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ORIGINAL PAPER

Zn and Se supplementation abrogated metals-(metaloids) mixture-mediated ocular-thymus toxicity via modulation of oxido-inflammatory and antiapoptotic mechanisms in female Sprague Dawley rats

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ABSTRACT

Introduction and aim. This is an evaluation of the protective effects of Zn and Se in the eye and thymus of rats exposed to cocktail noxious metal mixtures (CNMM) (Al, Pb, Hg and Mn) in ameliorating ocular pathologies due to autoimmunity.

Material and methods. Female Sprague rats were grouped into eight (n=5) and orally exposed to various treatments for a period of 60 days: (1): the control group receive deionized water only; (2): the CNMM only group received lead acetate $Pb(C_2H_3O_2)_2$ (20 mg/kg), $AlCl_3$ (35 mg/kg), $HgCl_2$ (0.40 mg/kg) and $MnCl_2$ (0.56 mg/kg); (3) received CNMM+$ZnCl_2$, 0.80 mg/kg; (4) received CNMM+Na_2SeO_3, 1.50 mg/kg; (5) received CNMM+$ZnCl_2$, 0.80 mg/kg and Na_2SeO_3, 1.50 mg/kg combination. Oxidative stress markers, nuclear factor erythroid 2- related factor 2, nuclear factor kappa B, interleukin 6, tumor necrosis factor alpha and caspase-3 and histopathological changes were determined.

Results. CNMM decreased antioxidants levels but increased malondialdehyde and nitric oxide concentrations. CNMM increased levels of nuclear factor erythroid 2- related factor 2, and nuclear factor kappa B, interleukin 6 and tumor necrosis factor alpha and caspase-3. There was moderate retinal degeneration and total cell loss at the ganglionic cell layer in the eye; severe degenerative thymus, lymphocyte depletion and multifocal necrosis in CNMM only.

Conclusion. Supplementation with Zn and Se reduced the biochemical and histopathological changes in the eye and thymus in response to CNMM exposure.

Keywords. ameliorative effects of zinc and selenium, cocktail noxious metal mixture, eye, thymus

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Introduction

The eye is a sensory organ of vision located in the orbit and consists of three coats, three compartments and three fluids. The coat comprises the outer fibrous layer: (cornea, sclera, lamina cribrosa); the middle vascular layer or uveal tract (iris, ciliary body, choroids) and, the inner neuronal layer [pigment epithelium of the retina, retinal photoreceptors, and retinal neurons].[1] The eye transmits sensory impulses (clear image of objects) in the environment to the brain through the optic nerve and the posterior visual pathways to form visions.[2] The functions of all regions of the ocular surface system are closely integrated therefore, dysfunctions of or injury to one or more components of this system can lead to system-wide sequelae such as cicatrizing diseases and dry eye.[3]

Preclinical and clinical studies show the importance of heavy metals like cadmium and lead in the pathogenesis of eye diseases as their excessive exposure can lead to the development of age-related macular degeneration, cataracts, and glaucoma.[4] Cadmium, lead and mercury cause oxidative stress, particularly in the cells of the retina and neurons possibly through reduced glutathione levels, which damage lipid membranes and DNA, thereby suggesting a possible route to increased risk of glaucoma.[5] However, various trace elements such as iron, copper, zinc, selenium, calcium, magnesium, molybdenum, sodium, potassium and manganese help in maintaining the balance of prooxidative and antioxidative processes, regulation of fluid and ion flow through cell membranes of the ocular tissues.[6]

The immune system is one of the complex networks of mechanisms the eye has employed to maintain homeostasis and healthy ocular surface environment necessary to preserve visual function.[7] Environmental toxins trigger the immunological events that shape the outcome of the diverse spectrum of autoimmune-based ocular surface disorders.[7] The ocular surface contains its own local lymphoid tissues: conjunctiva-associated lymphoid tissue situated to sample antigens and maintain tolerance to commensal flora, with evolution of several mechanisms by the eye to modulate inflammation after environmental or microbial stress on the ocular surface.[8,9] Therefore, abnormal activation of the immune system may result in autoimmunity to self-antigens localized to the ocular surface and associated tissues. Most challenges, physical and immunological, to the homeostasis of the eyeball usually arise due to defect in lubrication and/or host defense of the ocular surface.[2]

Ocular surface autoimmune diseases comprise various range of pathologies and manifest as ocular specific (dry eye, Mooren's ulcerative keratitis), systemic (Sjögren's syndrome, ocular cicatricial pemphigoid), or occur secondary to other common autoimmune diseases (rheumatoid arthritis, systemic lupus erythematosus).[7] It is predicted that a combination of excessive or atypical stimuli and/or immunoregulatory dysfunction, together with genetically predisposed factors and/or hormone imbalance provides an environment conducive to activation of autoreactive lymphocytes. The autoimmune response can be perpetuated by both T-cell-dependent and independent mechanisms.

Various subsets of regulatory T cells (Tregs) such as unconventional (CD8+, γδ and NKT cells) and conventional Tregs (CD4+) which can be thymus-derived, naturally (nTreg) induced in response to specific antigens (iTreg) have been suggested to modulate the immune response within the ocular surface tissues and regional lymphoid organs. CD8+ T cells, γδ T cells, and NKT cells present within the conjunctiva-associated lymphoid tissue of healthy subjects may provide protection against autoimmunity and likely contribute to both anti-microbial defense and suppression of autoreactive lymphocyte differentiation and/or function, the latter, by secreting TGF-β and/or IL-10.[7,10] Tregs have been suggested to mediate autoimmune suppression by releasing soluble factors (TGF-β, IL-10), cell–cell contact that disables effector T cells and/or antigen presenting cells (APCs), and/or competing for soluble factors (IL-2 sequestration through high-level expression of the IL-2 receptor, CD25).

Environmental pollution with heavy metals (cadmium, lead, mercury) is of significance in the etiology of many eye diseases such as cataract, intraocular eye pressure and glaucoma.[6,11] Reactive oxygen species (ROS)-NLRP3-IL-1β signaling pathway axis was upregulated in environment-induced ocular surface diseases such as dry eye and corneal toxicity.[12,13]

Certain metals like copper, zinc, and iron are utilized by the human cells to control significant metabolic and signaling functions making them essential for life while others such as the heavy metals: lead, cadmium, mercury, chromium, thallium, nickel, copper, zinc and bismuth; semi-metals like arsenic, tellurium and even non-metals-selenium are characterized by toxicity to humans or the environment.[6,14] Zinc and iron respectively reduced the development of advanced age-related Macular degeneration (AMD) and oxidative stress implicated in the development of AMD.[15]

High concentration of mercury has been reported in cornea, iris, retina and lens, aqueous humor of humans with organomercury poisoning.[16] Their toxic action is associated with their ability to accumulate in the body tissues and organs.

