Original Article

The Oxidative Stress Mediated Toxicity and Immunotoxic Effect of Copper Oxide Nanoparticles on Spleen of Adult Albino Rats

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ABSTRACT

The escalated use of copper oxide nanoparticles (CuO NPs) in various fields increased risk of human and environmental exposure. Spleen accounts for a crucial role in both innate and adaptive immunity and represents a potential target for nanoparticles toxicity. The aim of this study was to investigate the immunotoxic effect of sub-acute oral exposure to CuO NPs on spleen of adult male albino rats. Thirty rats were divided into two main groups. Group (I) control which was subdivided into two groups (10 rats each): (IA) negative control and (IB) positive control (received deionized water). Group II received CuO NPs (125 mg/kg). Animals were orally gavaged with treatments on a daily base for 28 days. At the end of experiment, blood samples on EDTA were used to assess number of red blood corpuscles (RBCs), hemoglobin (Hb%), platelets (PLT), white blood cells count (WBCs), and differential leucocyte count (neutrophils, monocytes, and lymphocytes). Serum samples were analyzed for total IgG and IgM levels. Thereafter, spleens were resected to assess levels of malondialdehyde (MDA), superoxide dismutase (SOD), tumor necrosis factor-alpha (TNF- α), and interleukin-4 (IL-4). Number of T-Lymphocyte subpopulation (CD4+, CD8+ and CD19+) and histopathology (H&E) were also determined. The results of the present study revealed that CuO NPs significantly decreased RBCs, Hb%, PLT, WBCs and lymphocytes% and significantly increased neutrophils% and monocytes%. Also, a significant reduction in total IgG and IgM in serum was detected. Furthermore, spleens of CuO NPs treated group demonstrated significant elevation in MDA, TNF-α., and CD8+. Meanwhile, SOD, IL4, CD4+, CD19+ and CD4+ /CD8+ ratio were significantly decreased. Structural damage was indicated by reduced number and cellularity of lymphoid follicles, as well as, congestion of red pulps. It can be concluded that the immune function of spleen was intoxicated by CuO NPs mostly due to oxidative stress mechanism and inflammation.

Keywords: Copper oxide nanoparticles, Spleen, ROS, Immunoglobulin, Lymphocytes, Flow cytometry.

Submission date:22 August 2020 **Revision date:** 22 September 2020
Acceptence date: 24 September 2020

I. INTRODUCTION

Copper is one of the essential trace elements involved in metabolic processes and maintenance of many enzymes' vitality (Tang et al., 2019). Copper oxide nanoparticles (CuO NPs) have gained too much attention owing to unique conductive, optical, magnetic, and electrical characteristics (Anreddy, 2018). CuO NPs are widely included in multiple applications including catalytic, optical sensors, electronics, superconductors, as well as biomedical field. The field of biomedicine encounters important applications of CuO NPs in foot wears and blankets to prevent nosocomial infections in hospitals. As food additives, they represent a preferable choice due to antimicrobial and growth promoting effects (Tang et al., 2019). Additionally, CuO NPs can be used in a wide range of products ranging from cosmetics to pesticides as potent antifungal and antimicrobial agents (Ezealisiji et al., 2019). Unfortunately, CuO NPs have been reported as the most toxic metal oxide nanoparticles (He et al., 2020).

The uptake of CuO NPs is mediated mainly via liver Kupffer cells as well as splenic macrophages (Cataldi et al., 2017). This can be attributed to the fact that both types of cells are essential parts of reticulo-endothelial system (RES) which is concerned with the removal of foreign bodies (De Jong et al., 2019).

Spleen is a major lymphoid organ in the reticulo-endothelial system. Once antigen is recognized, a cascade of immunological reactions take place in spleen involving activation and recruitment of more and more cells with production of antibodies and cytokines (Dipo, 2019). CuO NPs are mainly distributed to liver, kidney, and spleen where a toxic reservoir of Cu ions is built up in these organs (Lee et al., 2016 a). The high migration to spleen renders the immune system as a potential target for NPs toxicity. Accordingly, more specific immunological investigations are needed (De Jong et al., 2019).

Actually, the immune system is considered as a sensitive target for nanoparticles intoxication. In mice, low-dose of toxicants which are relatively safe for other organs can be extremely toxic for the immunity (Zhou et al., 2019). The immunomodulatory effect caused by nanoparticles is of great importance as it enhances infection and cancer development (Zolnik et al., 2010).

Data on the immunotoxic potentials of most metal nanoparticles are still rare. So, the aim of this study was to investigate the immunomotoxic effect of sub-acute oral exposure to CuO NPs on spleens of adult male albino rats.

II. MATERIALS AND METHODS

II.1. Chemicals:

Copper (II) oxide nanoparticles were obtained in the form of odorless dry black nano-powder with particle size <50 nm, molecular weight of 79.55 g/mol, CAS No. is 1317-38-0 (Product code: 544868), manufactured by Sigma-Aldrich chemical company, USA and purchased from Sigma-Egypt (Eltayaran St., Nasr City-Cairo).

II.2. Particle characterization:

Dry powder of CuO NPs was suspended in deionized water at concentration of 1 mg/mL and then sonicated using a sonicator at room temperature for 15 min. to form a homogeneous suspension. A drop of suspension was placed onto a carbon-coated copper grid, air-dried, and observed with transmission electron microscope (Model JEM-1400, JEOL Ltd., Tokyo, Japan) (Ahamed et al., 2010). This characterization was done in Electron Microscopy Unit, Faculty of Agriculture Research Park (FARP), Cairo University, Egypt.

II.3. Animals and grouping:

All rat experiments were performed in compliance with the relevant laws and guidelines of the Zagazig University of Medicine, Egypt, which are in accordance with the National Institutes of Health Guidelines for Animal Care. A total of 30 male albino rats (weighing 150–200 g) were bred and reared at the animal care center. Animals were acclimated for two weeks under standard laboratory conditions prior to commencement of treatment. Food and water were made available ad libitum. Room temperature was maintained at 23 ± 2 °C, 12 h light–dark cycles, with a relative humidity of 40–60%. Animals were assigned at random to one of two groups;

Group I (control): was subdivided into two subgroups (10 rats each); a negative control (IA, n= 10) received no treatment to measure basic parameters and a positive control (IB, n=10) received 1 mL of deionized water.

Group II (CuO NPs treated group): 10 rats had received CuO NPs (125 mg/kg) which was suspended in deionized water and sonicated with a probe sonicator. This dose represents 1/20 of oral LD50 of copper oxide nanoparticles in rats (2500 mg/kg) (Safety data sheet according to Regulation (EC), Sigma-Aldrich, 2020 and Assy et al., 2019). All treatments were administered via oral gavage on a daily base for 28 days.

II.4. Specimen collection:

II.4.1. Blood:

At the end of experiment, 24 hours after the last dose, rats were anesthetized by using intra-peritoneal injection of pentobarbital (50 mg/kg). Thereafter, blood samples (5 mL) were collected via cardiac puncture. Each blood sample was divided into two halves. One was evacuated into sterile tubes containing ethylenediamine tetraacetic acid for determining blood cells count. The other half was ejected into non-heparinized glass tubes and allowed to clot for 30 min. at 25 °C after which serum samples were separated by centrifugation (600×g, 15 min, 4 °C) and stored at -20 °C until analysis (Nemzek et al., 2001).

