Special Issue:

Veterinary Medicine between Sustainable Development and Public Health to Confront Global Changes

Maternal Copper Oxychloride Exposure Induces Immunological Alterations and Oxidative Damage in Male Rats Offspring

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Abstract | Copper oxychloride is a fungicide that preserving the freshness of fruits and vegetables in supermarkets for extended durations and can result in various illnesses. Consequently, consumers and agricultural laborers may encounter fungicide residues in food and water through oral ingestion, inhalation, and skin exposure. However, there have been only a limited number of research investigating the impact of prenatal exposure to COC on the animal's immune system. Therefore, three groups of pregnant rats, each consisting of 10 rats, were assigned. The 1st group was the control group, while the other two groups were administered doses 36.75 mg and 73.5 mg of COC from the 6th day of gestation until the 20th day of gestation. The immune system of the male progeny was assessed detecting the levels of interleukins (IL-2 and IL-6) and immunoglobulins (IgG and IgE). The total antioxidant capacity (TAC) and total oxidative status (TOS) were assessed. The spleen and thymus were preserved for histopathological and ultra structural analysis. The male offsprings, who were exposed to low and high COC levels throughout their mothers' pregnancy, statistically P≤0.05 exhibited a significant elevation in IL-2, IL-6, IgG, IgE and TOS levels in comparison to the control rats. On the opposite hand, IL-4 and TAC showed reduction in their level. Rat pups of the mothers that were given 73.5mg COC exhibited a statistical P≤0.05 increase in lymphocytes accompanied with hypertrophy in the white pulp of the spleen. The results suggested that when pregnant rats are exposed to moderate levels of COC, their offspring experience changes in their immune system and oxidative damage, which may be shown through alterations in the structure and function of the spleen. The immunologic alteration may be due exposure to pregnant rats to high concentration of copper in the COC treatment that transported to their male neonates and induced oxidative damage.

Keywords: Copper oxychloride, IgE, IgG, Spleen, TOS

Received | September 01, 2024; **Accepted** | October 11, 2024; **Published** | October 26, 2024
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Citation | Soliman MTA, Shosha HM, Ebaid HM, El-Hak HNG, Abdelrazek HMA (2024). Maternal copper oxychloride exposure induces immunological alterations and oxidative damage in male rats offspring. Adv. Anim. Vet. Sci. 12(s1): 284-293.
DOI | https://dx.doi.org/10.17582/journal.aavs/2024/12.s1.284.293
ISSN (Online) | 2307-8316; **ISSN (Print)** | 2309-3331

INTRODUCTION

Fungicides, broadly used in industry and agriculture to rheostat fungal diseases, have raised concerns about their potential impacts on human and animal health. They are chemical compounds used to control fungal diseases in agriculture and horticulture (Dhanasekaran, 2014). Studies have shown that fungicides can be teratogenic,

carcinogenic, mutagenic, and toxic to various organ systems (Oruç, 2010; Preeti *et al*., 2015). Their development has progressed through multiple generations, from early inorganic compounds to modern systemic fungicides with specific modes of action (Saha, 2023). While fungicides are designed to control fungal growth (Dhanasekaran, 2014), their use can have unintended consequences. Copper oxychloride (COC), one of the widely used fungicide in vineyards, has been shown to have significant ecological and toxicological effects. In human lymphocytes, COC exhibited genotoxic and cytotoxic potential, increasing chromosome aberrations, micronuclei formation, and altering genomic stability (Bayram *et al*., 2016). COC caused postnatal toxicity in lactating females rats and their offspring, affecting biochemical markers and organ histology (Abomosallam *et al*., 2020). Also, in mice resulted in immunotoxicity, evidenced by changes in thymus and spleen weights, cell death, and altered gene expression (Mitra *et al*., 2012).

The immunoprotected impact of interleukin-2 (IL-2) is demonstrated by its stimulation of immune cells and immune factor expression, which also improves the immunological response (Schijns *et al*., 1994). Interleukin-4 (IL-4) is a cytokine that controls humoral and adaptive immunity and increases B cell secretion (Ryan, 1997). Numerous investigations have demonstrated elevated IL-2 and IL-4 expression in the peripheral blood of immunocompromised patients (Vinante *et al*., 1999).

