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Histopathology (Gills and Stomach) and the Leukocyte Profiles of the Common Carp (*Cyprinus carpio*) Induced by the Waste from High-Efficiency Particulate Air (HEPA) Filter

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ABSTRACT

Due to the COVID-19 pandemic, the use of high-efficiency particulate air (HEPA) filters in air purifiers has been widely employed to reduce the transmission of the COVID-19 virus. The existence of the heavy metals silver (Ag) and copper (Cu) in HEPA filters will adversely affect both water quality and the survival of aquatic organisms. Further analysis is required to assess the effects of using HEPA filters on environmental pollution and identify any associated adverse effects. This study investigated the impact of HEPA filter exposure on the histopathology of the gill and stomach, as well as the leukocyte profile in common carp (*Cyprinus carpio*). In brief, common carp samples measuring 8– 9cm in length were subjected to various concentrations of HEPA filters over a duration of 96 hours. The concentrations tested included a control group, as well as concentrations of 5, 10, 20, 30, and 40%. The study revealed that the use of a HEPA filter on common carp had a substantial impact on the leukocyte profile of *Cyprinus carpio*. Specifically, it caused a considerable decrease in the number of leukocytes at a concentration of 40%, resulting in a reduction of around 11.3 cells/ mm^3. Particularly, there was a considerable increase in both total leukocyte count and phagocytosis activity at a concentration of 10% of the HEPA filter. In addition, the HEPA filter induced oedema, hyperplasia, congestion, hypertrophy, fusion, hemorrhagic, and lysis in the tissue structure of the gills. These effects were found at concentrations of 20, 30, and 40%, resulting in a total damage of approximately 12, 28.6, and 66.3%, respectively. The HEPA filter causes hemorrhagic, congestion, and lysis in the stomach organ, resulting in total damage of approximately 7, 13.3, and 32%, respectively. The HEPA filter induces significant alterations in the gill and stomach tissue, with concentrations ranging from 50– 60%.

INTRODUCTION

HEPA filters or air filters are a type of filter that is currently being widely used due to its ability to filter air efficiently. HEPA filters can clean the air from aerosols and droplets **(Salama *et al.*, 2022)**. HEPA filters have the ability to filter free particles in the air reaching 99.97% of aerosols with a size of 0.3µm, which is the most penetrating particle size (MPPS) **(Ku *et al.*, 2023)**. The usage of HEPA filter in a room can also increase the effectiveness of air change per hour (ACH), and remove particles, reducing the risk of transmission in general wards with insufficient isolation rooms, as is the case with the large, sequential waves of the COVID-19 pandemic **(Qian *et al.*, 2007)**. The high effectiveness of HEPA filters results in the increased use of air filters, which in turn also results in an increase in the amount of waste produced **(Lowther *et al.*, 2022)**. HEPA filters contain heavy metals (Ag and Cu) in the form of nanoparticles (NPs), which function as antimicrobial agents and antiseptics that can combat antibiotic resistant bacteria and nosocomial infections **(Fan *et al.*, 2021)**. The presence of heavy metals (Ag and Cu) in HEPA filters that are removed without going through a treatment will have an impact on the water quality and the life of aquatic organisms. The impact of using HEPA filters must be analyzed profoundly to figure out the side effects that are caused when they pollute the environment.

The heavy metals Ag and Cu contained in the HEPA filter can affect the number of white blood cells **(Koldera dan Malgozata, 2013)** and differential leukocytes, as well as the histopathology of exposed fish organs **(Ewa *et al.*, 2018)**. Copper (Cu) is a type of heavy metal that is important for vertebrate animals, especially fish. The heavy metal Cu has countless functions in cell biochemistry, such as its role in cellular activity in the respiratory system and being a cofactor for more than 30 types of enzymes **(Ajani & Akpoilih, 2010)**. However, if Cu levels in the waters are high, it will be acute or even chronic in aquatic organisms. Chronic effects can include reduced growth, increased mortality, reproductive problems, reduced fertility, and changes in fish behavior. In addition, the presence of the heavy metal Cu in the fish's body also has an impact on the accumulation in the organ tissue. Increased use of NPs and other chemical materials has also increased concerns about their exotoxin effects. Silver nanoparticles (Ag NPs) are widely recognized as a significant class of heavy metals employed in a nanoparticle form, boasting a substantial global annual production ranging from 135 to 420 tons **(Pulit-Prociak & Banach, 2016)**. Heavy metals silver (Ag) in ionic form can induce toxicity for bacteria and can affect aquatic organisms, both freshwater and marine fish, in low concentrations **(Waalewijn *et al.*, 2014)**. Studies have reported that accumulated Ag-NPs in tissues can induce oxidative stress which triggers the leukocytes and differential leukocytes number in fish **(Vali *et al.*, 2022)**.

Fish are widely used as an animal model in biomedical research **(Megarani *et al.*, 2020)**. The common carp *Cyprinus carpio* is a widely used fish species and highly popular in the Indonesian aquaculture, and it has been frequently studied as agriculturally relevant model of fish disease in Indonesia. Common carp is relatively insensitive and can survive and accumulate contaminants at heavily polluted sites, which is why it is used as a freshwater bioindicator in environmental toxicology **(Stoyanova *et al.*, 2020)**. The objective of this study was to examine the histopathology (gills and stomach) and leukocyte profile of common carp fish that were exposed to a HEPA filter.

MATERIALS AND METHODS

1. Fish preparation

A group of common carp fish (*Cyprinus carpio*) with a length size ranging from 8-9cm were kept in a freshwater aquarium with an adequate aeration at a temperature of 27°C. The carp fish were subjected to a one-week period of acclimatization prior to the commencement of the experiment. The fish specimens were provided with a daily ration of commercial feed, which was administered twice a day.