II.4.2. Organs:

Rats were sacrificed by cervical dislocation. Spleens of all test animals were excised, washed with ice cold saline, and checked for anomalies. Then, one half of spleen was homogenized for MDA, SOD, TNF-α, IL-4, CD4+, CD8+ and CD19+ analysis. The other half was fixed in 10% formalin for histopathological study.

II.5. Biochemical studies:

II.5.1. Hematological analysis:

Hematologic parameters including RBCs count, Hb%, platelets (PLT), WBCs count, and differential leucocyte count (neutrophils, monocytes, and lymphocytes) were assayed according to the method described by Chanarin et al. (1973), using Coulter T660 hematology analyzer, Beckman Coulter, Inc., USA.

II.5.2 Oxidative stress markers in the spleen:

Splenic malondialdehyde (MDA) and superoxide dismutase (SOD) levels were measured spectrophotometrically following the method described by Ohkawa et al. (1979) and Nagi et al. (1995) respectively, using Biodiagnostic kits (Biodiagnostic, Giza, Egypt).

II.5.3. Cytokine levels in the spleen:

Levels of TNF- α and IL-4 were assessed by using ELISA kits according to manufacturer's instructions (ELISA kit; Abcam Cambridge, UK; TNFα: Cat# ab100785 and IL-4: Cat# ab100770).

II.5.4. Serum immunoglobulin:

Total IgG and IgM levels were measured in rat serum using ELISA kits obtained from Abcam (ab189578, ab157738; Cambridge, MA), according to the method described by Salauze et al. (1994).

II.6. Lymphocyte subpopulation analysis:

Splenic tissues at size of 0.2 g from each rat through a cell strainer. Then, the red blood cells were removed from the suspension with red blood cell lysis buffer. Suspensions were then centrifuged and supernatants were removed. Afterward, cell sedimentations were suspended again with PBS at cell concentration of 106/mL. The cell suspensions were stained with antibodies (anti-CD4+, anti-CD8+ and anti-CD19), purchased from BD Biosciences, Shanghai, China). The cells (CD4+, CD8+ and CD19 cells) were analyzed by flow cytometry (BD FACSVerse, BD Biosciences, Shanghai, China) (Mitra et al., 2013).

II.7. Haematoxylin and eosin staining (H&E):

Paraffin-embedded blocks were sectioned out at 5 µm thick sections and stained with haematoxylin and eosin following the method described by Bancroft and Stevens (1996).

II.8. Statistics:

Data were analyzed by Statistical Package of Social Science (SPSS), software version 20. Quantitative data were summarized as mean ± SD (standard deviation). Student t-test was used for comparison between two independent quantitative data which are normally distributed. Probability (P value of >0.05 indicates non-significant results; P value <0.05 means significant difference, P value >0.001 for highly significant result).

III. RESULTS

III.1. Characterization of CuO NPs:

Morphological characterization of CuO NPs by transmission electron microscopy (TEM) revealed that most of CuO NPs were spherical in shape with different sizes ranging from 4.77 to 7.78 nm (Figure 1).

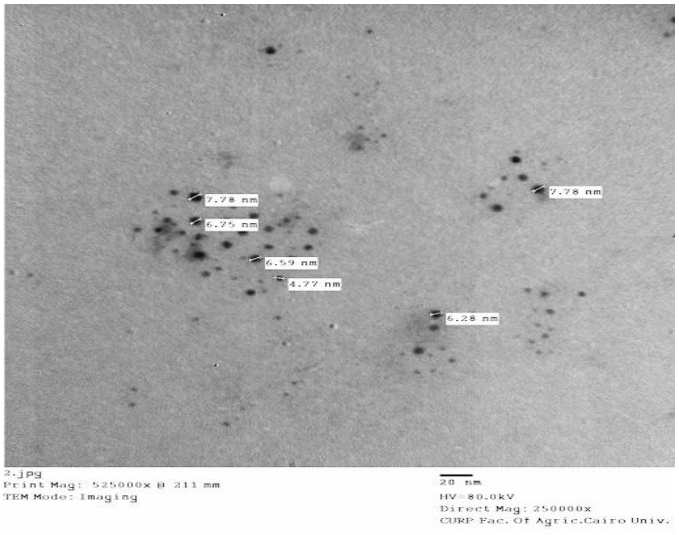

Figure (1): Transmission electron microscopy image of copper oxide nanoparticles showing spherical shape particles with different sizes ranging from 4.77 to 7.78 nm.

III.2. Biochemical results:

There was no statistically significant difference observed regarding the biochemical parameters of all control groups. So, the negative control group (IA) was used for comparison with CuO NPs treated groups.

III.2.1. Hematological findings:

Administration of CuO NPs significantly ($p< 0.001$) decreased mean values of RBCs count, Hb%, platelets count, WBCs count and lymphocytes%. Meanwhile, nano-CuO particles significantly ($p< 0.001$) increased mean values of neutrophils% and monocytes% compared to the control group (Table 1).

Table1: Statistical comparison between negative control and CuO NPs groups regarding the mean values of hematological findings using student t-test.

Parameter	Negative control group	CuO NPs group (125 mg/kg for 4 weeks)	t	p-value
RBCs (10^{12}/L)	7.07±0.13	5.65±0.37	11.590	<0.001**
Hb (%)	16.99±1.24	10.93±1.00	12.037	<0.001**
PLT (10^9/L)	1163.3±34.78	1088.7±29.91	5.143	<0.001**
WBCs (10^9/L)	11.49±0.25	8.31±0.51	17.661	<0.001**
Neutrophils (%)	11.64±0.15	28.79±1.76	30.653	<0.001**
Monocytes (%)	2.44±0.15	3.77±0.15	19.600	<0.001**
Lymphocytes (%)	83.2±1.20	55.4±3.63	22.955	<0.001**

** $p <0.001$(highly significant) compared to control group; Values are presented as mean ± SD (Standard deviation); Number of rats in each group equals to 10 rats; %= percent; RBCs= Red blood corpuscles; Hb= Hemoglobin; PLT= Platelet; WBCs= White blood cells.

III.2.2. Splenic oxidative stress markers:

Treatment with CuO NPs resulted in a highly significant ($p< 0.001$) increase in mean values of splenic MDA and highly significant ($p< 0.001$) decrease in mean values of splenic SOD when compared to the control group (Table 2).

Table 2: Statistical comparison between negative control and CuO NPs groups regarding the mean values of splenic SOD and MDA levels using student t-test.

Parameter	Negative control group	CuO NPs group (125 mg/kg for 4 weeks)	t	p-value
MDA (nmol/g tissue)	40.5±4.03	127.8±7.93	31.036	<0.001**
SOD (µ/mg protein)	38.05±4.63	15.35±4.26	11.410	<0.001**

** p<0.001 (highly significant) compared to control group; Values are presented as mean ± SD (Standard deviation); Number of rats in each group equals to 10 rats; MDA= Malondialdehyde; SOD= Superoxide dismutase.

III.2.3. Splenic inflammatory markers:

Administration of CuO NPs showed a highly significant (p< 0.001) elevation in mean values of TNF-α and a highly significant (p<0.001) decrease in mean values of IL-4 in comparison with the control group (Table 3).