Information on the influences of COC has been enlarged recently due to its worldwide use. However, there have been few studies handling prenatal COC exposure on the immunologic changes. Prenatal exposure to environmental toxicants, including pesticides and fungicides like COC, has been shown to have profound effects on the developing immune system (Holladay and Smialowicz, 2000). The immune system undergoes critical stages of development during gestation, and exposure to harmful substances during this time may disrupt normal immune system programming, potentially leading to long-term immune dysfunction or increased susceptibility to disease later in life. Fetal and neonatal immune systems are more vulnerable to external insults, as they are not fully developed, and their immune responses are still maturing (Goenka and Kollmann, 2015). Disruptions in this early developmental phase can lead to altered immune function, including immunosuppression, autoimmunity, or enhanced allergic responses. Thus, investigating prenatal exposure to COC is important to determine how it may affect immune system maturation and how these changes may manifest in adulthood (Shimizu *et al*., 2023). Fungicides, including COC, are widely used in agricultural settings, and their potential to impact human and animal health has raised concerns, especially considering pesticide residues in food, water, and air. While fungicides are designed to target fungal organisms, many have been found to have off-target effects on human and animal physiology particularly in terms of endocrine disruption and immune system modulation (Zubrod *et al*., 2019).

In order to signify the present study, it is important to contextualize the potential immunotoxic effects of COC within the broader literature on fungicides and their impacts on the immune system. Many other fungicides have been investigated for their toxic effects on immune function, and drawing comparisons between COC and these compounds can provide important insights into the potential risks of COC exposure. Triazole fungicides have been shown in studies to have strong immunosuppressive effects, such as lowering the production of immune cells, changing the way cytokines are made, and lowering antibody responses (Martínez-Matías and Rodríguez-Medina, 2018). These chemicals, commonly used in agricultural settings, have been shown to affect both humoral immunity (e.g., antibody production) and cell-mediated immunity (e.g., T-cell responses) (Antachopoulos and Roilides, 2005). Propiconazole has been shown to change T-cell responses and lower the ability of macrophages to eat other cells, especially in newborn rats. This shows that fungicide exposure during pregnancy can affect the development of the immune system (Lima *et al*., 2022). Chlorothalonil is another common fungicide that has been shown to change rats immune systems by lowering the number of T cells in the spleen and raising the levels of inflammatory cytokines like IL-6 and TNF-α. These alterations in immune function are important for understanding how prenatal exposure to fungicides may disrupt normal immune system development (Cestonaro *et al*., 2022). Mancozeb is a widely used fungicide. It has also been linked to changes in the immune system, such as less antibody production and weaker T-cell responses, in studies done before and after birth. Research on mancozeb has demonstrated that it can alter the cytokine profiles of exposed individuals, leading to an imbalance between pro-inflammatory and anti-inflammatory responses (Corsini *et al*., 2013). Many fungicides, including those mentioned above, have been shown to cause immune dysregulation, leading to either immunosuppression or paradoxically immune hyperactivity (e.g., increased production of pro-inflammatory cytokinesautoimmune-like responses).

Based on previous literatures, the influence of COC on immunity should be matched. Investigating the immunotoxicity of COC during prenatal exposure is crucial for understanding how these chemicals may affect health across the lifespan. Although there is growing interest in the effects of chemical exposures on the immune system, there is a limited amount of research specifically examining the immunotoxicity of COC. While studies on pesticides and

fungicides are becoming more common, COC, a copper-based fungicide, has not been as thoroughly studied for its impact on the immune system, especially during critical windows of development like prenatal exposure. This research gap warrants investigation, as it will help identify potential health risks associated with common fungicides that may be overlooked in existing risk assessments. This study posits that male rats exposed to COC at levels below lethal levels will have changes in their immune systems, including changes in the types of immune cells they have, problems with how their immune systems respond to challenges and immune system problems caused by oxidative stress. These expected changes are consistent with findings from previous studies on copper-based fungicides and other environmental toxicants and fungicides. By testing this hypothesis, the study aims to understand better the potential prenatal immunotoxic effects of COC exposure in a real-world context of environmental and occupational exposure. Consequently, this study aimed to investigate the influence of prenatal exposure of COC on the immunologic change and oxidative stress of the male rats' offspring through determination of IL-4, IL-2, IL-6, immunoglobulins (Ig G and Ig E), total antioxidant capacity (TAC) levels, total oxidative status (TOS), and examination of histopathological changes of the thymus, and spleen. In addition to the ultrastructure of the spleen.

MATERIALS AND METHODS

Chemicals

The commercial copper COC consists of 95% copper by weight. The substance is a pale green, finely textured powder with a smooth consistency. It consists of soft clusters and was acquired from the Central Agricultural Pesticide Laboratory, ARC, in Egypt. The chosen doses were determined based on the observed LD_{50} of COC in rats, which was found to be 1470 mg/kg body weight (Bayram *et al*., 2016).