Oxidative stress and mitochondrial dysfunction are implicated in conditions affecting both the anterior segment (dry eye disease, keratoconus, cataract) and posterior segment (age-related macular degeneration, proliferative vitreoretinopathy, diabetic retinopathy, glaucoma) of the human eye, autoimmune uveitis.[11,17-20] Increased susceptibility to disease may partially be at-

tributed to genetic and epigenetic changes that develop over time with continual environmental exposure and damage from endogenous and exogenous reactive oxygen species (ROS).[17]

The major chemical species associated with oxidative stress in the eye include Superoxide (O_2^-), Hydroxyl radical (-OH), Hydrogen peroxide (H_2O_2), malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE).[17] Thymus gland is a primary lymphoid organ of the adaptive immune system located in the upper anterior portion of the chest cavity, above the heart and behind the sternum.[21,22] It is bi-lobed with two subcomponents: the cortex and the medulla made up of epithelial, dendritic, mesenchymal, and endothelial cells.[22] The thymus gland is important in lymphoid cell homing and development, secretion of numerous cytokines, like IL-1 and -6, granulocyte colony stimulating factor (G-CSF), macrophage CSF (M-CSF) and GM-CSF essential during the various stages of thymocyte activation and differentiation and; the production and release of different hormones: thymosins, thymopoietin, thymulin.[22,23] Thymus instructs T-cells preventing autoimmunity and maintain self-tolerance; mature T-cells (naïve T-cells) leave the thymus to peripheral lymphoid tissues like spleen and lymph nodes to establish the peripheral T-cell repertoire. Thymus also produces other alternative T-cell lineages, including regulatory T-cells (Treg cells), natural killer T (NKT) cells and γδ-Tcells.[21] Treg cells help to maintain self-tolerance by actively suppressing immune responses.[24]

The thymus gland is a privileged site of T-cell generation, extremely vulnerable to toxic actions of chemicals.[25] The mechanisms of toxicity of thymus gland involve receptor binding (*Ah*, aryl hydrocarbon receptor); the Ca^{2+}-dependent activation of an endogenous endonuclease resulting in DNA fragmentation (programmed cell death or apoptosis) and interference with cell proliferation.[26] The consequences of thymus gland toxicity can be a decrease in output of newly generated T-lymphocytes (generation of a new T-cell repertoire), or induction of autoimmune symptoms by the creation of unwanted repertoire.

The physiological function of the thymus in mammals can be disturbed by introducing into the body increased doses of steroid hormones or by subjecting animals to stress. Salts of some heavy metals like cadmium, lead and mercury caused decreased DNA and vitamin C content *in-vitro,* reduced weight of thymus *in-vivo* with thymic involution, particularly visible in the cortical part of this gland.[27, 28] The thymolytic properties of the salts of these heavy metals are explained by their toxic effects on cell membranes, producing reactive oxygen species that result in lipid peroxidation, DNA damage, and depletion of cell antioxidant defense systems.[29,30]

Zinc and selenium are vital for cellular metabolism, able to reverse damage produced by some heavy metals such as cadmium on organs such as kidney and liver, possess anti-inflammatory, antioxidant, protective and immune properties.[31-34]

Aim

The literature seems to be inundated with toxicity studies of individual metals and there is paucity of information on the toxicity of metal mixtures on some organs like the thymus and the eye. We, therefore, posited that zinc and selenium supplementation would down-regulate the inflammatory biomarkers and up regulate antioxidant transcription factors in the eye and thymus of cocktail noxious metal mixture (CNMM) exposed rats.

Material and methods

Chemicals

Lead acetate, mercury chloride, aluminum chloride, and manganese dichloride were bought from Sigma Chemical Co. (St. Louis, MO, USA). Rat ELISA kit of tumor necrosis factor alpha (TNF – α), interleukin 6 (IL – 6), caspase-3, nuclear factor erythroid 2- related factor 2 (Nrf2), nuclear factor kappa B (Nf-kB) and heme oxygenase – 1 (Hmox-1) bought from Elab Science Biotechnology Company, (Beijing, China) were used.

Animals and treatments

In vivo study

Female (weight-matched 100-200 g and aged 8-10 weeks, n=25) Sprague Dawley rats from the Department of Pharmacology, Animal House, University of Port Harcourt, Nigeria were kept under standardized conditions, with water and food *ad libitum*, in accordance with the ethical principles on animal research adopted by the University of Port Harcourt institutional Centre for Research Management and Development Animal Care and Use Research Ethics Committee (UPH/CEREMAD/REC/18). Animals were housed in standard polypropylene cages under room temperature 25±2°C with a 12-h light/dark cycles throughout the duration of the experiment. The animals were acclimatized for two weeks before the commencement of the study. The animals received standard feed and deionized water *ad libitum*. All protocols were approved by the University of Port Harcourt institutional Centre for Research Management and Development Animal Care and Use Research Ethics Committee (number: UPH/CEREMAD/REC/18).

Experiment was conducted in accordance with the "Guide for the Care of Laboratory Animals" approved by the National Academy of Science.

Experimental design

Animals were weight matched and divided into 5 groups (n=5) and various treatments were administered by oral gavage. The animals' treatment protocol lasted for 60 days as follows:

Group 1 received deionized water only and served as the control.
Group 2: Rats were given Cocktail Noxious Metal Mixture (CNMM) only Pb, (20mgkg^{-1}), Hg (0.40mgkg^{-1}), Mn (0.560mgkg^{-1}) and Al (35mgkg^{-1}).
Group 3 rats received CNMM+$ZnCl_2$, 0.80 mg/kg.[35]
Group 4 were administered CNMM+Na_2SeO_3, 1.50 mgkg^{-1}.[36]
Group 5 received CNMM+$ZnCl_2$, 0.80 mgkg^{-1} and Na_2SeO_3, 1.50 mgkg^{-1} combined.

Body, eye and thymus weights, feed and fluid consumption

The body weight of rats was measured weekly and at the end of the 60th day of experiment; the eye and thymus weight was recorded directly after the sacrifice on day 60. The absolute weight of the eye and thymus (g)=mean of eye and thymus weight for each group taken.
The relative weight of eye and thymus
(g/100 g body weight)=

$$= \frac{\text{eye and thymus weight for each group}}{\text{final body weight}} \times 100.$$

Feed (g) and fluid consumption (ml) were recorded daily.

Harvesting and necropsy of eye and thymus

At the end of 60 days of treatment, animals in each group were euthanized using pentobarbital (50mg/kg) intra-peritoneally. The eye and thymus of each rat were harvested, rinsed in cold saline water, weighed and used for both biochemical parameters and heavy metal analyses.

Analysis of heavy metals

The eye and thymus tissues (20 mg) were digested separately using 2 ml of perchloric acid and 6 ml of nitric acid. The samples were subsequently kept for 30 min before heating at 105°C until digestion was completed and the solutions made up to 15 mL (final volume) with deionized water. The Solar thermo elemental flame Atomic Absorption Spectrometer (Model SG 71906) was used to determine Lead (Pb), Aluminum (Al), Mercury (Hg) and Manganese (Mn) concentrations.[37] Limits of detection (LoD) were 0.001 mgkg^{-1} for Aluminum (Al), Mercury (Hg) and Manganese (Mn) and 0.01 mgkg^{-1} for Pb, whereas the limits of quantification (LoQ) were 0.0033 mgkg^{-1} for Al, Hg and Mn and, 0.033 mgkg^{-1} for Pb.

Assay of antioxidant enzymes

GPx activity was measured using a well-established method of Rotruck, et al.[38] A known amount of enzyme preparation was allowed to react with hydrogen peroxide and GSH for a specified time period. The GSH content remaining after the reaction was measured by Ellman's reaction.[39] This method is based on the development of yellow color when dithionitrobenzoic acid (DTNB) is added to compounds containing sulfhydryl groups.