Table 3: Statistical comparison between negative control and CuO NPs groups regarding the mean values of splenic TNF-α and IL-4 using student t-test.

Parameter	Negative control group	CuO NPs group (125 mg/kg for 4 weeks)	t	p-value
TNF-α (pg/ mg protein)	7.92±1.05	40.45±3.15	31.003	<0.001**
IL-4 (pg/ mg protein)	18.34±3.31	5.48±1.6	11.069	<0.001**

** p<0.001 (highly significant) compared to control group; Values are presented as mean ± SD (Standard deviation); Number of rats in each group equals to 10 rats; TNF-α= Tumor necrosis factor-alpha; IL-4= Interleukin-4.

III.2.4. Serum immunoglobulins:

Results revealed a highly significant reduction in total IgG and IgM in serum of CuO NPs treated group when compared to control group (Table 4).

Table 4: Statistical comparison between negative control and CuO NPs groups regarding the mean values of serum total IgG and IgM using student t-test.

Parameter	Negative control group	CuO NPs group (125 mg/kg for 4 weeks)	t	p-value
Total IgG (g/L)	1.735±0.161	0.92±0.046	15.325	<0.001**
Total IgM (mg/L)	282.1±13.44	152.5±12.30	22.487	<0.001**

** p<0.001(highly significant) compared to control group; Values are presented as mean ± SD (Standard deviation); Number of rats in each group equals to 10 rats; IgG= Immunoglobulin G; IgM= Immunoglobulin M.

III.3. Lymphocyte subpopulation in splenic tissue:

Administration of CuO NPs significantly (p<0.001) decreased mean values of CD4+ (T-helper cells), CD19+ (B lymphocytes) and CD4+ /CD8+ ratio. Contrary, CuO NPs significantly (p<0.001) increased mean value of CD8+ (T-cytotoxic cells) compared to control group (Table 5 and Figure 2&3).

Table 5: Statistical comparison between negative control and CuO NPs groups regarding the mean values of CD4+, CD8+, CD4+ /CD8+ ratio, and CD19+ using student t-test.

Parameter	Negative control group	CuO NPs group (125 mg/kg for 4 weeks)	t	p-value
CD4+ (%)	17.8±2.39	12.04±1.35	6.624	<0.001**
CD8+ (%)	11.34±0.92	19.8±2.29	10.802	<0.001**
CD4+ /CD8+ ratio	1.58±0.26	0.62±0.11	10.724	<0.001**
CD19+ (%)	17.6±2.01	10.59±1.36	9.132	<0.001**

** p<0.001(highly significant) compared to control group; Values are presented as mean ± SD (Standard deviation); Number of rats in each group equals to 10 rats; %= percent; CD4+= T-helper cells; CD8+= T-cytotoxic cells; CD19+= B lymphocytes.

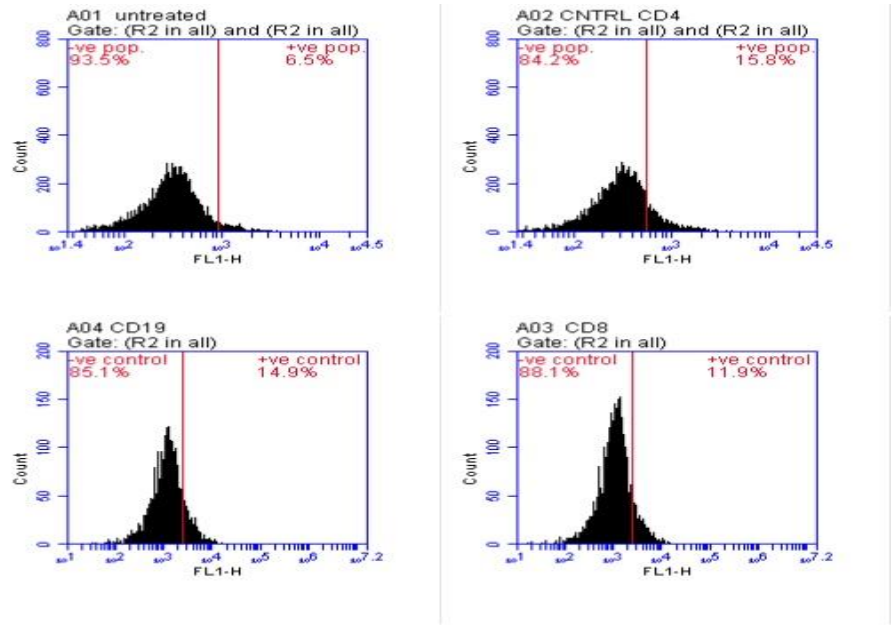

Figure (2): Flow cytometry analysis of CD4+, CD8+ and CD19+ in the spleen of adult male albino rat from the control group.

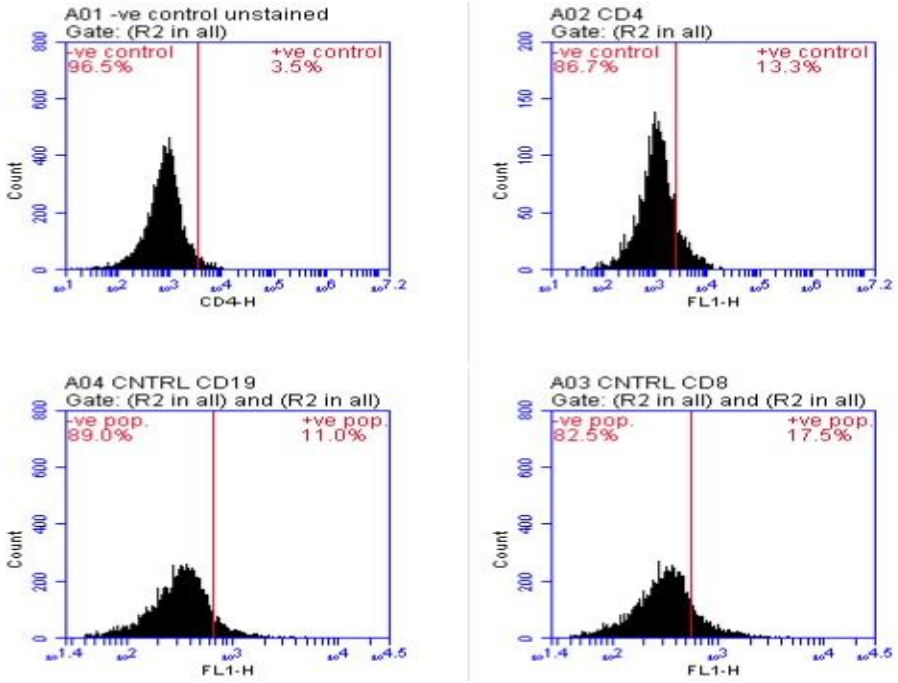

Figure (3): Flow cytometry analysis of CD4+, CD8+ and CD19+ in the spleen of adult male albino rat orally gavaged by CuO NPs (125 mg/kg/day) for 28 days.