Animals

A total of 40 rats, consisting of 30 females and 10 males, were got from the animal house at the Faculty of Veterinary Medicine, Suez Canal University, Egypt. The rats had an average weight of 155±10g. Prior to the experiment, metallic cages containing rats (5 each cage) were kept for a duration of 2 weeks for acclimatization. The cages were floored using wood shavings. The rats are kept in a controlled environment with a temperature range of 26˚C (±1˚C) and a relative humidity 55±5%. They were exposed to natural daylight. The animals were given unrestricted access to both water and food. Ethical approval for this study was obtained from the Faculty of Science, Port Said University, Egypt (PSU.Sci.68). The ethical considerations for this study were robustly addressed at every research stage. Special care was taken to minimize harm to both the pregnant dams and their developing offspring, particularly about the potential impacts of COC exposure on fetal development. Ethical approval was granted based on a clear scientific rationale, a commitment to minimizing suffering, and ongoing monitoring to ensure that the animals were treated by established ethical standards. The potential implications for public health, especially about prenatal pesticide exposure, justify the moral justification for the study while still upholding the principle of humane treatment throughout the experimental process. Each female undergone daily collection of vaginal smears to check the regularity of the estrous cycle (Ekambaram *et al*., 2017). Only females in the regular cyclic state and specifically in the proestrus stage have been selected to be introduced to a male for the purpose of mating. The ratio of males to females will be maintained at a rate of 1 male for every 3 females. The zero day of pregnancy is established by the existence of spermatozoa in a vaginal smear.

Experimental design

A total of thirty pregnant females were aligned into 3 groups, with ten animals in each group. The number of animals was calculated according to the requirement of the ethical committee by resource equation method (Arifin and Zahiruddin, 2017). The rats were treated with varying doses of COC from the 6th day of gestation (GD6) to the 20th day of gestation (GD20). Pregnant females are categorized into three groups: Group I consisted of a control group that was administered carboxymethyl cellulose (CMC) by gavage. Group II: The high COC group was administered a dose of COC equivalent to 1/20 LD_{50} (73.5 mg/kg Bwt) of COC in CMC via gavage. Group III: The low COC group was administered a dose of COC equivalent to an 1/40 LD_{50} (36.75 mg/kg Bwt) of COC in CMC via gavage. The selection of 1/20 and 1/40 of the LD_{50} of COC was based on a combination of factors: Ensuring the doses were high enough to elicit measurable immune responses while still within the range of environmentally relevant and occupationally plausible exposures according to Epa (2009).

Throughout the testing period, all experimental animals were consistently observed daily for any indications of poisoning. This experiment utilized exclusively male progenies, and female offspring were excluded and assigned for another experiment. Females tend to have stronger innate and adaptive immune responses than males, potentially due to the influence of estrogen and other sex hormones, and females show more pronounced immune changes or reactions due to hormonal influences (Dunn *et al*., 2024). Following the 30-days weaning phase, the rats were euthanized by tetrahydrofuran inhalation anesthesia.

Blood and tissue preparations

Blood samples of rats offspring were obtained from eye venous plexus that was placed retroorbital (Sanford, 1954) under effect of tetrahydrofuran inhalation anesthesia into two parts. One part of blood with anticoagulant (EDTA) was castoff for the hematological parameters estimation, and a second part was got without anticoagulant to obtain sera. The sera were isolated and stored at a temperature of -20°C for biochemical analyses. The thymus and a large portion of the spleen were treated with neutral 10% formalin for fixation, then preserved in 70% ethyl alcohol and prepared as paraffin sections. The remaining smaller portions of the spleen were preserved in glutaraldehyde for the purpose of preparing ultrastructural samples.

Hematological parameters

The blood samples in EDTA were subjected to manual routine hematological examination of red and white cells count (RBCs and WBCs), hemoglobin (Hb) and differential white cells count according to Washington and Van Hoosier (2012).

Biochemical analysis

All serum biochemical assays were accomplished with specific enzyme-linked immunosorbent assay (ELISA) commercial kits Jingkang Biological Engineering Co., Ltd. (China) and high-purity reagents according to the manufacturer's instructions. The serum cytokines levels of IL-2, IL-6 and IL4 were estimated according to Gu *et al.* (2018) and Nonogaki *et al.* (1995), respectively. The TAC and TOS were determined according to Herken *et al.* (2009). Immunoglobulins; IgG and IgE were determined according to Bazin *et al.* (2017).