Catalase (CAT) activity was measured using slight modification of the technique by Claiborne.[40] This technique is based on the principle that catalase in the sample will split hydrogen peroxide which can be estimated at 240 nm using a spectrophotometer.[40] Superoxide dismutase (SOD) activity was estimated with the technique previously illustrated by Misra, et al.[41] This method is based on the principle that at pH 10.2, SOD has the capacity to inhibit the autoxidation of epinephrine.

Assessment of oxidative stress markers

The lipid peroxidation marker, malondialdehyde (MDA) level was assayed using the standard method.[42] MDA reacts with the chromogenic reagent, 2-thiobarbituric acid (TBA) under acidic medium to produce a pink colored complex with 532 nm absorbance.

Nitric oxide (NO): this assay adapted the Griess reaction technique.[43-44] One microliter 100 µl of heart and lungs (separately) supernatant was added to 100 µl acidic Griess reagent (1% sulfanilamide and 0.1% naphthlethylenediamine dihydrochloride in 2.5% phosphoric acid). The absorbance was read at 540 nm against blank.

Enzyme-linked immunosorbent assay

The inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), heme oxygenase and Hmox-1, apoptotic marker caspase 3, and transcription factors Nf-kB, Nrf2 in the homogenized eye and thymus cell supernatant was detected using commercially available ELISA kits following the manufacturer's instructions. All experiments were conducted in triplicate.

Assessment of inflammatory markers and transcription factor markers

IL-6, TNF –α, Nr2, Hmox-1, Nf-kB and Caspase 3 activities were measured with IL-6, TNF –α, Nrf2, Hmox-1, Nf-kB and Caspase 3 Activity Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer's directions. Eye and thymus samples were lysed for 15 min on ice. The ovarian and thyroid homogenate were centrifuged at 16,000g for 10 min at 4°C.
Standards or samples were added to the micro-ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for rat IL-6 and avidin-horseradish peroxidase (HRP) conjugate were added successively to each micro plate well and incubated. Free components were washed away. The substrate solution was added to each well; only those wells that contain rat IL-6, biotinylated detection antibody and avidin-HRP conjugate appeared blue in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turned yellow. The

optical density was measured spectrophotometrically at a wavelength of 450 nm±2 nm. The optical density value was proportional to the concentration of rat IL-6. Concentrations of rat IL-6 in the samples were calculated by comparing the optical density of the samples to the standard curve. This procedure was repeated for TNF –α, Nrf2, Hmox-1, Nf-kB and caspase 3.

Histopathological examinations

After fixing with 10% neutral buffered formalin for 72 hours, eye and thymus were separately embedded in paraffin by standard histological method. The tissues were sectioned coronally in 5 µm thickness, and then slices were dewaxed followed by hydration procedures. The sections were stained with hematoxylin-eosin stain kit (Vector Laboratories, USA) according to the manufacturer's instructions. The sections of the eye and thymus were evaluated under a light microscope and photographed with image acquisition parameters settings at 100× and 400×.

Statistical analysis

All the results were expressed as Mean±Standard deviation (std). Microsoft Xlstat 2014 was used in performing analysis of variance and Tukey multiple comparison pairwise tests to check if the concentration of the biomarkers were significantly ($p<0.05$) different between groups. Pandas was used in obtaining the descriptive statistical parameters (biomarkers and heavy metals mean conc.). Graph Pad Prism 5© was used in plotting all graphs (GraphPad Software, California, USA).

Results

Effect of zinc and selenium on body weight, absolute and relative weight of eye and thymus in CNMM

The absolute weight of the eye was significantly reduced ($p< 0.05$) in the various metal mixtures compared to the control group (deionized water); in the thymus, significant weight reduction was observed only in CNMM+Zn and CNMM+Se groups compared to the control (Table 1). However, there was no difference in the relative weights of the eye and thymus (Table 1). A significant reduction in body weight was observed in the metal mixture only and the different combinations with trace elements; feed intake was non-significantly reduced compared to the control group with Table 1). The percentage body weight of the rats treated with essential trace elements is shown in Figure 1. The weight of the rat in the control group ranged from 126-200 g with an increase of 7.4%, the CNMM group ranged from 117–181 g with an increase of 6.35%, the group treated with CNMM+Zn ranged from 60 -163 g with an increase of 10.25%, the rat treated with CNMM+Se ranged from 76–125 g with a weight increase of 7.95% and rats exposed to CNMM+Zn +Se had a range of 40–127 g with an increase of 11.5%.

Table 1. Effect of zinc and selenium on the body weight, absolute and relative weight of eyes and thymus of female albino rats exposed to CNMM*

Treatment	Absolute weight eyes (g)	Relative weight eyes (%)	Absolute weight thymus (g)	Relative weight thymus (%)	Feed intake	Fluid intake
Deionized H_2O (only)	0.80 ± 071a	0.40 ± 5.02	0.30 ± 0.0^{0}a	0.15 ± 0.00	154.10 ± 23.42	242.98 ± 35.35
CNMM (only)	0.55 ± 0.^{0}7a	0.30 ± 9.90	0.30 ± 0.14^{a}	0.17 ± 19.8	148.27 ± 16.42	210.07 ± 33.46
CNMM+Zn	0.53 ± 0.0^{4}a	0.33 ± 0.33	0.23 ± 0.1^{0}a	0.14 ± 0.83	147.94 ± 22.13	195.62 ± 43.20
CNMM+Se	0.46 ± 0.0^{6}a	0.30 ± 8.45	0.21 ± 0.0^{4}a	0.14 ± 5.63	127.63 ± 35.47	209.74 ± 51.27
CNMM+Zn+Se	0.51 ± 0.1^{4}a	0.33 ± 1.98	0.34 ± 0.0^{8}a	0.22 ± 1.13	127.82 ± 29.85	203.85 ± 53.26

* values expressed as mean±standard deviation, n=5, different superscripts (a, b, c) are significantly different from each other at $p<0.05$

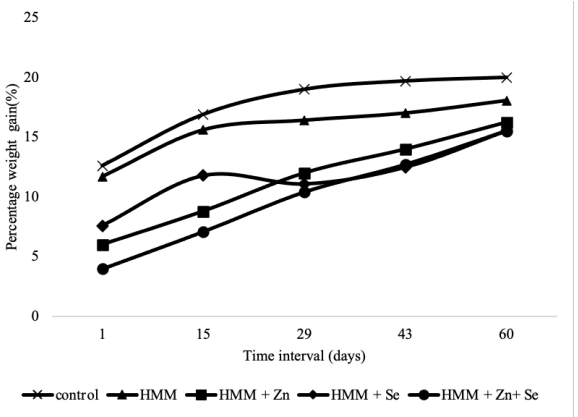

Fig. 1. Effect of Zn and Se on the percentage body weight gain

Antioxidants profile in the eyes of rats treated with zinc and selenium extract after CNMM exposure

SOD in the eyes was significantly reduced ($p<0.05$) in CNMM only group when compared to deionized water and increased in the CNMM+Zn, CNMM+Se and CNMM+Se+Zn groups treatment groups compared to deionized water and HMM only. There was a significant SOD increase in compared to CNMM+Zn only (Fig. 2A); GPx was reduced in CNMM only and CNMM+Zn only groups but increased in CNMM+Zn+Se group; a significant increase was observed in CNMM+Se only and CNMM+Zn+Se groups compared to CNMM +Zn only group (Fig. 2B).

