tremendous improvements in grain preservation, food security and financial gain (1). When AlP is exposed to moisture in the air or in contact with hydrochloric acid in the stomach following oral ingestion, phosphorous trihydride (PH_3), is liberated, which noncompetitively inhibits mitochondrial glutathione peroxidase and cytochrome C oxidase (2), resulting in disruption of cellular redox status, pro-inflammatory cytokine levels, damage to multiple organs and death (3). AlP poisoning is highly fatal and increasing in many countries where there is no strict control of the pesticide. Nowadays, reported cases have shown that about 300, 000 people die yearly due to pesticide poisonings globally (4), of which AlP is the leading culprit.
Studies have shown that vitamins C and D (5, 6) have antioxidant and anti-inflammatory activities. Vitamin C is the common hydro-soluble ketolactone vitamin with strong antioxidant properties (7). It forms a synergy with tocopherol, a lipophilic vitamin for stronger and more efficient antioxidant functions (8, 9). Ascorbate is effective in scavenging and preventing initiation and propagation of free radicals by excellently donating one- or two-electrons to oxidants (10), thus preventing oxidative damage. Moreover, vitamin D is a lipid-soluble vitamin having two different forms: 1, 25$(OH)_2$D3 and 25(OH)D3 circulating in the plasma. The enzyme, 25-hydroxyvitamin D3-1-Alpha-hydroxylase (CYP27b1), converts 25(OH) D3 into 1, 25$(OH)_2$D3, the hormonally active metabolite of vitamin D. Increased levels of vitamin D have been reported to markedly increase glutathione and total antioxidant capacity, and lower lipid peroxidation and oxidative stress (11, 12). Consequently, we hypothesised that combined vitamins C and D supplementation may synergistically or complementarily protect against AlP-induced oxidative damage of organs, and reduce fatality.
Evidence from previous experimental animals and clinical studies using different substances, including vitamin C, as a component of a supportive regimen for AlP toxicity, shows unsatisfactory outcomes. The fact that an antidote for AlP is yet to be discovered, and there is no effective supportive treatment. This present study sought to address the knowledge gap. This study, therefore, investigated the effects of AlP on key intracellular antioxidant enzymes, and the modulatory–role of combined vitamins C and D supplementation on interleukin-6 (IL-6) and tumour-necrosis factor-alpha (TNF-α) in lungs and kidneys of AlP-induced toxicity in Wistar rats.

Materials and Methods
Kits for GPx and SOD activities were purchased from R&D Systems, Inc., Minneapolis, MN 55413, USA. Enzyme-linked immunosorbent assay (ELISA) kits for IL-6 and TNF-α were supplied by Elabscience Biotechnology, B18, Phase 11, Biomedical Park, Wuhan, Hubei, China. Ascorbic acid (SPARTAN-C) tablets manufactured by Kunimed Pharmachem Limited, Ikeja, Lagos, Nigeria, and Cholecalciferol (Health Aid vitamin D, 1000 IU) produced by Health Aid plus Limited, Nigeria, were purchased from Mediplus Pharmacy, Victoria Island, Lagos, Nigeria. Aluminium phosphide (*Quickphos* tablets) was procured from a licensed Agrochemical distributor in Owo, Ondo State, Nigeria. 10% neutral buffer formalin, phosphate-buffered saline (PBS) solution, and Haematoxylin and Eosin (H&E) were prepared from stock reagents of analytical grade.

Experimental animals and design
Twenty-five adult male *Rattus norvogicus* (Wistar) rats weighing between 150 and 210g were used in this study. The rats were obtained from the Animal Holding of the Department of Anatomical Sciences, Achievers University, Owo, Ondo State, Nigeria. Using the Resource Equation Approach (13), five rats were randomised into five groups (A-E):
A = Unexposed control
B = AlP-exposed not treated (AlP)
C = AlP-exposed and treated with 100 mg vitamin C (AlP+Vit.C)
D = AlP-exposed and treated with 10 mg vitamin D (AlP+Vit.D)
E = AlP-exposed and treated with combined vitamins (C 100 mg) and D (10 mg) (AlP+Vit.C+D).
Rats were kept in a 12-hour light/dark cycle and at 23 ± 2 °C, allowed to acclimatise for a week before the commencement of the study. They were fed with normal rat pellets and free access to clean water throughout the experimental period.