Histopathological and ultrastructure preparation

The paraffin sections of the thymus and spleen ready for histopathological assessment were imperiled to Hematoxylin-Eosin-stained (Drury and Wallington, 1980). The small samples of spleen were prepared according to Gad El-Hak *et al.* (2024) and examined using a transmission electron microscope (JEOLJEM-2100, Japan).

Statistical analysis

The data was subjected to one-way analysis of variance (ANOVA) followed by Duncan's test (IMB-SPSS version 28.0 for Mac OS) to compare the mean values obtained from the different groups (Knapp, 2017). The data were expressed as the mean ± standard error (SE), and statistical significance was regarded as a P-value less than 0.05. The sample size was denoted as n.

RESULTS AND DISCUSSION

The administration of high and low doses of COC to pregnant dams induced statistical (P = 0.000) reduction in RBCs and WBCs count in male offspring. The high group males were statistically (P = 0.000) lower in RBCs count than the low ones however, there was non-significant variation between high and low group males WBCs count. The Hb level of high group males revealed significant reduction (P = 0.001) than control. The low group revealed non- significant non- significant variation when compared to control and high groups. Monocytes and basophils percent revealed non-significant variation (P = 0.871 and P = 0.088, respectively) among tested groups. Eosinophils demonstrated statistical (P = 0.000) promotion in high and low COC treated groups than control with high value in the high COC group (P = 0.000) in comparison to the low one. Neutrophils were statistically (P < 0.05) amplified in high COC group than the control and low groups. However, lymphocytes revealed statistical (P = 0.000) decline in high and low groups with lesser value in the high group (P = 0.000) as shown in Table 1.

Table 1: Effects of COC on the hematology parameter of the male weaned male rats.

Parameters	Control group	Low dose COC	High dose COC
RBCs(10^3/µL)	5.63^{a}±0.50	3.506^{b}±0.3	3.68^{b}±0.3
Hb (g/100 mL)	13.25^{a}±0.50	12.68^{ab}±0.6	11.26^{b}±0.5
WBCs (10^3/µL)	4.55^{a}±0.30	2.58^{b}±0.30	2.26^{bc}±0.20
Monocytes (%)	4.6^{ab}±0.40	5.20^{ab}±0.30	6.10^{a}±0.40
Eosinophils (%)	3.48^{c}±0.20	4.10^{b}±0.30	7.71^{a}±0.60
Neutrophils (%)	29.8^{b}±1.10	32.80^{b}±1.90	37.0^{a}±1.00
Basophils (%)	0.55±0.20	0.55±0.30	0.36±0.20
Lymphocytes (%)	61.10^{a}±1.40	55.31^{b}±0.80	48.60^{c}±1.60

Data was expressed as means ± SEM, n=6 per sex. Data was statically analyzed using One-way ANOVA followed by Duncan multiple comparisons test $p \leq 0.05$. Different letters showed data of the same row which is statistically significant $p \leq 0.05$. High dose COC group: maternal rats was given by gavage 73.5 mg/kg body weight copper oxychloride (COC) from the 6th day of gestation (GD6) to the 20th day of gestation (GD20) and low dose COC group rats were given 36.75 mg/kg body weight COC from the 6th day of gestation (GD6) to the 20th day of gestation (GD20).

Biochemical analysis

Analysis revealed that IL-2, IL-6, IgE, and TOS levels were statistically elevated in the two COC administered groups in comparison to the control group (P = 0.000). IgG was statistically (P = 0.000) low in high COC maternally treated males than control and low group. Furthermore, the levels of IL-4 and TAC were reported to be considerably (P = 0.000) lower in the two groups treated with COC than the control group as shown in Table 2. The high COC group revealed a statistical (P = 0.000) variation than the low one in all tested parameters.

Table 2: Effects of copper oxychloride (COC) maternal exposure on the immunological parameters and oxidative status of the male weaned offspring.