III.4. Haematoxylin and eosin staining (H&E):

Spleens resected from all animals revealed no gross anomalies. The control group demonstrated normal spleen structure formed of white and red pulps. The white pulp showed well-circumscribed lymphoid follicles. The red pulp was made up of anastomosing and branching splenic cords with blood sinusoids in between (Figure 4 a). The CuO NPs treated group showed reduction in number of follicles (Figure 4 b), atrophy of white pulps (Figure 4 c), and reduced cellularity (Figure 4 d). Red pulp showed also signs of inflammation as congestion (Figure 4 e). Apoptotic changes were also demonstrated in some areas of spleen (Figure 4 f).

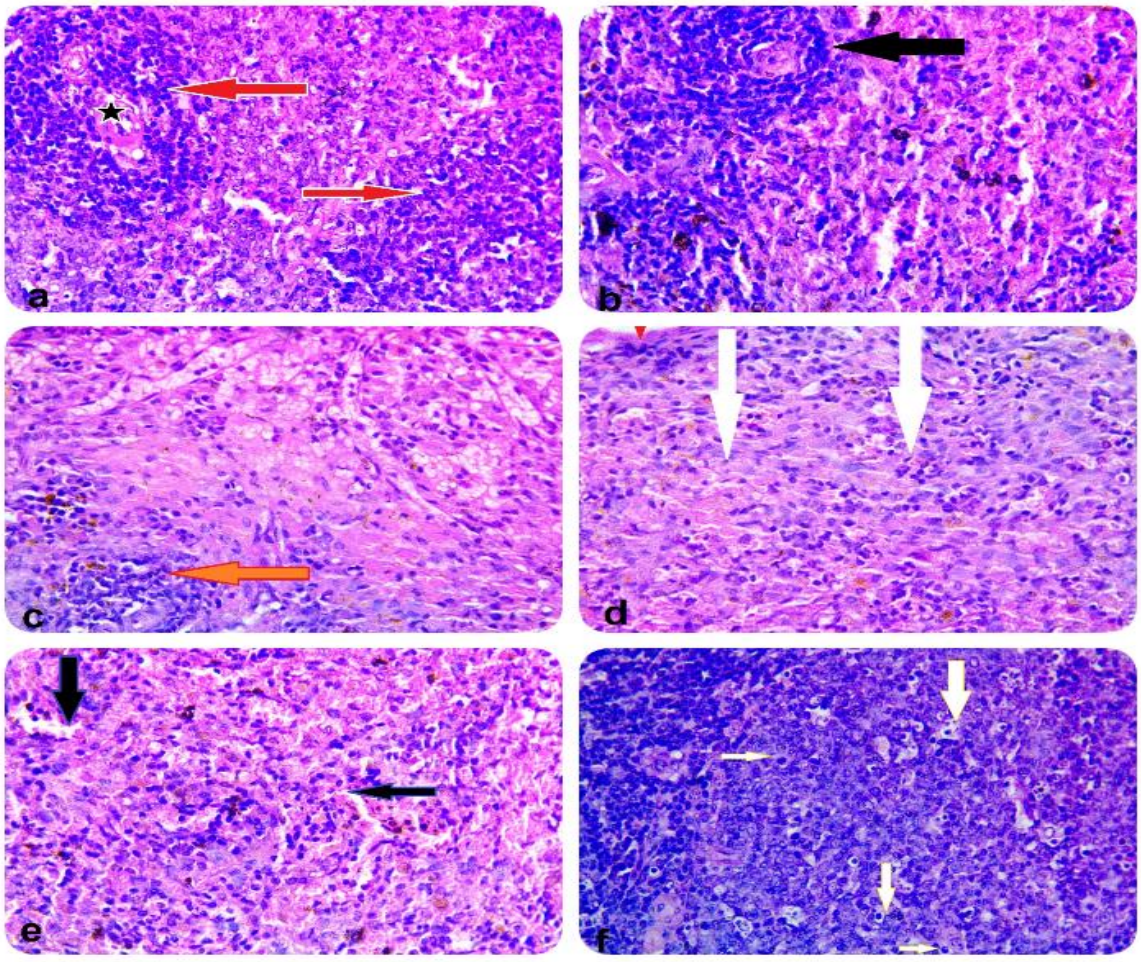

Figure (4): H&E staining micrographs of spleen tissues showing: a) Section from control group showing normal spleen with intact cellular white pulp (red arrow) and central arteriole (asterisk) (H&E x400). Sections from CuO NPs treated group showing b) atrophied white pulps (black arrow)(H&E x400). c) Reduced number (only one referred by arrow) and cellularity of white pulp (H&E x400). d) Wide area (white arrow) under the capsule (red arrow) without follicles denoting reduced follicle number (H&E x400). e) Congestion of red pulp (black arrow) (H&E x400). f) Numerous apoptotic cells in the spleen (white arrow) (Total magnification = x400).

IV. DISCUSSION

Results of the present study revealed alteration in hematological profile and immune reaction as was indicated by the significant reduction in RBCs count, Hb%, platelets, and total WBCs count. The differential WBCs revealed decreased lymphocytes and increased neutrophils and monocytes in treated group when compared to the control.

The reduced RBCs, Hb, and platelets are consistent with hematological findings reported in previous studies (Lee et al., 2016 b; Taha et al., 2017; Attia et al., 2019). Similarly, rats orally treated by ZnONPs showed significant decrease in RBCs, platelets, and Hb% (Abass et al., 2017). The reduced WBCs is consistent with the results demonstrated by Chung et al., (2009), Al-Naimi et al. (2013), and Taha et al. (2017). Also, rats treated by AgNPs had revealed immunological suppression as was indicated by grave decline in total WBCs and lymphocytes (Hassanen et al., 2019).

After being administered orally and upon reaction with H+ of gastric juice, CuO NPs are transformed into their ionic states (Strauch et al., 2017). So, the Cu2+ content in cells and tissues (cellular cytoplasm and nucleus) is markedly increased (Sivakumar et al., 2020). The high level of Cu2+ can competitively inhibit iron absorption and utilization resulting in decreased serum iron (Arredondo et al., 2005). The reduced serum iron leads to reduction in Hb synthesis and anemia (Attia et al., 2019).

Besides, the prolonged exposure of RBCs to the excess copper enhances destruction of cell membrane and leakage of internal enzymes with shortened RBCs lifespan (Mitra et al., 2013). Also, the oxidative stress and lipid peroxidation (LPO) generated by CuO NPs cause anemia either directly by destroying erythrocytes or indirectly by increasing the vulnerability of RBC to destruction (Attia et al., 2019). Collectively, anemia reported with CuO NPs can be attributed to defective Hb synthesis and the reduced RBCs count due to excess Cu2+ and lipid peroxidation.

In the same line, the decreased platelets associated with CuO NPs can be ascribed to the oxidative stress mediated damage of platelet membranes (Attia et al., 2019). The significant reduction in platelet count indicates the ability of CuO NPs to cause thrombocytopenia and impaired clot formation (Maciel-Magalhães, 2020).

As regards the diminished WBCs count, it can be attributed to the depletion of these cells in the immune response (Attia et al., 2019). Also, the decreased lymphocyte implies the detrimental effect of CuO NPs on the immune cells. On the other hand, the increased percentages of neutrophils and monocytes are mainly related to the inflammatory response as well as being a compensatory response to the decreased lymphocytes (Lee et al., 2016 b). Meanwhile, this compensation could not overcome the overall depletion of WBCs in the present study.