AlP-exposure and treatment
AlP-exposed rats received 2 mg/kg (w/v) body weight of AlP orally daily. Groups C, D and E were treated 45 min afterwards with vitamin C (100 mg), Vitamin D (10 mg), and combined vitamins (C 100 mg and D 10 mg) per kilogram body weight, respectively, using oral gavage for 28 days.
AlP was dissolved with distilled water in a plastic bottle well capped, in a safety fume cabinet and used immediately after preparation. Preparation was precautionary and done on a daily basis.
The handling of animals and the experimental protocols of the study strictly adhered to the Experimental Animal Care and Use Regulation provided by the National Institutes of Health (14) and other approved protocols of the research ethics committee, Achievers University, Owo, Ondo State, Nigeria.

Tissue samples collection and preparation
At the end of the experimentation, all animals were humanely euthanised using ketamine (75mg/kg/bw) and xylazine (10mg/kg/bw) intraperitoneally. They were laid down on the dissecting board in a supine position, and their anterior thoracoabdominal and pelvic wall with peritoneum was carefully dissected in the midline to expose the organs of interest. They were dissected using student dissecting sets, and the lungs and kidneys were excised and immediately transferred into 10% neutral buffered formalin for histopathological analysis. For the biochemical analysis, the organs (lung and kidneys) in each group were rinsed twice in freshly prepared 0.1 mol L^{-1} phosphate-buffered saline (PBS, pH 8.0), and an equal portion was then homogenised and centrifuged at 3000 revolutions per minute for 20 min at room temperature. The obtained supernatant was kept at -80°C till the assay of pro-inflammatory cytokines and enzyme antioxidants.

Histopathological analysis
Portions of the lungs and kidneys transferred into 10% neutral buffered formalin were allowed to fix for at least 72 hours before further histological protocols and analysis. Tissues were processed for light microscopic study using an automatic tissue processor machine (Thermo Scientific Spin Tissue Processor, STP120, Frankfurt, Germany). Tissues were dehydrated in various grades of alcohol and cleared in two changes of xylene, infiltrated in two changes of wax bath, and finally embedded in paraffin wax. Thin sections of 4 microns were stained with Haematoxylin and Eosin (H&E) and were studied histologically using an Olympus binocular light research microscope (XSZ-107BN, No. 071771). Micrographs of the sections were taken with a Kodak Digital Camera (Kodak Easyshare C183).

Biochemical assays
Activities of GPx and SOD were determined using the methods described by (15) and (16), respectively. Levels of IL-6 and TNF-α were evaluated by Sandwich enzyme-immunosorbent assay (ELISA) kits following the protocols described by the manufacturer.

Statistical analysis
Data were analysed using the PASW Statistics version 18, a statistical package from SPSS Inc (Chicago, IL, United States of America) and results were presented as mean ± standard deviation. Student t-test was used to compare means of two groups, while multiple comparisons of group means were done using analysis of variance (ANOVA) and Post hoc analysis by least significant difference (LSD). The level of significance was set at 95% confidence interval, and a p-value less than 0.05 ($p < 0.05$) was considered statistically significant. Charts were drawn using the Microsoft Excel 2010 software application.

Results
Biochemical findings
Tables 1 and 2 present levels of SOD, GPx, IL-6 and TNF-α in the kidneys and lungs homogenates of rats in groups A and B. There were significant variations in the activities of SOD and GPx, and levels of IL-6 and TNF-α in the kidneys (Table 1)

and lungs (Table 2) in group B compared to group A. Group B had significantly low SOD (p = 0.000) and GPx (p = 0.000) activities and elevated IL-6 (p = 0.000) and TNF-α (p = 0.000) levels compared to group A.