Parameters	Control group	Low dose COC	High dose COC
IL-2 (pg/mL)	127.40^{c}±0.50	144.26^{b}±0.50	190.95^{a}±0.90
IL-4 (pg/mL)	111.26^{a}±0.90	100.33^{b}±0.90	82.04^{c}±0.30
IL-6 (pg/mL)	6.226^{c}±0.01	6.79^{b}±0.050	8.18^{a}±0.02
IgG (ng/mL)	325.30^{a}±1.20	324.50^{ab}±1.00	320.60^{c}±0.50
IgE (ng/mL)	117.70^{c}±2.60	141.30^{b}±1.10	174.11^{a}±1.00
TAC (nmol/mL)	2.60^{a}±0.014	2.37^{b}±0.009	2.20^{c}±0.008
TOC (nmol/mL)	0.84^{c}±0.006	0.99^{b}±0.008	1.12^{a}±0.002

Data was expressed as means ± SEM, n=6 per sex. Data was statically analyzed using One-way ANOVA followed by Duncan multiple comparisons test $p \leq 0.05$. Different letters showed data of the same row which is statistically significant $p \leq 0.05$. (interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), immunoglobulin G (IgG), immunoglobulin E (IgE), total antioxidant capacity (TAC), total oxidative capacity (TOC), high dose COC group: maternal rats was given by gavage 73.5 mg/kg body weight copperoxychloride (COC) from the 6th day of gestation (GD6) to the 20th day of gestation (GD20) and low dose COC group rats were given 36.75 mg/kg body weight COC from the 6th day of gestation (GD6) to the 20th day of gestation (GD20).

Histopathological and ultrastructure preparation

The H & E spleen-stained sections of the control group and low exposure group with COC exhibited the typical structure of the spleen, characterized by the existence of clearly defined red and white pulp Figure 1a, b. The H&E spleen-stained sections of higher exposure dose of COC showed hyperplasia of lymphoid follicles forming white pulp and clearly defined red pulp Figure 1c.

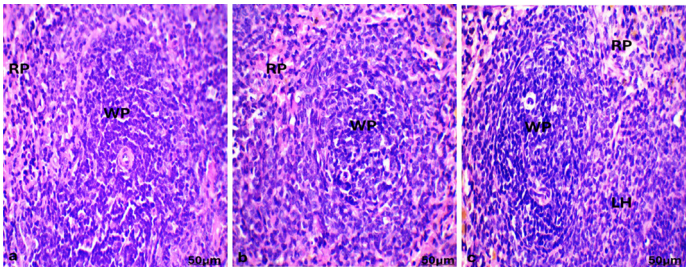

Figure 1: Photomicrograph of spleen of male albino rat offspring from (a) control rats (b) Low dose COC group in which dams rats were given 36.75 mg/kg body weight COC from the 6th day of gestation (GD6) to the 20th day of gestation (GD20) showed normal histological structure of the white pulp (WP) area and the red pulp (RP). (c) High dose COC group: maternal rats were given by gavage 73.5 mg/kg body weight copperoxychloride (COC) from the 6th day of gestation (GD6) to the 20th day of gestation (GD20) showed hyperplasia of the lymphocyte surrounded the whit pulp (WP) and normal histological structure of the red pulp (RP) (X400).

The H & E spleen-stained sections of the control group, low exposure group with COC and higher exposure group to COC exhibited typical structure of the thymus characterized by the presence of clearly defined cortex and medulla (Figure 2).

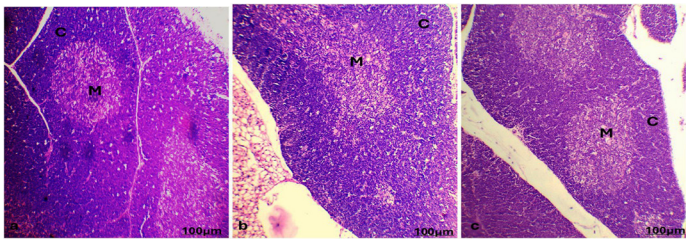

Figure 2: Photomicrograph of thymus of male albino rat offspring from (a) control rats (b) Low dose COC group in which dams rats were given 36.75 mg/kg body weight COC from the 6th day of gestation (GD6) to the 20th day of gestation (GD20) (c) High dose COC group: maternal rats were given by gavage 73.5 mg/kg body weight copper oxychloride (COC) from the 6th day of gestation (GD6) to the 20th day of gestation (GD20) showed normal histological structure of the thymus cortex (C) and medulla (M). (X 100).