Contrary to the results of the present study, the increased WBCs reported by Khabbazi et al. (2015), Taha et al. (2017), and Attia et al. (2019) was explained by the acute inflammatory response caused by the short term interaction between CuO NPs and the immune system. Furthermore, De Jong et al. (2019) attributed the increased WBCs count to the increased neutrophilic granulocytes in short term exposure to CuO NPs. However, after a certain time, the exaggerated immune response becomes weakened and atrophy of lymph nodes takes place depleting immunity and reducing WBCs (Attia et al., 2019).

The lipid peroxidation and oxidative stress have been cited as the most accepted mechanism for NPs toxicity (Alarifi et al., 2013). This was further supported by various in vivo and in vitro studies (Niska et al., 2015; Griffitt, et al., 2007; Assadian et al., 2018). Oxidative stress with metal NPs like CuO NPs may be related to the surface properties, the elaborated metal

ions, or both (Wang et al., 2016). Oxidative stress is associated with over production of reactive oxygen species (ROS) (He et al., 2020).

The excessive ROS can be attributes to the small size and large surface area of NPs, the interaction of NPs with the cellular components like mitochondria, and the excessive O2 production upon activation of NADPH-oxidase enzyme in membrane of phagocytic cells (Alarifi et al., 2013). The generated ROS enhance inflammation and damage to lipid membranes, proteins, and DNA (Yang et al., 2009 and Akhtar et al., 2016).

On the other hand, the elaborated Cu2+ from CuO NPs was demonstrated by He et al. (2020) as the major cause for the oxidative stress and excess superoxide anions in cells. Interestingly, He et al. (2020) had recommended copper chelating agents instead of usual antioxidants to alleviate the oxidative damage mediated by CuO NPs.

In the present study, the ability of CuO NPs to induce oxidative stress was evaluated by measuring the levels of lipid peroxidation marker (MDA) and the antioxidant enzyme (SOD) in spleen tissues. Superoxide dismutase (SOD) represents the first detoxification enzyme and the most powerful antioxidant in the cell. Also, it stands for an essential component of the first line defense system antagonizing ROS (Ighodaro and Akinloye, 2018). In the present study, oxidative stress was indicated by the elevated MDA and decreased SOD levels.

In support of this, nano-Cu particles caused significant elevation of MDA levels in spleens of treated rats (Zhou et al., 2019). In livers of rats orally exposed to CuO NPs, Anreddy (2018) and Tang et al. (2019) had reported dose dependent reduction in activity of hepatic antioxidant enzymes like reduced glutathione, catalase and superoxide dismutase, whereas MDA levels were significantly increased. Also, Elkhateeb et al. (2020) had demonstrated increased MDA levels and decreased SOD activity and other antioxidants in kidney of CuO NPs treated rats. In the same line, addition of NAC (N-acetyl-cysteine) to the epithelial cells of kidney had been proven to alleviate CuO NPs-induced cytotoxicity (Srikanth et al., 2016).

The levels of cytokines are measured as biomarkers for the immunomodulation caused by NPs (Elsabahy et al., 2013). Results of this study revealed increased levels of pro-inflammatory cytokine (TNF-α) in spleen of CuO NPs treated rats when compared to the control. In contrast, a significant decrease of anti-inflammatory cytokine (IL-4) was detected. This means that CuO NPs can cause apparent inflammatory response in spleen.

In the same line, CuO NPs increased expression of pro-inflammatory cytokines and decreased anti-inflammatory ones in both mouse respiratory tract (Ko et al., 2018) and Hela cells (Dey et al., 2018). Also, Hanley et al. (2009) reported similar results for ZnO nanoparticles in primary immune cells of human. Furthermore, Khan et al. (2013) demonstrated a significant increase in the pro-inflammatory cytokines with gold nanoparticles after 1 day of treatment.

Based on the mechanism of action, immune system is divided into two sub-systems; the first one is the innate system which represents the first line of defense against foreign particles. This system is regulated by phagocytic cells (macrophages and dendritic cells), neutrophils, and mast cells which serve as antigen-presenting cells (APCs) to the cells of the second subsystem (Akira et al., 2006).

The second subsystem is the adaptive system (cell mediated immunity) which encounters more specialized cells (T and B cells) for degradation or neutralization of engulfed nanoparticles. T lymphocytes are subdivided into: a) T helper (Th) cells which include (Th1, Th2, Th17); b) T regulatory cells (T regs); and c) cytotoxic T cells. As regards T helpers, Th1 mediates the pro-inflammatory response

via pro-inflammatory cytokines including interferon (IFN)-γ, interleukin (IL)-2, and tumor necrosis factor (TNF) – α. On the other hand, Th2 mediates the antagonistic anti- inflammatory immune response via anti-inflammatory cytokines (IL-4, IL-5, IL-10, and IL-13). Additionally, Th2 facilitates production of antibodies from B cells (Muhammad et al., 2020).

After gastric tract absorption, high levels of CuO NPs are sequestrated in spleen by macrophages of marginal zone and red pulp. These cells elaborate excess ROS to remove the intruder resulting in oxidative stress (Zhou et al., 2019). Actually, ROS act as secondary messenger to enhance the inflammatory immune response in spleen (Muhammad, et al., 2020). The pathophysiological potential of NPs is determined by the extent of the oxidative stress, ranging from inflammation to cell death and even cancer (Petrarca et al., 2015). Moreover, it was speculated that inflammation caused by nano-Cu oxide particles could be attributed to Cu2+ ions themselves that trigger oxidative stress and accordingly activate inflammatory response (He et al., 2020).

The inflammatory response mediated by NPs is modulated according to the specific pattern of cytokines production by T cells. Homeostasis of T cells (Th1/Th2 balance) represents a critical factor for the adaptive immunity (Luo et al., 2015). Some metal NPs modulate immunity by disturbing homeostasis of T cells via polarizing the cytokine balance towards Th1 cytokines; increasing production of pro-inflammatory cytokines (IL-2, IFN-, and TNF-), and decreasing the production of Th2 cytokines (IL-4, IL-5, and IL-6) (Petrarca et al., 2015). Amongst these nanoparticles, CuO NPs have been reported to evoke the most powerful inflammatory response (Karlsson et al., 2008). Contrary, some other metal NPs disturb Th1/Th2 balance by polarizing cytokine balance towards expression of Th2 cytokines and decreasing pro-inflammatory cytokines like AgNPs (Li et al., 2018).

The spleen accounts for a crucial role in both innate and adaptive immune response organization. The spleen is organized into regions called the red pulp and white pulp. Red pulp contains the macrophages and dendritic cells while white pulp contains T and B lymphocytic cells. The white pulp mimics the structure of lymph nodes and allows generation of antigen-specific immune response (Bronte and Pittet, 2013).