Table 1: SOD, GPx, IL-6 and TNF-α in kidney homogenate of AIP-exposed and unexposed rats

Parameter	Group		p-value
	Unexposed rats	AIP-exposed rats	
SOD (U/mg protein)	9.67 ± 1.55	5.28 ± 1.18*	0.000
GPx (U/mg protein)	44.12 ± 2.27	22.27 ± 1.21*	0.000
IL-6 (Pg/mg protein)	25.67 ± 0.69	211.56 ± 5.12**	0.000
TNF-α (Pg/mg protein)	179.66 ± 3.49	382.06 ± 10.28**	0.000

Key: SOD = Superoxide dismutase; GPx = Glutathione Peroxidase; IL-6 = Interleukin 6; TNF-α = Tumour necrosis factor alpha; * = Significantly decreased compared to control; ** = Significantly increased compared to control

Table 2: SOD, GPx, IL-6 and TNF-α in lung homogenate of AIP-exposed and unexposed rats

Parameter	Group		p-value
	Unexposed rats	AIP-exposed rats	
SOD (U/mg protein)	7.05±1.43	3.58±0.35*	0.000
GPx (U/mg protein)	68.60±1.72	25.42±1.52*	0.000
IL-6 (Pg/mg protein)	13.27±2.32	88.78±6.20**	0.000
TNF-α (Pg/mg protein)	140.16±13.23	434.87±68.80**	0.000

Key: SOD = Superoxide dismutase; GPx = Glutathione Peroxidase; IL-6 = Interleukin 6; TNF-α = Tumour necrosis factor alpha; * = Significantly decreased compared to control; ** = Significantly increased compared to control

Figures 1 and 2 show the effects of supplementation of sole vitamins C, D and in combination on IL-6 and TNF-α in kidneys and lung homogenate of AIP-exposed rats.

Kidney of rats in group E had significant reductions in IL-6 (p = 0.006) and TNF-α (p = 0.000) compared to group B (Figure 1).

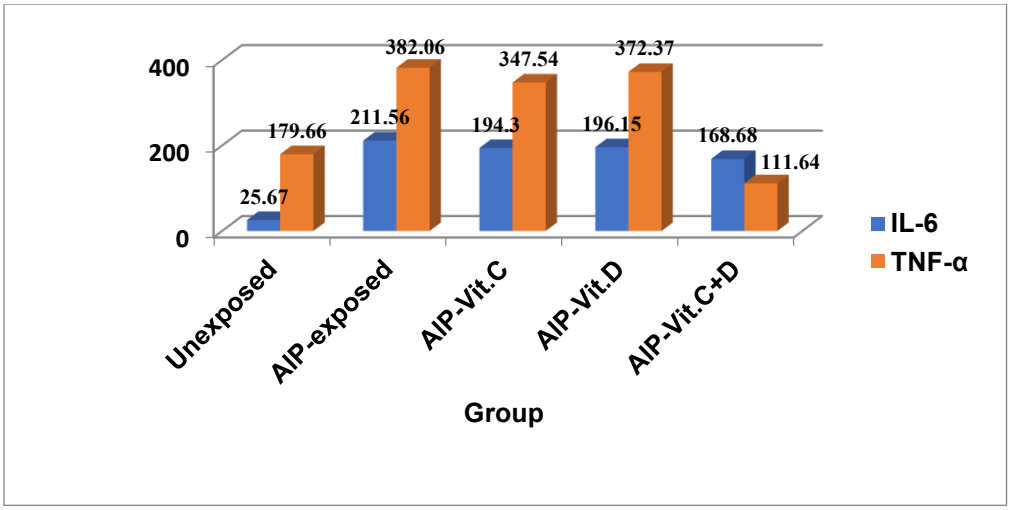


Figure 1: Kidneys of AIP-exposed rats treated with combined vitamin C and D (group E) had significantly low IL-6 (p = 0.006) and TNF-α (p = 0.000) levels compared to AIP-exposed rats in group B

In group C, lungs of AIP-exposed rats treated with vitamin C had reduced TNF-α (p = 0.046), while in group E, AIP-exposed rats treated with combined vitamin C and D had reduced IL-6 and TNF-α (p = 0.005, p = 0.022) compared to group B (Figure 2).