GSH significantly decreased in CNMM and CNMM+Zn only groups compared to deionized water group; significantly increased in CNMM +Se and CNMM+Se+Zn groups compared to CNMM+Zn only but

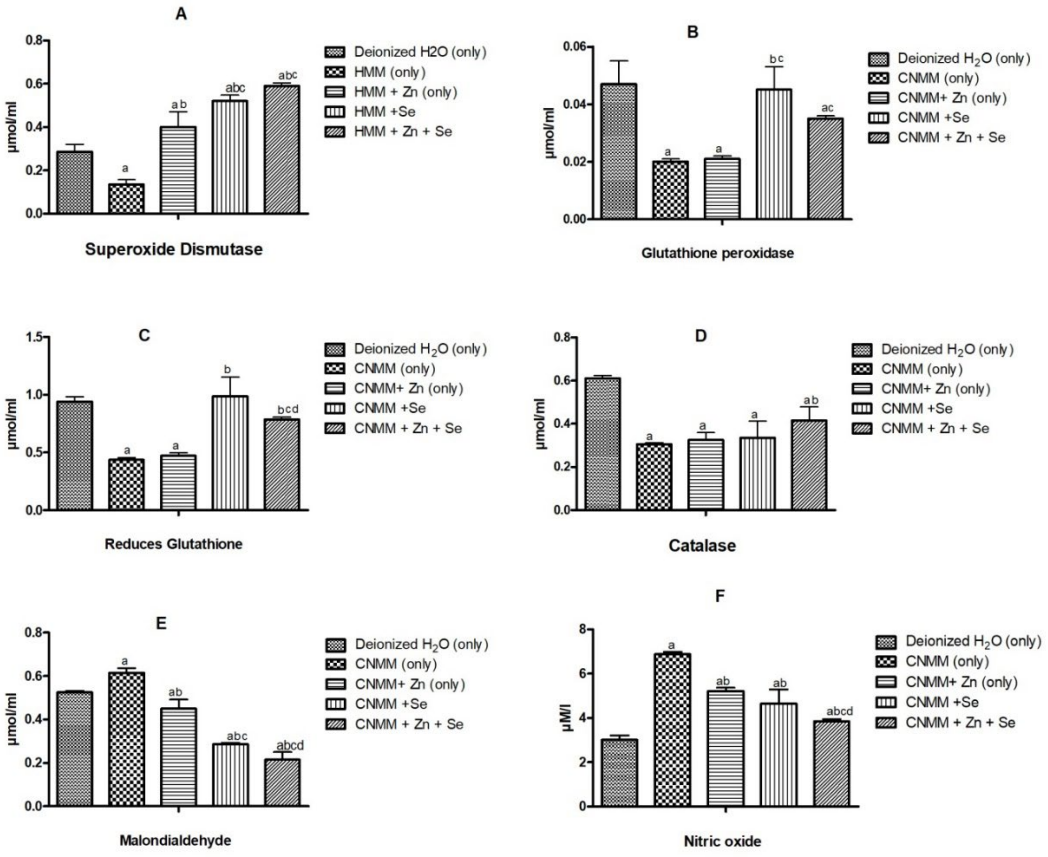

Fig. 2. Essential elements (Zn and Se) on antioxidants (SOD, GPx, CAT, GSH) MDA and NO levels in eye of female albino rats after exposure to CNMM, a – $p<0.05$ compared to deiodized H_2O, b – $p<0.05$ compared to CNMM, c – $p<0.05$ compared to CNMM+Zn, d – $p<0.05$ compared to CNMM+Se

decreased in CNMM+Se+Zn group compared to CNMM+Se (Fig. 2C).

A significant reduction in CAT was seen in all the various metal treatment groups compared to deionized water but an increase in CNMM+Se+Zn compared to CNMM only (Fig. 2D). Similarly, significant reduction of MDA in all metal treated groups except CNMM only group compared to deionized water was observed; MDA decreased in the various metal treated groups compared to CNMM only and also in CNMM +Se and CNMM+Se+Zn groups compared to CNMM+Zn only (Fig. 2E).

However, NO increased significantly in all the metal treated groups compared to the control but decreased in the other metal treatment groups compared to CNMM only; a reduction was observed in CNMM +Zn+Se compared to CNMM+Zn and CNMM +Se (Fig. 2F).

Antioxidants profile in the thymus of rats treated with zinc and selenium after CNMM exposure

In the thymus, the level of the SOD significantly increased in CNMM+Se and CNMM+Se+Zn groups compared to the control and in all the other metal treated groups compared to CNMM only; CAT also increased in CNMM+Zn+Se compared to CNMM+Se (Fig. 3A).

A significant decrease of GPx was seen in all the metal treated groups compared to the control and an increase in CNMM+Zn+Se compared to CNMM only group (Fig. 3C). GSH level was reduced and increased respectively compared to CNMM only group and CNMM+Se (Fig. 3D). MDA level was significantly reduced in CNMM+Se and CNMM+Se+Zn compared to control and CNMM only groups (Fig. 3E).

NO significantly increased in all the heavy metal treated groups compared to control but decreased in the other metal treated groups compared to CNMM only group (Fig. 3F).

Pro-inflammatory markers (IL-6, TNF-α and Casp 3) and transcription factors (NF-κB and Nrf2) in the eyes of rats treated with zinc and selenium following CNMM exposure

In the eyes, pro-inflammatory cytokines Il-6, TNF-α and Casp 3 were significantly increased in CNMM only compared to the control but decreased in the other metal treated groups compared to CNMM only (Fig. 4A, 4B and 4E). Similarly, Nf-kβ was significantly increased in CNMM only compared to the control but decreased