Results of the present study revealed a significant reduction in total IgG and IgM in serum of CuO NPs treated group when compared to the control group reflecting immune suppression. These results are consistent with Zhou et al. (2019) and in the same line with other metal NPs like AgNPs (Hassanen et al., 2019), TiO(2) NPs (Sang et al., 2012), and Fe2O3 NPs (Shen et al., 2011). The reduction in serum immunoglobulins had been attributed to the decreased number of B cells due to cytotoxic damage caused by NPs (Zhou et al., 2019). Additionally, under normal conditions, Th2 cells drive the humoral immunity and up regulate the production of antibodies via anti-inflammatory cytokines (IL-4, IL-6, IL-10, and IL-13). These cytokines signal B-cells to proliferate and differentiate to most classes of antibodies (IgG and IgE) (Zhu, 2015). Accordingly, impaired Th2 cell function can reduce IgG just as impaired B-cells (Luo et al., 2015 and Hassanen et al., 2019).

The T cells including T helper (T) cells, regulatory T cells, and cytotoxic T cells express surface proteins including CD4+ on T helper cells and CD8+on cytotoxic T cells (Luo et al., 2015). B cells express CD19+ on their surface. Many studies have proven the ability of NPs to alter T cells expansion, activation, and proliferation (Dobrovolskaia et al., 2016).

In the present study, assessment of lymphocyte subpopulation in spleen tissues revealed decreased CD4+,

CD4+/CD8+, and CD19+ levels which are consistent with the impaired cellular and humoral immunity. Meanwhile, CD8+ was significantly increased. The reduced lymphocyte subpopulation can be ascribed to the oxidative stress as well as the excessive exposure to copper which have detrimental effect on immune cells leading finally to immunosuppression (Mitra et al., 2012). The increased CD8+ cells promote cytotoxicity and apoptosis with excess copper (Mitra et al., 2013), while the decreased Cd19+ is consistent with the diminished immunoglobulins reported in present study. Similarly, in Zhou's study, number of CD4+ and ratio of CD4+/CD8+ were reduced indicating the imbalance of cellular immunity induced by nano-Cu particles (Zhou et al., 2019).

Sections obtained from spleens of CuO NPs treated group revealed structural alterations as was indicated by lymphoid depletion, atrophy of white pulps, reduced cellularity, inflammatory congestion, and apoptosis. These findings are further supporting the disturbed immunological function and reduced T lymphocytes subpopulations reported in our study. Results of the present study are parallel to the previous studies conducted by Zhou et al. (2019), El Bialy et al (2020), and Lee et al. (2016 b) where the structural alterations were ascribed to the oxidative stress mechanism. Additionally, apoptosis detected in this study is a well-documented form of programmed cell death caused by CuO NPs mediated DNA damage. The damage of DNA is caused either via direct penetration of NPs to the nucleus or indirectly via generating ROS which react with the nucleus causing DNA damage (Alarifi et al., 2013).

V. CONCLUSION AND RECOMMENDATIONS

Sub-acute exposure to CuO NPs exerts oxidative stress mediated damage and immunotoxic effect on spleens of treated rats. Oxidation was reflected by high lipid peroxidation and reduced SOD. Suppression of immunity was indicated by diminished WBCs count, reduced serum immunoglobulins, altered T cells subpopulation, and the depleted lymphatic nodules in CuO NPs treated group. Attention should be paid to the immunotoxic effect caused by repeated exposure to CuO NPs in food additives and daily used products like cosmetics. Also, a simple investigation like full blood picture should be routinely done for workers occupationally exposed to nano-copper oxide to detect toxicity.

VI. CONFLICTS OF INTEREST

Authors declared no conflict of interest.

VII. REFERENCES

Abass MA, Selim SA, Selim AO, El-Shal AS and Gouda ZA (2017): Effect of orally administered zinc oxide nanoparticles on albino rat thymus and spleen. IUBMB Life. 69 (7): 528-539.

Ahamed M, Siddiqui MA, Akhtar M J, Ahmad I, Pant AB and Alhadlaq H A (2010): Genotoxic potential of copper oxide nanoparticles in human lung epithelial cells. Biochem Biophys Res Commun. 396 (2): 578-583.

Akhtar MJ, Kumar S, Alhadlaq HA, Alrokayan SA, Abu-Salah KM, Ahamed M (2016): Dose-dependent genotoxicity of CuO nanoparticles stimulated by reactive oxygen species in human lung epithelial cells. Toxicol Ind Health. 32 (5): 809-821.

Akira S, Uematsu S and Takeuchi O (2006): Pathogen recognition and innate immunity. Cell. 124: 783-801.

Alarifi S, Ali D, Verma A, Alakhtani S and Ali BA (2013): Cytotoxicity and genotoxicity of copper oxide nanoparticles in human skin keratinocytes cells. Int J Toxicol. 32 (4): 296-307.

Al-Naimi RA, Al-Tayar NH, Alsoufi LAM and Al-Taae EHY (2013):

Hematological and biochemical evaluation after different orally doses of copper sulfate in rats. Iraqi J Vet Med. 38 (1):83-91.

Anreddy RNR (2018): Copper oxide nanoparticles induces oxidative stress and liver toxicity in rats following oral exposure. Toxicol Rep. 5: 903-904.

Arredondo M and Núñez MT (2005): Iron and copper metabolism. Mol Aspects Med. 26: 313-27.

Assadian E, Zarei MH, Gilani AG, Farshin M, Degampanah H and Pourahmad J (2018): Toxicity of copper oxide (CuO) nanoparticles on human blood lymphocytes. Biol Trace Element Res. 184 (2): 350-357.

Assy W, Wasef M, Abass M and Elnegris H (2019): A study of short term chronic pulmonary toxicity, neurotoxicity and genotoxicity of copper oxide nanoparticles and the potential protective role of vitamin E on adult male albino rats. ZJFMT. 17(2): 1-18.

Attia A, El-Banna S, el-trass E, Yahya R, Azab A, Jbireal J and Shkal K (2019): Hematotoxicity induced by copper oxide and/or zinc oxide nanoparticles in male albino rats. J Biotechnol. 3: 1-7.

Bancroft JD and Stevens A (1996): Theory and practice of histological techniques, Churchill Livingstone Press, Edinburgh, London, Melbourne, New York.

Bronte V and Pittet MJ (2013): The spleen in local and systemic regulation of immunity. Immunity. 39 (5): 806-818.

Cataldi M, Vigliotti C, Mosca T, Cammarota M and Capone D (2017): Emerging role of the spleen in the pharmacokinetics of monoclonal antibodies, nanoparticles and exosomes. Int J Mol Sci. 18 (6):1249.

Chanarin I, Cairns J, and Waters D (1973): Coulter blood count. J Clin Pathol. 26: 978.

Chung MK, Baek SS, Lee SH, Kim H, Choi K and Kim JC (2009): Combined repeated dose and reproductive/developmental toxicities of copper monochloride in rats. Environ Toxicol. 24: 315-26.

De Jong WH, De Rijk E, Bonetto A, Wohlleben W, Stone V, Brunelli A, Badetti E, Marcomini A, Gosens I and Cassee FR (2019): Toxicity of copper oxide and basic copper carbonate nanoparticles after short-term oral exposure in rats. Nanotoxicology. 13(1): 50-72.

Dey A, Manna S, Chattopadhyay S, Mondal D, Chattopadhyay D, Raj A, Das S, Bag B and Roy S (2018): Azadirachta indica leaves mediated green synthesized Copper oxide nanoparticles induce Apoptosis through activation of TNF-α and Caspases signalling pathway against cancer cells. J Saudi Chem Soc. 23:10.