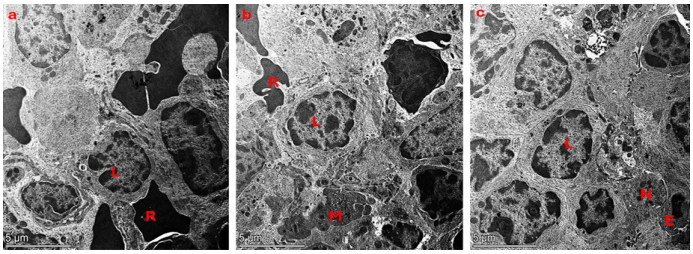

Figure 3: An electron micrograph of the spleen from (a) the control group showed normal ultrastructure of multiple lymphocytes of nuclei with chromatin that are homogenously distributed (L). The red pulp is lined by endothelial cells with red blood cells (R) between them. (b) Low dose COC group in which dam's rats were given 36.75 mg/kg body weight COC from the 6th day of gestation (GD6) to the 20th day of gestation (GD20) showed normal ultrastructure of multiple lymphocytes of nuclei with chromatin that homogenously distributed (L) and macrophage (M). The red pulp is lined by endothelial cells with red blood cells (R) between them. (c) High dose COC group: maternal rats were given by gavage 73.5 mg/kg body weight copper oxychloride (COC) from the 6th day of gestation (GD6) to the 20th day of gestation (GD20) showed normal ultrastructure of multiple lymphocytes of nuclei with chromatin that homogenously distributed (L), neutrophils (N) and eosinophils (E). The red pulp is lined by endothelial cells with red blood cells (R) between them.

Ultra-structural examination of the spleen of male albino rat offspring from the control and treated dam rats revealed normal ultrastructure of multiple lymphocytes (Figure 3). Regarding the lower dose group of COC showed

megakaryocytes (Figure 3B). Moreover, the higher dose of COC showed neutrophil and eosinophil cells infiltration (Figure 3C).

The immune system is an intricate system consisting of immune organs, immune cells, and immunological reactive substances that participate in the immune rejection of foreign infections and the elimination of foreign agents (Ahmad *et al*., 2022). Environmental pollution exposure might heighten the likelihood of disturbing the balance of the immune system and leading to pathological conditions in immune function (Suzuki *et al*., 2020).

The COC has been used globally as antifungal with several toxicity outcomes (López-Prieto *et al*., 2023). It is widely recognized that copper has the ability to pass through the placenta of a pregnant mother (McArdle *et al*., 2008). The offspring from the groups exposed to higher and lower dosages of COC exhibited a disturbance in the amount of white blood cells, lymphocytes and erythropoiesis (RBCs and Hb). The present finding according to Tomaszewska *et al*. (2022) depend on the fact that there was a disruption in pluripotent stem cell differentiation into all blood cell types and in the repopulation of cells in the bone marrow and spleen. The increases of neutrophil agreed with Al-Naimi *et al*. (2013) who observed a promotion in neutrophils in rats orally exposed to high copper dose in the form of copper sulphate.

The changes in haematological parameters can be directly linked to immune system alterations: variations in WBCs count particularly those of neutrophils and lymphocytes, indicate immune activation, which could imply that COC exposure causes an inflammatory response, possibly by activating the innate immune system. Following COC exposure, immune suppression (shown by drops in WBC counts, particularly lymphocytes, or changes in platelet counts) may indicate downregulation of immune function, potentially increasing the susceptibility of the animals to infections or lowering their capacity to mount an adaptive immune response (Ekiz *et al*., 2005).

The present study showed COC-induced changes in Hb which may lead to the progress of microcytic anemia (Jain and Kamat, 2009). The decline in RBCs number noted in the neonates of the exposure groups in the present study could be outcome of a further mechanism intricated in the action of COC in-inducing impairments in bone marrow function (Travlos, 2006). Lymphocyte revealed a statistical decrease in the neonates from treated groups with COC. The later neutropenia may be a hallmark for immunosuppression (Naeim *et al*., 2008). Moreover, the observed neutrophilia and lymphopenia with COC prenatal exposure may suggest glucocorticoids overproduction in a stressful condition (Boes and Durham, 2017). The COC induced an increased in the eosinophilic count which is an indicator of the allergic reaction that may be inherited from their mother (Wardlaw *et al*., 2000).