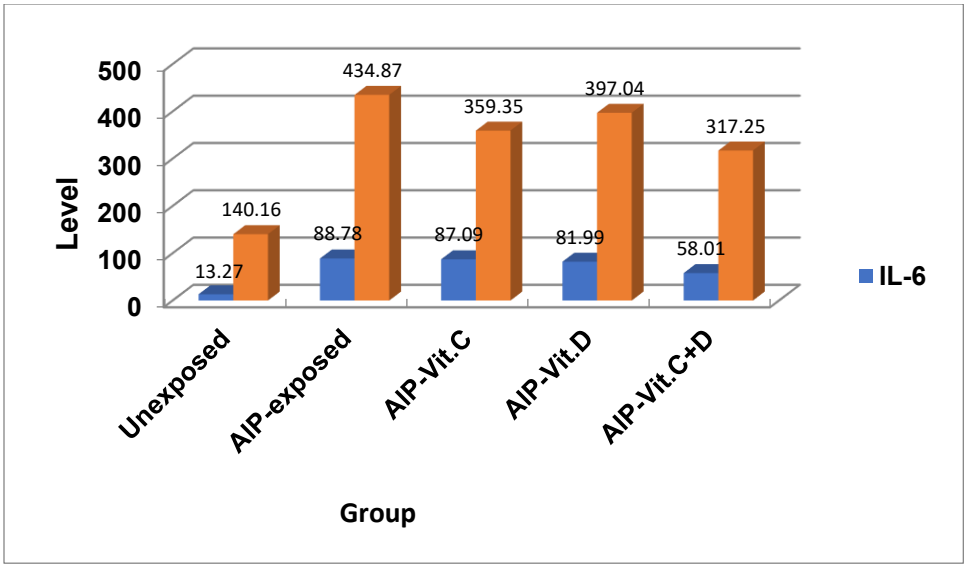


Figure 2: Treatment with vitamin C (group C) caused low TNF-α (p = 0.046) level, while combined vitamins C and D (group E) caused low IL-6 (p = 0.005) and TNF-α (p = 0.022) levels in the lung homogenate compared to AIP-exposed rats (group B)

Histopathological Findings

1 A &B (Group A) reveal renal cortex consisting of normal glomeruli (black arrow), renal tubules (yellow arrow), and the interstitial spaces (slender arrow). 1C&D (Group B) show glomeruli (black arrow), renal tubules (yellow arrow, blue) and interstitial spaces mildly infiltrated by inflammatory cells (red arrow, slender arrow) 1E&F (Group C) show a renal cortex consists of normal glomeruli with capsular spaces (black arrow), renal tubules (yellow arrow) and interstitial spaces mildly infiltrated by inflammatory cells (red arrow). 1G&H (Group D) show a moderate architecture with the renal cortex revealing normal glomeruli with mesangial cells and capsular spaces (black arrow), renal tubules (yellow arrow) and interstitial spaces with mild vascular congestion (green arrow). 1I&J (Group E) show normal tissue architecture, but interstitial spaces are mildly congested.

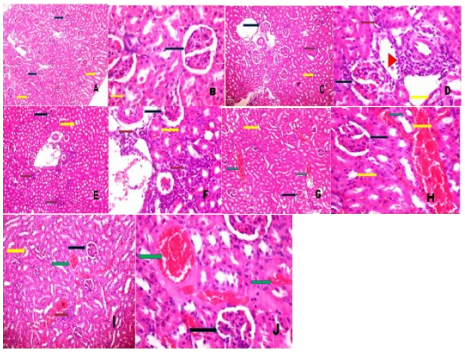


Plate 1(A-J) shows the kidneys of rats stained with Haematoxylin and Eosin. Each pair of letters represents histoarchitecture viewed under x100 and x400

2 A&B (Group A) show normal bronchiole (black arrow), alveoli sacs (yellow arrow), intra-alveolar spaces (slender arrow) and alveolar epithelium (blue arrow). 2C&D (Group B) show the bronchiole with perilymphocytic inflammation (black arrow), the intra-alveolar spaces mildly infiltrated by inflammatory cells (slender and red arrows). 2E&F (Group C) reveal a peri-bronchiole inflammation (black arrow) with moderate vascular congestion (green arrow) and alveolar spaces mildly inflamed (slender arrow). 2G&H (Group D) show peri bronchiolar lymphocytic follicle (black arrow), intra-alveolar spaces mildly infiltrated by inflammatory cells (slender arrow) and normal alveolar epithelium (red arrow and blue arrow). 2I&J (Group E) show the bronchiole (black arrow) and the

presence of a lymphocytic aggregate (black arrow head) with the intra-alveolar spaces mildly inflamed (slender arrow) while the alveolar epithelium appears normal (blue arrow).