Dipo A (2019): Lymphatic system 2. Available at: (URL: https://www.researchgate.net/publication/332353982_Lymphatic_system_2/citation/download). Access date [Aug (4th/2020)].

Dobrovolskaia MA, Shurin M and Shvedova AA (2016): Current understanding of interactions between nanoparticles and the immune system. Toxicol Appl Pharmacol. 299: 78-89.

El Bialy BE, Hamouda RA, Abd Eldaim MA, El Ballal SS, Heikal HS, Khalifa HK and Hozzein WN (2020): Comparative toxicological effects of biologically and chemically synthesized copper oxide nanoparticles on mice. Int J Nanomedicine. 15: 3827-3842.

Elkhateeb SA, Ibrahim TR, El-Shal AS, Abdel Hamid OI (2020): Ameliorative role

of curcumin on copper oxide nanoparticles-mediated renal toxicity in rats: An investigation of molecular mechanisms. J Biochem Mol Toxicol. 2020; e22593.

Elsabahy M and Wooley KL (2013): Cytokines as biomarkers of nanoparticle immunotoxicity. Chem Soc Rev. 42 (12): 5552-5576.

Ezealisiji KM, Siwe-Noundou, Krause RWM (2019): Copper Oxide Nano-Hydrogel Composite and their toxicology studies: A green chemistry approach. J Mater Sci Nanotechnol. 7(1): 105.

Griffitt RJ, Weil R, Hyndman KA, Denslow ND and Powers K (2007): Exposure to copper nanoparticles causes gill injury and acute lethality in zebrafish (Danio rerio). Environ Sci Technol. 41: 8178-8186.

Hanley C, Thurber A, Hanna C, Punnoose A, Zhang J and Wingett DG (2009): The influences of cell type and ZnO nanoparticle size on immune cell cytotoxicity and cytokine induction. Nanoscale Res Lett. 4(12):1409-1420.

Hassanen EI, Khalaf AA, Tohamy AF, Mohammed ER and Farroh KY (2019): Toxicopathological and immunological studies on different concentrations of chitosan-coated silver nanoparticles in rats. Int J Nanomedicine. 14: 4723-4739.

He H, Zou Z, Wang B, Xu G, Chen C, Qin X, Yu C, and Zhang J (2020): Copper oxide nanoparticles induce oxidative DNA damage and cell death via copper ion-mediated p38 mapk activation in vascular endothelial cells. Int J Nanomedicine. 15: 3291-3302.

Karlsson HL, Cronholm P, Gustafsson J and Möller L (2008): Copper oxide nanoparticles are highly toxic: a comparison between metal oxide nanoparticles and carbon nanotubes. Chem Res Toxicol. 21:1726-1732.

Khabbazi M, Harsij M, Hedayati SAA, Gholipoor H, Gerami MH and Ghafari FH (2015): Effect of CuO nanoparticles on some hematological indices of rainbow trout Oncorhynchus mykiss and their potential toxicity. Nanomed J. 2: 67-73.

Khan HA, Abdelhalim MAK, Alhomida AS and Al Ayed MS (2013): Transient increase in IL-1beta, IL-6 and TNF-alpha gene expression in rat liver exposed to gold nanoparticles. Genet Mol Res. 12(4): 5851-5857.

Ko JW, Shin NR, Park JW, Park SH, Lee IC, Kim JS, Kim JC, Ahn KS, Shin IS (2018): Copper oxide nanoparticles induce collagen deposition via TGF-β1/Smad3 signaling in human airway epithelial cells. Nanotoxicology. 12 (3): 239-250.

Ighodaro OM and Akinloye OA (2018): First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. Alex. J. Med. 54: 287-293.

Lee IC , Ko JW, Park SH, Lim J, Shin I, Moon C, Kim SH, Heo JD and Kim JC (2016 a): Comparative toxicity and biodistribution of copper nanoparticles and cupric ions in rats. Int J Nanomed. 11: 2883-2900.

Lee IC, Ko JW, Park SH, Shin NR, Shin I, Moon C, Kim JH, Kim HC and Kim JC (2016 b): Comparative toxicity and biodistribution assessments in rats following subchronic oral exposure to copper nanoparticles and microparticles. Part Fibre Toxicol. 13(1):56.

Li WT, Wang LY, Chang HW, Yang WC, Lo C, Pang VF, Chen MH and Jeng CR (2018): Th2 cytokine bias induced by silver nanoparticles in peripheral blood mononuclear cells of common bottlenose

dolphins (Tursiops truncatus). Peer J. 6: e5432. https://doi.org/10.7717/peerj.5432

Luo Y, Chang LW and Lin P (2015): Metal-Based Nanoparticles and the Immune System: Activation, Inflammation, and Potential Applications. Biomed Res Int. 2015: 12.

Maciel-Magalhães M, Medeiros RJ, Bravin JS, Patricio BFC, Rocha HVA, Paes-de-Almeida EC, Santos LMG, Jacob SC, Savignon TCM and Amendoeira FC (2020): Evaluation of acute toxicity and copper accumulation in organs of Wistar rats, 14 days after oral exposure to copper oxide (II) nano- and microparticles. J Nanopart Res. 22: 2.

Mitra S, Keswani T, Ghosh N, Goswami S, Datta A, Das S, Maity S and Bhattacharyya A (2013): Copper induced immunotoxicity promote differential apoptotic pathways in spleen and thymus. Toxicol. 306.

Mitra S, Keswani T, Dey M, Bhattacharya S, Sarkar S, Goswami S, Ghosh N, Dutta A and Bhattacharyya A (2012): Copper-induced immunotoxicity involves cell cycle arrest and cell death in the spleen and thymus. Toxicol. 293: 78-88.

Muhammad Q, Jang Y, Kang SH, Moon J, Kim WJ and Park H (2020): Modulation of immune responses with nanoparticles and reduction of their immunotoxicity. Biomater Sci. 8: 1490-1501.

Nagi MN, al-Bekairi AM and al-Sawaf HA (1995): Spectrophotometric assay for superoxide dismutase based on the nitrobluetetrazolium reduction by glucose-glucose oxidase. Biochem Mol Biol Int. 136 (3): 633-638.

Nemzek JA, Bolgos GL, Williams BA and Remick D G (2001): Differences in normal values for murine white blood cell counts and other hematological parameters based on sampling site. Inflamm Res. 50: 523-527.

Niska K, Santos-Martinez MJ, Radomski MW and Inkielewicz-Stepniak I (2015): CuO nanoparticles induce apoptosis by impairing the antioxidant defense and detoxification systems in the mouse hippocampal HT22 cell line: Protective effect of crocetin. Toxicol in Vitro. 29: 663-671.

Ohkawa H, Ohishi N and Yagi K (1979): Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 95: 351-358.

Petrarca C, Clemente E, Amato V, Pedata P, Sabbioni E, Bernardini G and DiGioacchino M (2015): Engineered metal based nanoparticles and innate immunity. Clin Mol Allergy. 13:13.

Safety data sheet according to Regulation (EC) Copper Oxide Nanoparticles. Sigma-Aldrich [Web page] [2020] Available at: (URL: https://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=EG&language=en&productNumber=544868&brand=ALDRICH&PageToGoToURL=https%3A%2F%2F. Access date [Jan (29th/2020)].