The immune system is susceptible to oxidative stress during prenatal development, a period of critical immune cell differentiation and maturation (Martini *et al*., 2023). Exposure to COC could disrupt this process by causing oxidative damage to developing immune cells, which may result in immune dysregulation later in life. Oxidative stress can impair the proliferation, differentiation, and function of immune cells, including T-cells, B-cells, and regulatory T-cells (Tregs), all essential for normal immune function. This could lead to an imbalanced immune response and reduced immune tolerance, making individuals more susceptible to autoimmune diseases or allergies (Wójcik *et al*., 2021). COC induced oxidative stress could likely interfere with immune signaling pathways like NF-KB, MAPK, and JAK-STAT. These pathways control how immune cells work and how many cytokines they make. This disruption could explain the changes in the study's IL-6, TNF-α, and IL-4 levels, shifting the immune system toward a more pro-inflammatory phenotype (Chen *et al*., 2018) this was similar to results of obtained by Fu *et al*. (2024). Moreover, prenatal exposure to COC can cause oxidative damage that can affect immune memory, autoimmune susceptibility, and allergic diseases in later life. Disruptions in immune system development could impair responses to infections and vaccines (Wójcik *et al*., 2021; Martini *et al*., 2023). The existing study explored the prenatal COC exposure on immunological response of male albino rats offspring via determination of IL-2, IL-4, IL-6, IgG, Ig E, TOS and TAC levels beside the histopathological inspection of the thymus and spleen. There is general agreement that COC treatment can be harmful to adults, however the influence of prenatal exposure of it has not been deliberated. The reduction in IL-4 and TAC observed in the study could indicate immune suppression and increased oxidative stress following COC exposure. The drop in IL-4 points to a possible shift away from a balanced Th2-type immune response. This could make it harder for the body to mount a strong antibody-mediated immune response (Chtanova and Mackay, 2001; Segerstrom and Miller, 2004). Meanwhile, the reduction in TAC points to elevated oxidative stress, which can negatively affect immune cell function and contribute to chronic inflammation and immune system dysregulation (Chokkalingam *et al*., 2013).

As both mediators of the immune response and destroyers of cells expressing certain antigens, T lymphocytes are essential components of the immune system (Magez *et al*., 2020). The present research aimed to explore the impact of COC exposure on different cytokine profiles. The Th1/Th2 paradigm is established as crucial for maintaining proper immune function (Kidd, 2003). Th1 cells show a

vital part in the immunological response against infections, while Th2 cells are recognized for their significance in the humoral immune response and their contribution to the production of antibodies by B cells (Harris and Gause, 2011). Th1 cells release IL-2 which suppress the Th2 immune responses, whereas Th2 cells release IL-4 which regulate the Th1 immune responses (Romagnani, 1999). Deviation from the normal equilibrium of Th1 and Th2, can result in immunological dysfunction (Romagnani, 2004). T lymphocytes that have been stimulated can generate IL-4. The later immune cytokine-4 (IL-4) is a crucial determinant of lymphocytes survival in the adaptive immune system. It is also implicated in the progress of antibody-mediated autoimmune disorders (Fitzgerald *et al*., 2001). The results of this investigation demonstrated that exposure to COC greatly decreased the concentrations of IL-4. In contrast, the serum IL-2 and IL-6 levels of rat's exposure to their mother with COC showed significant increase compared to the control rats.

Studies have declared that IL-6 acts a crucial part in controlling the equilibrium between Th17 cells and Treg cells (Samson *et al*., 2012; Tao *et al*., 2019). Significant increase in IL-6 levels following treatment with COC indicated that exposure to COC may lead to an imbalance of Th17/Treg cells, so increasing the immunological and inflammatory response. Collectively, these findings indicate that dam exposure to COC may disturb the equilibrium of these cytokines, providing evidence that COC has an immunotoxic impact on rats by increasing the activity of immune cells. Dysregulation of cytokines levels in the blood and the abnormal equilibrium of T lymphocytes can result in impaired immunological activity (Elenkov *et al*., 2005).

The normal histological architecture of the spleen and thymus, functioning as a lymphoid organ, is crucial for maintaining effective infection management by the immune system (Elmore, 2012). This structure enables sufficient rummaging of pathogens and activation of adaptive immune system (Mešťanová and Varga, 2016). The chief constituent of COC is copper. The former is recognized as troublesome toxins, its accretion in the body results in alteration in the spleen and thymus as described by Mitra *et al*. (2013). This study investigated the histological changes in rat spleens and thymus gland following treatment their dams with COC in order to advance a deeper comprehension of the pathological changes. Regarding the spleen of neonates from treated dams with high dose of COC showed hyperplasia of white pulp lymphocytes of the spleen with disappearance of the demarcation line between the white pulp and the red pulp. Such results were in harmony with Al-Naimi *et al*. (2010). The infiltration of lymphocytes into red pulp according to Amer (2022) may be due to the elevation of the intracellular free calcium levels by copper. Additionally, copper enhanced the Ca ATPase activity, resulting in disturbances in cell-cell and cell matrix adhesion. Consequently, this led to the infiltration of lymphocytes. The hypertrophy of the white pulp in the spleen observed in this study could have various immunological implications, depending on whether it represents acute immune activation or chronic immune dysregulation (Lewis *et al*., 2019). At first, it suggests an immune response to the COC exposure, possibly as part of an adaptive immune reaction to neutralize the toxicant. However, if the hypertrophy is persistent, it could signal chronic immune activation, leading to immune exhaustion, autoimmunity, or other forms of immune dysregulation (Lewis *et al*., 2019).