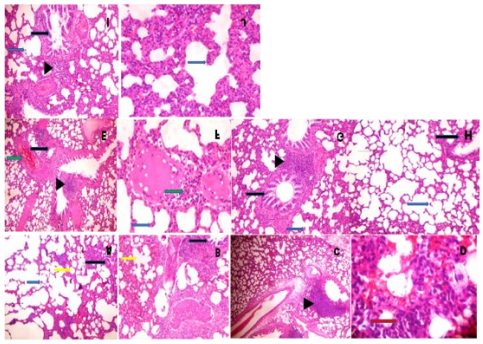


Plate 2 (A-J) shows lungs of rats stained with haematoxylin and eosin. Each pair of letters represents histoarchitecture viewed under x100 and x400

Discussion

The active form of aluminium phosphide, PH_3, is a potential stressor and mediator of excessive mitochondrial ROS through enzyme inhibition and disruption of oxidative phosphorylation. PH_3 perfuses both cellular and subcellular levels, rapidly impairs endogenous antioxidant defence systems by inhibiting key inner mitochondrial antioxidant enzymes, including SOD and GPx (17). In this present study, the significantly low SOD and GPx activities in the lungs and kidneys of AlP-exposed rats agree with the reports of Mehrpour et al (18) and Afolabi et al. (19). The observed low SOD and GPx activities may be due to noncompetitive inhibition of GPx by PH_3, resulting in the accumulation of hydroxyl and peroxynitrite radicals and suppressed induction of SOD.

SOD and GPx are key antioxidant enzymes actively operating at the forefront of defence against reactive species. Superoxide dismutases (SODs) are expressed in both prokaryotes and eukaryotes, and they catalyse two-electron reduction of superoxide radicals, the mutated products of oxygen metabolism. SODs exist in different types as defined by a specific metal cofactor (copper, iron, manganese, or nickel) present in the active sites of the enzymatic antioxidants (20). While Cu/Zn-SOD is present in extracellular fluids (ECF) and cytosol of eukaryotic cells, Mn-SOD is predominantly localised in the mitochondrial inner membrane. Mn-SOD is the major ROS scavenger not affected by Fenton reactions. However, the activity of Mn-SOD can be influenced by oxidative stress, resulting in a variety of diseases (20). Moreover, the buildup of superoxide ions (O_2-) in the lung can speedily react with nitric oxide (NO), resulting in peroxynitrite (ONOO-), another very reactive radical (21). Glutathione peroxidase (GPx) is a selenoprotein and an important enzymatic antioxidant. It primarily scavenges and catalyses the reduction of hydrogen peroxide and organic hydroperoxides (ROOH) to water and molecular oxygen using glutathione as a reducing substrate (22). Expression of GPx varies in different cells, tissues, or organs, but predominantly presents in the mitochondria (23). Activity of GPx has been reportedly inhibited by PH_3 (24). Excess H_2O_2 following GPx inhibition can interact with iron to form hydroxyl radical, a highly damaging ROS. Altogether, non-competitive binding of PH_3 to SOD and GPx might have created intra- and extracellular oxidising environments with increased reactive oxygen and nitrogen species (ROS/NOS) and increased release of pro-inflammatory cytokines, including TNF-α and IL-6.

In the present study, significantly elevated TNF-α and IL-6 levels in the lung and kidneys of rats exposed to a sublethal dose of AlP suggest an immunologic response to PH_3-induced oxidative damage to the organs and mitochondrial DNA (mtDNA). Oxidative stress occurs when antioxidant defence mechanisms cannot offset excessive ROS/NOS production. This damaging event is usually followed by an inflammatory response. Impaired Mn-SOD activity in the mitochondria can result in increased hydroxyl radical through the reaction between iron and hydrogen peroxide (11). Hydroxyl and peroxynitrite radicals are more injurious than the parent radicals and can oxidise mitochondrial DNA (mtDNA), causing damaged mtDNA and mitochondrial dysfunctions (25). Release of damaged mtDNA fragments into the cytosol can escape autophagy, accumulate and circulate as a damage-associated molecular pattern (DAMP) (25). DAMP signals recruitment of neutrophils, toll-like receptor 9 (TLR9-T) cells and macrophages to the localised injured sites, which

initiate the release of pro-inflammatory cytokines, including TNF-α and IL-6 (25). IL-6 and TNF-α are small signalling molecules released by immune or endothelial cells to initiate and regulate immunologic responses. Unregulated cytokine release in a microenvironment due to an imbalance between oxidising and reducing agents can lead to cytokine storm (26).