Salauze D, Serre V and Perrin C (1994): Quantification of total IgM and IgG levels in rat sera by a sandwich ELISA technique. Comp Haematol Int. 4:30-33.

Sang X, Zheng L, Sun Q, Li N, Cui Y, Hu R, Gao G, Cheng Z, Cheng J, Gui S, Liu H, Zhang Z and Hong F (2012): The chronic spleen injury of mice following long-term exposure to titanium dioxide nanoparticles. J Biomed Mater Res (Part A). 100: 894-902.

Shen CC, Wang CC, Liao MH and Jan TR (2011): A single exposure to iron oxide nanoparticles attenuates antigen-specific antibody production and T-cell reactivity

in ovalbumin-sensitized BALB/c mice. Int J Nanomedicine. 6: 1229-1235.

Sivakumar S, Velmurugan C, Ebenezer Jacob Dhas DS, Brusly Solomon A and Leo Dev Wins K (2020): Effect of nano cupric oxide coating on the forced convection performance of a mixed-mode flat plate solar dryer. Renew Energy. 155: 1165-1172.

Srikanth K, Pereira E, Duarte AC and Rao JV (2016): Evaluation of cytotoxicity, morphological alterations and oxidative stress in Chinook salmon cells exposed to copper oxide nanoparticles. Protoplasma. 253: 873-884.

Strauch BM, Niemand RK, Winkelbeiner NL and Hartwig A (2017): Comparison between micro- and nanosized copper oxide and water soluble copper chloride: interrelationship between intracellular copper concentrations, oxidative stress and DNA damage response in human lung cells. Part Fibre Toxicol. 14(1): 28.

Taha MN and Taha ST (2017): The effect of copper nanoparticles on liver function and some hematological parameters in rat. Int J Sci Res (IJSR) ISSN (Online): 2319-7064. Index Copernicus Value (2015): 78-96.

Tang H, Xu M, Luo J, Zhao L, Ye G, Shi F, Lv C, Chen H, Wang Y and Li Y (2019): Liver toxicity assessments in rats following sub-chronic oral exposure to copper nanoparticles. Environ Sci Eur. 31: 30.

Wang D, Lin Z, Wang T, Yao Z, Qin M, Zheng S and Lu W (2016): Where does the toxicity of metal oxide nanoparticles come from: the nanoparticles, the ions, or a combination of both? J Hazard Mater. 308:328-334.

Yang H, Liu C, Yang DF, Zhang HS and Xi ZG (2009): Comparative study of cytotoxicity, oxidative stress and genotoxicity induced by four typical nanomaterials: the role of particle size, shape and composition. J Appl Toxicol. 29: 69-78.

Zhou X, Zhao L, Luo J, Tang H, Xu M, Wang Y, Yang X, Chen H, Li Y, Ye G, Shi F, Lv C and Jing B (2019): The toxic effects and mechanisms of nano-cu on the spleen of rats. Int j mol sci. 20(6): 1469.

Zhu J. (2015): T helper 2 (Th2) cell differentiations, type 2 innate lymphoid cell (ILC2) development and regulation of interleukin-4 (IL-4) and IL-13 production. Cytokine. 75(1): 14-24.

Zolnik BS, González-Fernández A, Sadrieh N and Dobrovolskaia MA (2010): Nanoparticles and the immune system. Endocrinol. 151:45

الملخص العربي

التأثير السام الناتج عن الإجهاد التأكسدي والسمية المناعية لجزيئات أكسيد النحاس متناهية الدقة على طحال الجرذان البيضاء البالغة

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أدى الاستخدام المتزايد لجزيئات أكسيد النحاس متناهية الدقة في مختلف المجالات إلى زيادة خطر التعرض البشري والبيئي لهذه الجزيئات. يلعب الطحال دورًا هاما في كل من المناعة الفطرية والتكيفية للجسم فيما يمثل هدفًا محتملاً لسمية الجزيئات متناهية الدقة. ولذلك أجريت هذه الدراسة لفحص التأثير المناعي الناتج عن التعرض الفموي شبه الحاد لجزيئات أكسيد النحاس متناهية الدقة على طحال ذكور الجرذان البيضاء البالغة. تم تقسيم ثلاثين جرذا إلى مجموعتين رئيسيتين. المجموعة (I) الضابطة التي تم تقسيمها إلى مجموعتين (10 فئران لكل منهما): (IA) مجموعة ضابطة سالبة و (IB) مجموعة ضابطة موجبة . أما المجموعة الثانية تم اعطائها 125مجم / كجم من جزيئات أكسيد النحاس متناهية الدقة . تم إعطاء العلاج بواسطة انبوب عن طريق الفم مرة واحدة يوميا لمدة 28 يومًا. في نهاية التجربة، تم استخدام عينات الدم المسحوبة في انابيب تحتوي على إديتا لقياس عدد كريات الدم الحمراء، نسبة الهيموجلوبين، الصفائح الدموية، عدد خلايا الدم البيضاء، وعدد الكريات البيضاء التمييزي (العدلات، الوحيدات، والخلايا الليمفاوية). تم تحليل عينات المصل لتحديد المستوى الإجمالي للأجسام المناعية (IgG وIgM). بعد ذلك ، تم استئصال الطحال لقياس مستوى المالوندهايد، انزيم السوبر اوكسيد دسميوتيز ، عامل نخر الورم -ألفا ، و انترلوكين-4. كما تم تحديد نسبة الخلايا الليمفاوية الفرعية في الطحال (CD4+,CD8+, CD19+)وكذلك فحص نسيج الطحال بالميكروسكوب الضوئي. أظهرت نتائجنا أن جزيئات أكسيد النحاس متناهية الدقة أدت إلى انخفاض ذات دلالة إحصائية في عدد كرات الدم الحمراء ، نسبة الهيموجلوبين ، الصفائح الدموية ، عدد خلايا الدم البيضاء ، ونسبة الخلايا الليمفاوية ، و زيادة ذات دلالة إحصائية في نسبة العدلات والوحيدات. كما تم الكشف عن انخفاض ذات دلالة إحصائية في إجمالي الاجسام المناعية (IgG وIgM) في الدم. علاوة على ذلك ، أظهر طحال المجموعة المعالجة بجزيئات أكسيد النحاس متناهية الدقة ارتفاعًا ذات دلالة إحصائية في مستوى المالوندهايد ، عامل نخر الورم -ألفا والخلايا الليمفاوية (CD8+). وفي الوقت نفسه ، انخفض مستوى انزيم السوبر اوكسيد دسميوتيز, انترلوكين-4 والخلايا الليمفاوية (CD4 + و CD19+) و نسبة CD4+ / CD8+ بشكل ملحوظ . و قد أستدل على تلف انسجة الطحال من خلال انخفاض عدد وخلوية البصيلات الليمفاوية ، وكذلك احتقان اللب الأحمر. واخيرا فيمكن استنتاج أن الوظيفة المناعية للطحال قد تأثرت سلبًا بجزيئات أكسيد النحاس متناهية الدقة على الاغلب بسبب آلية الإجهاد التأكسدي والالتهاب.