The ultrastructure examination of the spleen of neonates from treated dams with lower dose of COC showed macrophage cell. Macrophages are large leukocytes with dual roles of phagocytosis and antigen presentation, crucial for both innate and adaptive immune responses. The primary function of T-helper cells is to facilitate the immune response of adaptive type. Following antigen identification and recruitment of Th cells, naive Th cells undergo activation and differentiation into cells with the ability to secrete cytokines, therefore enhancing and directing the immune response (Calder and Sonnenfeld, 2017).

The ultrastructure finding of the spleen from the high dose exposure of COC showed neutrophils and eosinophils which may be mirrors the cell damage of the organs owing to decreased level of antioxidants and increased oxidative stress that were consistent with Abd El-Rahman *et al*. (2020) who ascribed these variations of the spleen to the oxidative stress injury. Increased TOS and decreased TAC were demonstrated in COC administered groups of the herein study. Gad El-Hak and Mobarak (2020) found that COC encouraged oxidative damage. Evidence displays that reactive oxygen species (ROS) indirectly or directly subsidize to copper toxicity machineries (El-Hak and Mobarak, 2019).

The present study showed exposure to the dam during pregnancy to COC decreased the level of IgG. The herein result was consistent with (Hasssnin, 1999) who remarked the protein synthesis inhibitory effect of COC that led to reduction of IgG and other immunoglobulins. The IgG is responsible for passive immunity and plays a substantial role in regulation of B cells mediated humeral immunity (Niewiesk, 2014) therefore, the role of COC in suppressing humeral immunity is suggested. IgG can pass through placenta of dams to their offspring and in dams milk (Palmeira *et al*., 2012; Mimoun *et al*., 2020) that may be suggestive to COC reducing influence on maternal IgG where the half-life of IgG was assessed to be seven days

in rats (Niewiesk, 2014). The IgE level in the neonates demonstrated significant elevation in COC groups that are suggestive to allergic or immunosuppressive responses (Hashim, 2024) in offspring. This came in harmony with the observed eosinophilia.

CONCLUSIONS AND RECOMMENDATIONS

It was found that COC induced health problem to the male offspring with the most toxic influences on the immune system. Prenatal exposure to COC produced immune-toxic consequence on the spleen as it caused loss of normal histological construction of the spleen and also it produced an increase in the lymphocytes, neutrophils, and monocytes with amplification of the levels of TOS, IL-2, IL-6, Ig E and decreased the level of IL-4, IgG, and TAC. Furthermore, more research work are needed to find a safe and an effective supplement for depressing and immunomodulating the COC adverse influence in the neonates off springs. Studying long lasting effect across several postnatal intervals of cytokines and immunoglobulins could be a limitation of the present study that needs future research to explore whether the COC immune response could resolve or last allover life. Moreover, these future studies should focus on clinical signs of immune suppression or autoimmune diseases. Investigations of immune cell subsets (such as Tregs, Th17 cells, etc.) to clarify whether the immune response is appropriate or skewed remain required. A more comprehensive analysis of immune endpoints beyond just cytokine levels, such as immune cell function, tissue histopathology, and serological markers of autoimmunity, would allow for a more comprehensive interpretation of COC prenatal exposure induced immune perturbations.

ACKNOWLEDGEMENT

The authors would like to thank Prof. Amal El Nahla , Physiology Department for revising the original script.

NOVELTY STATEMENT

The study provides new insights about copper oxychloride prenatal exposure and its influence on offspring immune system.

AUTHOR'S CONTRIBUTION

Conceptualization,H.N.G., H.M.A.A., H.M.E, M.T.A.S ; Methodology,H.M.S., H.M.A.A, H.N.G.; Formal analysis, H.M.S., H.M.A.A, H.N.G, M.T.A.S, H.M.E.; Investigation, H.M.S., H.M.A.A, H.N.G, M.T.A.S, H.M.E.; Resources, H.M.S., H.M.A.A, H.N.G, M.T.A.S, H.M.E.; Writing- original draft preparation, H.M.S; Writing-review and Editing, H.M.S., H.M.A.A, H.N.G, M.T.A.S, H.M.E.; all authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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