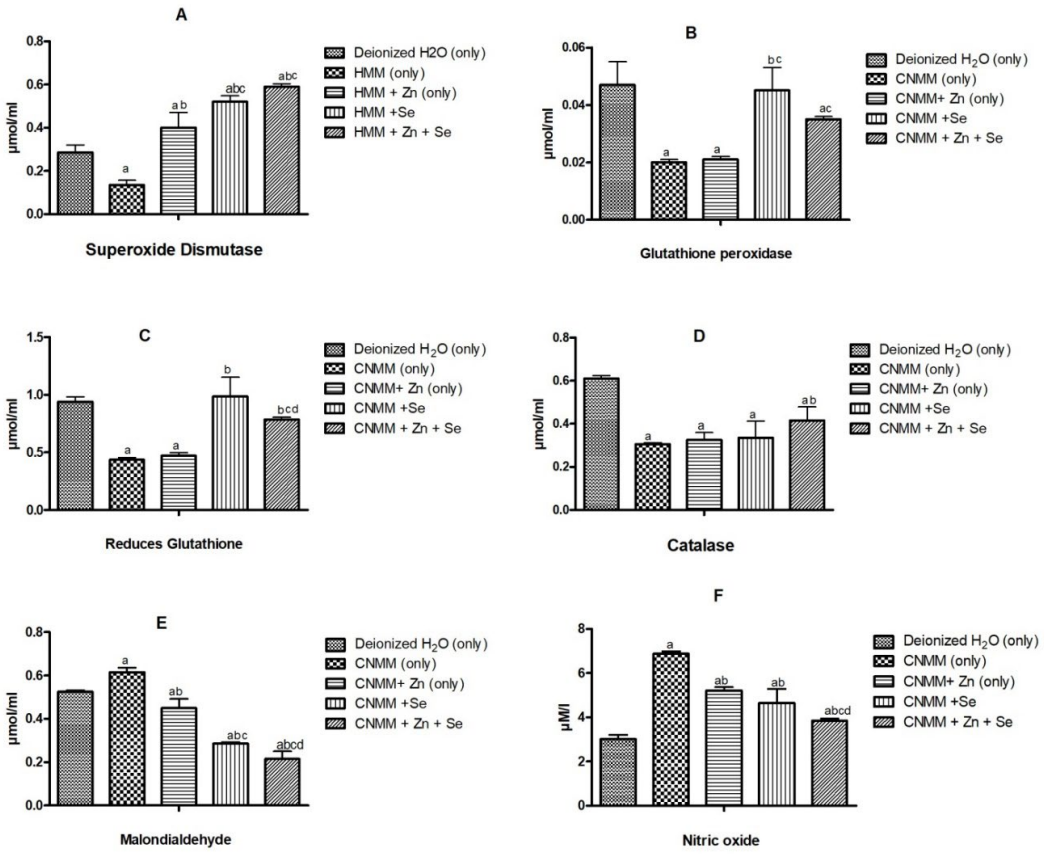

Fig. 3. Essential elements (Zn and Se) on antioxidants (SOD, GPx, CAT, GSH) MDA and NO levels in thymus of female albino rats after exposure to CNMM, a – p<0.05 compared to deiodized H_2O, b – p<0.05 compared to CNMM, c – p<0.05 compared to CNMM+Zn, d – p<0.05 compared to CNMM+Se

only in CNMM+Zn and CNMM+Se groups compared to CNMM only (Fig. 4D).

Nrf2 significantly increased in the various heavy metal treatment groups compared to control but decreased significantly in the other metal treated groups compared to CNMM only; reduced in CNMM+Se compared to CNMM+Zn (Fig. 4C).

Pro-inflammatory markers (IL-6, TNF-α and Casp 3) and transcription factors (NF-κB and Nrf2) in the thymus of rats treated with zinc and selenium following CNMM exposure

A significant increase in thymic IL-6, TNF-α and Casp 3 was observed in CNMM only compared to the control but decreased in the other metal treated groups compared to CNMM only; also, significant reduction of TNF-α in CNMM+Zn+Se compared to CNMM+Zn and CNMM+Se groups was observed (Fig. 5A, 5B and 5E).

Nrf2 and Nf-kβ were increased in the CNMM only and CNMM +Zn+Se groups compared to the control but decreased in the other metal treated groups compared to HMM only, however, Nf-kβ was increased in CNMM +Se and CNMM+Zn+Se groups compared to CNMM+Zn group (Fig. 5C an 5D).

Histology of the eye of rats treated with zinc and selenium following CNMM exposure and treatment

The eye of the rats that received deionized water only showed normal architecture of the retina layers: retina pigmented epithelium (RPE), lamina of the rods and cones (LRC), external limiting membrane (ELM), outer nuclear layer (ONM), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL), optic nerve fiber layer (OFL) and vitreous chambers (VC) (Fig. 6A).The CNMM only treated group showed moderate retinal degeneration seen as less distinct lamination of the retinal layers; considerably cell loss at the nuclear layer (NL) with no demarcation of the outer and inner nuclear layers; the IPL is enlarged and: there is total cell loss at the ganglionic cell layer GCL (Figure 6b).

The eye of CNMM+Zn treated rats showed mild retinal degeneration with decrease in thickness and/or density of cells at the nuclear and GCL. The lamination of the retinal layers was still well preserved and the RPE showed slight disruption (Fig. 6C) while the CNMM+Se group showed normal retinal layer (R) and VC (Fig. 6D).

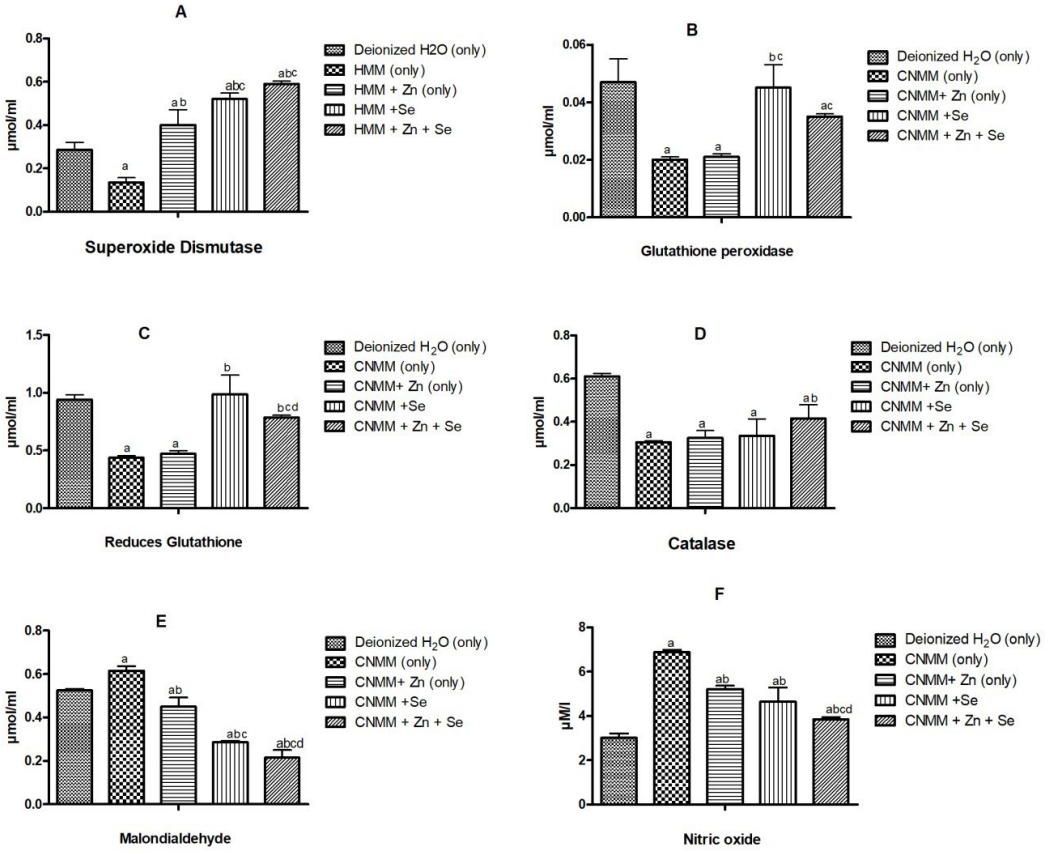

Fig. 4. Effect of essential elements on the pro-inflammatory cytokines (IL-6, TNF-a, transcription factors (Nrf2, Nfkb), and caspase 3 in eye of female albino rats after CNMM (Pb, Hg, Mn and Al) exposure, a – $p<0.05$ compared to deiodized H_2O, b – $p<0.05$ compared to CNMM, c – $p<0.05$ compared to CNMM+Zn

The group exposed to CNMM and combination of essential trace elements (Zn+Se) showed moderate retinal degeneration (Fig. 6E). The lamination of the retinal layers is less distinct at the outer segment (OS), the outer photoreceptor segments were absent; the thickness of the outer nuclear layer (ONL) was considerably reduced; at the inner segment (IS) the ganglion cells were reduced and there is associated retinal wall disruption (RWD) and retinal detachment (RD).