However, treatment with vitamin C supplementation significantly lowered TNF-α levels in the lung compared to the kidney of AlP-exposed rats. This may be due to the much higher ascorbic acid level or stronger antioxidant defences in the lung compared to the kidney (27, 28). Ascorbic acid and vitamin D are important anti-inflammatory compounds (29, 30). Zhu et al. (31) show that vitamin D supplementation promoted anti-inflammatory cytokines by markedly lowering TNF-α and IL-6 levels. High plasma circulating 1, 25$(OH)_2$D3 was also found to down-modulate IL-6 and TNF-α production (32, 33). Significant negative associations between 25 $(OH)_2$D3, TNF-α, and IL-6 levels were equally reported (34). In agreement with our present findings, significantly low IL-6 and TNF-α levels in both the kidneys and lungs of rats in the AlP-exposed group treated with combined vitamins C and D, compared to sole vitamin C or D, could be attributable to a therapeutic synergy between the vitamins. Both vitamins might have jointly deactivated the nuclear factor activated kappa B-cell (NFkB)/TNF-α common pathways. Deactivation of the NFkB/TNF-α pathways has been observed to down-regulate TNF-α and IL-6 release (35).

In addition, histopathological analysis of the kidneys of rats exposed to AlP revealed inflammation within the interstitial space, perivascular infiltration by inflammatory cells and alteration in the histoarchitecture due to PH3-mediated injury (Plates 1C&D). These findings are in agreement with Anand et al. (36). Similarly, the bronchial congestion, peribronchiolar lymphocytic infiltration and aggregation, and inflammation of the alveolar spaces in the lungs of rats exposed to AlP (Plates 2C&D) are consistent with previous findings (37, 38). The reduction of lymphocytic infiltration and moderate histoarchitecture observed in group E (Plates 1 I&J and 2 I&J) affirmed the biological synergy between the two vitamins in mitigating oxidative damage and down-regulating pro-inflammatory signalling in the lung and kidney of rats exposed to AlP. Histopathological analysis buttresses the chemical findings.

Conclusion

Prolonged exposure to sublethal doses of AlP caused lung and kidney damage. However, continuous and sufficient supplementation with combined vitamins C and D down-regulated pro-inflammatory cytokines and attenuated AlP-induced respiratory and renal damages. Clinically, vitamins C and D could potentially serve as an antidote in occupationally exposed subjects.

List of Abbreviations

AlP: Aluminum phosphide
DAMP: damage-associated molecular pattern
ELISA: Enzyme-linked immunosorbent assay
GPx: Glutathione peroxidase
H_2O_2: Hydrogen peroxide
H&E: Haematoxylin and Eosin stains
IL-6: Interleukin 6
PH_3: Phosphine gas
MtDNA: Mitochondrial deoxyribonucleic acid
NFkB: Nuclear factor activated kapper B-cell
NIH: National institute of health
O_2-: Superoxide ions
ONOO-: Peroxynitrite superoxide ions
ROS: Reactive oxygen species
ROOH: hydroperoxides
SOD: Superoxide dismutase
TLR9-T: toll-like receptor 9
TNF-α: Tumour necrosis factor alpha

Declarations

Ethical approval and consent to participate

This study was approved by the Research and Ethics Committee of the Achievers University, Owo, Ondo State (AUREC/07/2023).

Consent for publication

Not applicable

Data availability

All data generated or analysed in this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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The authors received no external funding for the study

Authors' contributions

MA conceived and designed the study, collected kidneys and lungs for histological examination and biochemical assays, and contributed to writing the manuscript.

UEF analysed and interpreted biochemical data and was the major contributor in writing the manuscript.
IMC and ATD performed histological examination of the studied organs with tissue micrographs.
AEI was involved in the experiment and tissue processing.
OAA interpreted the histological changes and proofread the manuscript.
All authors read the final manuscript and approved it for submission.

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