Histology of the thymus of rats treated with zinc and selenium following CNMM exposure and treatment

The photomicrograph of the thymus of rat in the control group showed moderate atrophy with lymphocyte depletion accompanied by multifocal areas of necrosis (arrows), thickened interlobular septum (S) and prominent blood vessel (BV) is noticed (Fig. 7A). The group that received CNMM only showed severe degenerative thymus, lymphocyte depletion, multifocal necrosis (arrows) and thickened interlobular septum with associated fatty change (S) (Fig. 7B).

CNMM+Zn treated group showed mild atrophy with lymphocyte depletion and multi-focal necrosis (arrows) (Fig. 7C). Similarly, photomicrograph of thymus of rats that received CNMM+Se showed mild atrophy with lymphocyte depletion and diffuse necrosis (Fig. 7D).

The group treated with CNMM and combination of essential trace elements (Zn+Se) showed moderate atrophy, lymphocyte depletion and diffuses necrosis (Fig. 7e).

Discussion

Heavy metals share common mechanisms of toxicities such as oxidative stress and inflammation.[45] ROS production is regulated by enzymatic antioxidants, including GPx, CAT, and SOD, and other non-enzymatic antioxidants like reduced glutathione, ascorbic acid and tocopherol.[46] SOD is a major part of the antioxidant system while GSH sustains cellular redox balance. Reduction in GSH and SOD levels leads to excessive utilization of superoxide and hydrogen peroxide causing lipid peroxidation by hydroxyl radicals and increased cellular content of MDA.[46]

This study showed that treatment with CNMM (Al, Pb, Mn and Hg) resulted in decreased CAT, SOD, GPx and GSH levels with increased MDA and nitric oxide (NO) levels in the eye and thymus of rats. HMM co-treatment with essential elements (zinc and selenium) singly or in combination, increased CAT, GPx and

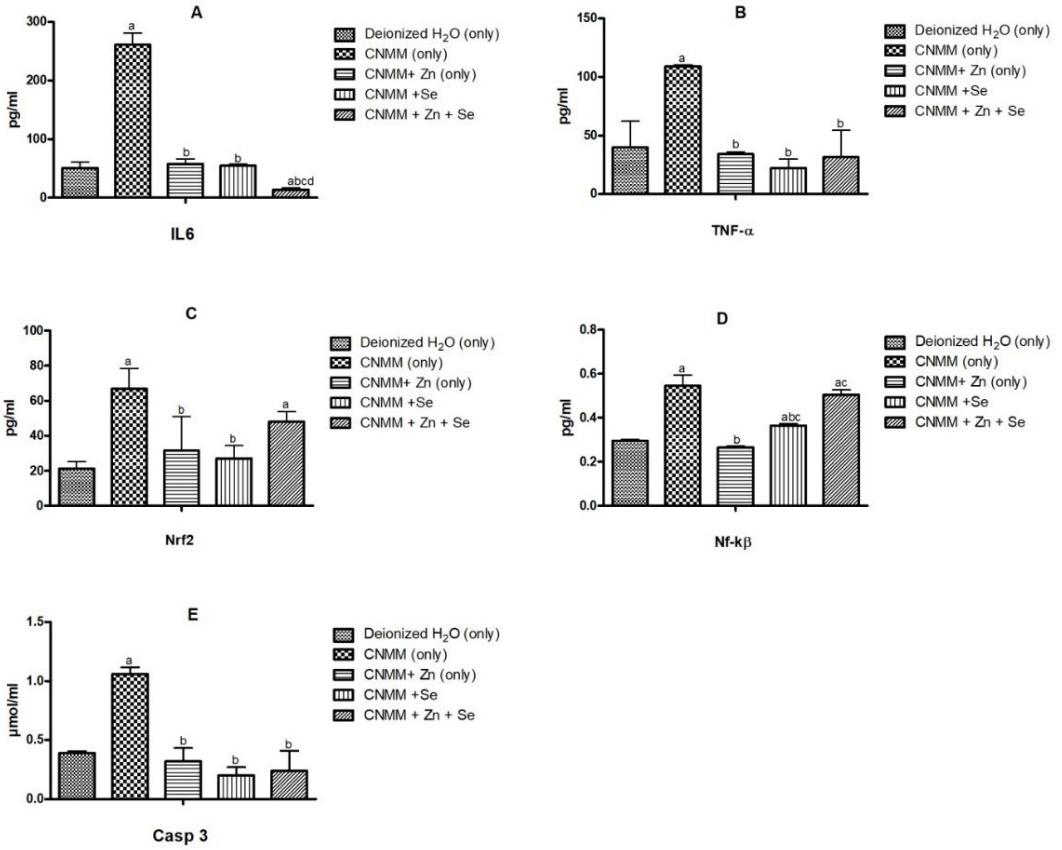

Fig. 5. Effect of essential elements on the pro-inflammatory cytokines (IL-6, TNF-a, transcription factors (Nrf2, Nfkb), and caspase 3 in thymus of female albino rats after CNMM (Pb, Hg, Mn and Al) exposure, a – $p<0.05$ compared to deionized H_2O, b – $p<0.05$ compared to CNMM, c – $p<0.05$ compared to CNMM+Zn, d – $p<0.05$ compared to CNMM+Se

GSH levels with decreased levels MDA and nitric oxide (NO) in these organs but not as with the control.[45] noted that essential elements like selenium (Se), cobalt (Co), copper (Cu), and zinc (Zn) are cofactors or structural components of some antioxidant enzymes. Superoxide dismutase is an isoenzyme that contains Cu and Zn (CuZn-SOD) and so, Zn supplementation might increase CuZn-SOD activity. Zn also reduces the toxicity of cadmium and lead by displacing them from the bonds to important enzymes.[47] GSH is an essential component of the antioxidant system and serves as a cofactor for GSH transferase (GST), which helps remove certain drugs and chemicals as well as other reactive molecules from the cells.[48] GPx is an important selenium-containing enzyme that protects many cells from oxidative damage caused by hydrogen peroxide and other reactive oxygen intermediates.

GPx, is also capable of neutralizing lipid hydroperoxides and this possibly explains the significant reduction of MDA and NO levels in groups co administered with the Se or its combination in our work.[49] Formation of endogenous aldehydes and their derivatives, such as MDA due to lipid peroxidation causes high reactivity and toxicity for cell components and so, causes oxidative stress and tissue damage.[49] The increased nitric oxide production can be attributed to increased nitrate/nitrite level upregulated by iNOS.[50] Inhibitors of ROS and NOX generation, modification of cellular signaling pathways that regulate ROS production and antioxidant defense may reduce ROS levels.[51] Our study showed that Zn and Se have potent antioxidant activity against metal mixture induced oxidative stress and also, provided cellular protection through scavenging hydroxyl radicals and inhibiting lipid peroxidation evidenced in reduced MDA and NO levels.

In this study, rats exposed to CNMM showed increased levels of pro inflammatory cytokines (IL-6, TNF-α), Caspace 3 proteins and transcription factors: nuclear factor erythroid 2-related factor 2 (Nrf2) and nuclear factor kappa B (NF-κB) in the eye and thymus. This is in line with the work of with significantly increased serum levels of IL-1, IL-6 and TNF-a in the group of workers chronically exposed to lead compared to control values.[52] However, Nrf2 expression was decreased in the testicular tissue of mice exposed to Cd and in cerebellum and cerebral cortex of rats challenged with heavy metal mixture.[30,49] Several cytokines, transcription factors like NF-κB and Nrf2 are regulated by

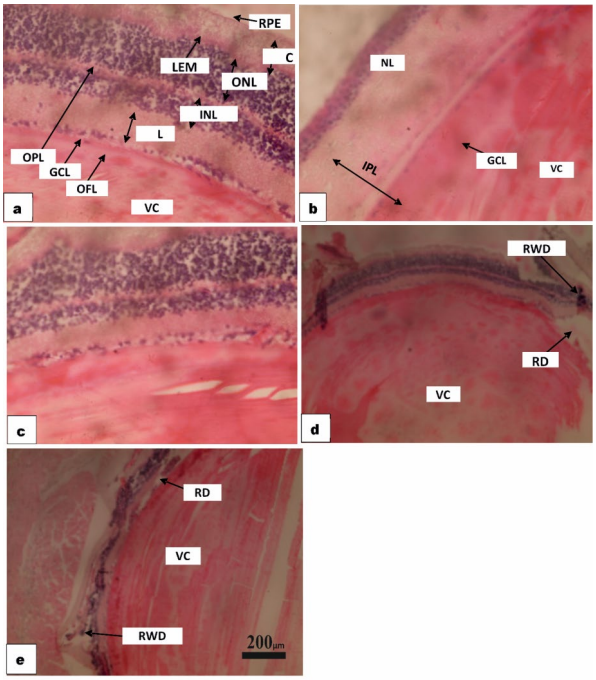

Fig. 6. Photomicrographs of light microscope of rat thymus of various treatment groups, cross sections of eyes were stained with hematoxylin and eosin (400X). A: rat eye treated with iodized water only showing normal architecture of the retina layers, B: rat eye treated with CNMM only exhibited moderate retinal degeneration seen as less distinct lamination of the retinal layers, C: rat eye treated with CNMM+Zn showed mild retinal degeneration with decrease in thickness and/or density of cells at the nuclear and ganglion cell layers, D: rat eye treated with CNMM+Se showed RPE with slight disruption, E: rat eye treated with CNMM+Zn+Se showed moderate retinal degeneration

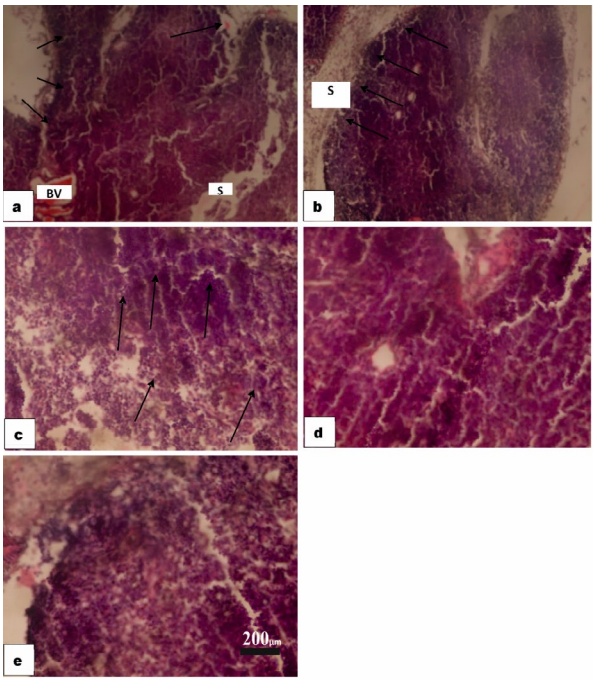

Fig. 7. Photomicrographs of light microscope of rat thymus of various treatment groups, cross sections of eyes were stained with hematoxylin and eosin (400×). A: rat thymus treated with iodized water only showed moderate atrophy with lymphocyte depletion, B: rat thymus treated with CNMM only showed severe degenerative thymus, lymphocyte depletion, C: rat thymus treated with CNMM+Zn showed mild atrophy with lymphocyte depletion, D: rat thymus treated with CNMM+Se showed mild atrophy with lymphocyte depletion, E: rat eye treated with CNMM+Zn+Se showed moderate atrophy, lymphocyte depletion

ROS and their up regulation could be attributed to increased lipid peroxidation observed in our work.[31,49,52] As an adaptive mechanism, Nrf2 is quickly up-regulated in cells and tissues in response to oxidative stress at early stage.[53]

Heavy metal mixtures can affect the Nrf2 pathway which is important in cellular defense against oxidative stress.[54] A luciferase assay also indicated that the levels of activator protein-1 and Nf-κB transcription factors were upregulated in metal mixture *in vitro*.[55] Heavy metals mediate germ cell apoptosis with oxidative stress and CNMM in our study potentiated apoptotic effect in the eye and thymus by upregulating the pro-apoptotic proteins, caspase-3.[50]

Oxidative stress is known to induce the release of proinflammatory cytokines that in turn, triggers signaling cascades of the inflammatory processes.[52] This study demonstrated the ability of CNMM to increase the levels of potent pro-inflammatory cytokines such as TNF-α, IL-6 and transcription factors (NF-κB and Nrf2) consistent with in vitro and animal studies which showed that various heavy metals induced high level expression of cytokines.[56] The up regulation of TNF-α, IL-6, NF-κB and Nrf2 by the treatment with heavy metal mixture in our study increased MDA and NO levels with down regulation of the antioxidant system in both eye and thymus. The activation of Nrf2 in the CNMM treated rats could be an initial compensatory mechanism in response to the toxic effect of the heavy metals evidenced by increased inflammatory cytokines.

However, co-treatment with Zn and Se reduced the levels of IL-6, TNF-α, Caspase 3 proteins and NF-κB but Nrf2 was activated.[57] reported that zinc decreased the expression of inflammatory cytokines and molecules by inhibition of NF-κB activation via A20 and PPAR-a pathways. Nrf2, an important cellular defense factor against oxidative stress regulates intracellular antioxidants and other proteins to neutralize reactive oxygen and/or nitrogen species (RNS) and also acts as target gene for NAD(P)H quinone oxidoreductase-1 (NQO-1), heme

oxygenase-1 (Hmox-1), superoxide dismutase (SOD) and glutathione S-transferase.[53] Activation of Nrf2 can trigger antioxidant enzyme expression and diminish oxidative stress.[58] Nrf2 has the ability to inhibit the expression of pro-inflammatory cytokines, like TNF- α and IL-6, as well as inducible nitric oxide synthase (iNOS) as Nrf2 knockout in mice significantly aggravated acute inflammation.[59] Activation of Nrf2 can trigger antioxidant enzyme expression and diminish oxidative stress.[58] Our work demonstrated the up regulation of antioxidants, reduced lipid peroxidation and down regulation of inflammatory cytokines with the activation of Nrf2 with Zn and Se supplementation. Nrf2 activation also induces cytoprotective gene expression to counteract the toxic effect of ROS and so, an important regulator of processes leading to toxicity and inflammatory diseases.[59] Zn stimulates the Nrf2 expression and transcription, likely by activation of Akt-dependent inhibition of Fyn nuclear translocation. The anti-inflammatory effect of Nrf2 can be due to the ability to inhibit the expression of pro-inflammatory cytokines, like TNF- α and IL-6, as well as inducible nitric oxide synthase.[59]

This study also showed that heavy metal mixture exposure potentiated apoptotic effect in the eye and thymus by upregulating the pro-apoptotic protein (caspase-3). Histological findings in this work showed moderate retinal degeneration and total cell loss at the ganglionic cell layer in the eye; severe degenerative thymus, lymphocyte depletion and multifocal necrosis in rats exposed to CNMM only. It has been proposed that heavy metals may mediate germ cell apoptosis with oxidative stress.[60] Oxidative stress impairs calcium ion channels and alters the mitochondrial membrane potential, leading to cytochrome C release, which enhances caspase cascade and fragmentation of DNA. Co-treatment with Zn and Se in this study counteracted the apoptotic cascade produced by metal mixture exposure therefore; the anti-apoptotic effect of Zn and Se might be due to their antioxidant capacity.[61]

Excessive ROS formation is closely related to inflammation and contributes to the pathogenesis of numerous diseases.[62] Oxidative stress triggers the pathophysiology of various ocular disorders, like dry eye disease, uveitis, pterygium, keratoconus, Fuchs endothelial corneal dystrophy, diabetic keratopathy.[61-65] The visual system is very vulnerable to oxidative stress due to its composition of several susceptible tissues with high metabolic activities.

Heavy metals exposure affects innate and adaptive immune systems through activation of inflammatory events such as release of cytokines and chemokines that results in pathologic conditions like autoimmune diseases.[66] Lymphatic organs are an important part of the body's immune system and play roles in preventing many diseases.[46] TNF-α is important in inflammation, innate and adaptive immune responses and has been implicated in a wide variety of human diseases.[45,56] Lead is related to the induction of oxidative stress, and alteration of immune response that could influence on suppression of T-helper type 1 cells and enhancement of T helper type 2 (Th2) cells which are significantly related to IL-6 production.[67]

Zinc and selenium have the ability to modulate immune response through the production of antibodies or anti-inflammatory cytokines. Selenium is vital in the functioning of enzymes involved in antioxidant system of the immune cells.[68]

Heavy metals affect immune response by altering the relative distribution of different T cell subpopulation such as $CD4^+$, $CD8^+$, Th1, Th2, Th17, Treg.[69] Treg cells help to maintain self-tolerance by actively suppressing immune responses.[24] The immune privilege of the ocular surface is actively maintained through a variety of immunoregulatory mechanisms that prevent the disruption of immune homeostasis.[70] Suppression of effector T cells in the local lymphoid compartment is an essential mechanism maintaining the integrity of the ocular surface; Treg in the lymph nodes draining the ocular surface have been shown to potently suppress sensitization of naive T cells and function of activated T cells, thus preventing the loss of ocular surface immune quiescence.[70-72]

Reception of signal from proinflammatory cytokines (e.g., IL-6) by the immune cells inhibits the function of Foxp3 with the induction of Th17 differentiation.[73] Mature antigen presenting cells bearing self-antigen migrate from the thymus to regional lymph nodes through afferent lymphatic vessels where they prime naive T cells, which then differentiate into the $CD4^+$ T helper cell subsets, T_H1 and T_H17. The effector T_H1 and T_H17 cells migrate through efferent blood vessels to the ocular surface, where they are thought to induce epithelial damage and tear dysfunction via proinflammatory cytokine release. IL-6 inhibits Treg differentiation and induces the development of Th17 cells.[74] The conversion of immunosuppressive Foxp3+ Tregs to pro-inflammatory Th17 cells has been identified as a critical factor in the pathogenesis of autoimmune diseases. T_H1 cells secrete the proinflammatory cytokines TNF-α, interferon-γ (IFN-γ), and IL-2, which activate macrophages. T_H17 cells secrete the cytokine IL-17, which stimulates the production of other proinflammatory molecules, recruits neutrophils, and has been shown to promote corneal epithelial barrier disruption.[49] There is also a correlation between IL-6 levels, disease severity and ocular surface parameters. In this study, the up regulation of these inflammatory cytokines in CNMM exposure would cause lack of tolerance in the eye which could be mitigated by Zn and Se supplementation as evidenced by reduced levels of these cytokines.

Zinc is required for thymic development, production of naive T lymphocytes, clonal expansion, Th1/Th2,

differentiation and normal T lymphocyte function. Zn modulates the activity of several kinases and phosphatases that enhance Nrf2 activity and selenium enhances Nrf2 target gene expression.[75] The cross-talk between Nrf2 and other transcription factors, including the aryl hydrocarbon receptor, NF-kB, tumor suppressor protein p53, and Notch making Nrf2 is important in regulating immune defense, differentiation, and tissue regeneration, as well as cell death. NF-kB is one of the major immune response transcription factors.[57,64] RelB, an NF-κB transcription factor subunit is essential for the development and differentiation of medullary thymic epithelial cells and changes in thymic function may decrease peripheral tolerance and hasten autoimmune disease.[76] TNF- α is one of the most potent physiologic inducers of NF-κB in human lens epithelial cells and this contributes to the amplification of inflammation.[64]

One limitation of the present which will be addressed by immunoblotting in future study is demonstration of increased Nrf2 nuclear trafficking from the cytosol following co-treatment with essential elements.

Conclusion

Taken together the present study has demonstrated that CNMM only exposed rats showed increased levels of MDA and NO, IL-6 and TNF-α, attenuated antioxidants and caspase 3 in the eye and thymus. Zn and Se attenuated adverse effects of HMM exposure. Zinc and selenium supplementation ameliorated CNMM ocular-thymus intoxication in female rats. Our study demonstrated the role of zinc and selenium as anti-oxidant and anti- inflammatory agents and can mediate in immunosuppression and ocular dysfunctions in rats. Zinc and selenium are involved in regulation of redox reactions and several molecular signaling pathways such as Caspase 3, NF-κB and Nrf2 in heavy metal cocktail toxicity in rats.

Declarations

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Author contributions

Conceptualization, O.E.O and C.N.O.; Methodology, O.E.O, A.N.E.; Software, X.X.; Validation, O.E.O, A.N.E. and C.N.O.; Formal Analysis, M.A, O.E.O, A.N.E; Investigation, M.A, A.N.E. and C.N.O..; Resources, X.X.; Data Curation, M.A, A.N.E. and C.N.O; Writing – Original Draft Preparation, T.U, O.E.O, AC, D.N.A.; Writing – Review & Editing, T.U, AC,AC, D.N.A, OEO; Visualization, O.E.O, A.N.E. and C.N.O.; Supervision, O.E.O, A.N.E; Project Administration, O.E.O.

Conflicts of interest

Authors confirm that there was no conflict of interest.

Data availability

All data have been provided.

Ethics approval

All the procedures involving the animals and the experimental protocol followed guidelines for the safe use of animals in research and were approved by the University of Port Harcourt animal research committee (UPH/CEREMAD/REC/18).

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