2. Haematology analysis

Haematological analysis of peripheral blood parameters is a useful and relatively inexpensive tool often used in fish toxicology. Haematology measurements include the number of leukocyte (WBC) and leukogram, also know as differential leukocyte (DLC – percentage of various types of leukocytes). White blood cell (WBC) and differential leukocyte (DLC) counts are among commonly evaluated hematologic parameters and are applied as biomarkers in the assessment of fish health status after exposure to various compounds **(Butrimavičienė *et al.,* 2021)**.

a) Leukocyte

The measurements of leukocyte was performed following the method described by **Donald Hunter and Bomford (1968)**. Briefly, 0.5ml blood sample was collected from caudal vein using a capillary thoma pipette. Further turk solution (Merck, USA) for fixation leukocyte was added. The sample was immediately homogonized with a gentle mixing. The WBC (white blood cells) were measured under microscope using the counting chamber and calculated with total cell leukocyte multiplied by 50 cell per mm^3.

b) Basophils

The functions of basophils in the immune system have not been fully understood since basophils are the least abundant leukocyte in most vertebrate species. Fish basophils in May-Grünwald stained smears showed dense blue-black granules. The nucleus is usually difficult to observe due to the abundance of granules **(Bianchi *et al.,* 2014)**. Basophils are extremely rare in most teleosts but are sometimes found in low numbers in peripheral blood smears.

c) Monocyte

Blood monocytes contribute to tissue-resident macrophage populations during inflammatory conditions and the depletion of resident macrophages in their environment **(Witeska *et al.,* 2022)**. The monocytes are the largest leukocytes, usually round to oval or sometimes irregular-shaped cells with an abundant light gray-blue cytoplasm, often containing vacuoles. The nucleus can be round, oval, kidney-shaped or bilobed with loose chromatin **(Vazquez & Guerrero, 2007; Bianchi *et al*., 2014)**.

d) Lymphocyte

Fish lymphocytes are small round cells with a narrow rim of light homogenous basophilic cytoplasm around the large round (or sometimes slightly oval or indented) condensed nucleus, similar to those found in other vertebrates **(Clauss *et al.,* 2008)**. Lymphocytes size is different among the fish; in *Cyprinus carpio*, the average size of lymphocyte was 6.6– 11.8.

e) Neutrophils

Neutrophils is the second most common leukocytes in fish blood. Neutrophils are granulocytes identified as round cells larger than lymphocytes, with abundant clear (slightly acidophilic or polychromatophilic) cytoplasm and eccentric, condensed nucleus that is either round, oval, kidney-shaped or lobed **(Witeska *et al.*, 2022)**. Neutrophils are capable of eliminating pathogens through multiple complementary mechanisms.

f) Eosinophils

The eosinophils are effective in controlling parasitic infections **(Balla *et al.*, 2010)**. Eosinophils are large round cells with pale blue-gray cytoplasm, filled with large round to oval eosinophilic granules (the color of the granules varies with species, from bright red or orange to pale pink), with round or lobed nucleus.

3. Histology (Gills dan Stomach)

Histopathological examination was performed following the method described by **Elsayed *et al.* (2006)**, gill and stomach tissue of *Cyprinus carpio* in each treatment were collected and immediately fixed in neutral buffered formalin (10%) for a week; dehydration was done using ascending grades of ethanol (70, 80, 90, and 100% for 1 hour each). The specimens were then cleared in 2 changes of xylene. After blocking using soft paraffin, serial sections of 4µm thickness were done. The sections were stained using haematoxylin-eosin stain and conducted the calculation of the amount of damage following this formula:

$$\text{Histology damage (\%)} = \frac{\text{The total (square) damage of tissue}}{\text{The total (square) of tissue}}$$

The percentage of tissue damage was calculated based on the methods used as the amount of damaged tissue divided by the amount of tissue analyzed in percent. The formula was obtained from tissue observations using one field of view using the OlyVIA 2.9.1 application (Olympus, USA) instead of a microscope. In one field of view, it is divided into several squares, and each square that is damaged is counted and then recorded as the number of damaged tissue. The square representing the damaged tissue is recorded as the number of damaged tissue analyzed.

4. Data analysis

Data are expressed as mean ± SD. Statistical significance of pairwise differences among three or more groups were determined using the one-way analysis of variance (ANOVA), followed by LSD test. $P < 0.05$ was considered statistically significant. Analysis was performed using SPSS for Windows (SPSS Inc., Version 20.0, Chicago, USA). Graph was performed using GraphPad Prism 7 (GraphPad Software, Inc. USA).

RESULTS

1. Profile haematology

To observe the effect of HEPA filter on aquatic organisms, haematological analysis was performed. In this study, further analysis of leukocyte total and differential leukocytes were assessed. The white blood cells (WBC) of *Cyprinus carpio* were divided into 2 types, granular and agranular cells. The leukogram, or also known as differential leukocytes, consisted of five types, namely basophils, eosinophils, neutrophils, lymphocyte, and monocyte that were identified. Agranular leukocytes, such as lymphocytes and monocytes, have cytoplasm that appears without granules **(Nussey *et al.*, 1995)**. Both size of lymphocytes (large and small) were noted in the blood of *Cyprinus carpio*, as depicited in Fig. (1). The HEPA filter induced an increase in several haematological profiles. Notably, the HEPA filter induced a significant decrease in the number of leukocyte, reaching an approximately 11.3 × 10^4 cells/ mm^3 at the concentration of 40% .

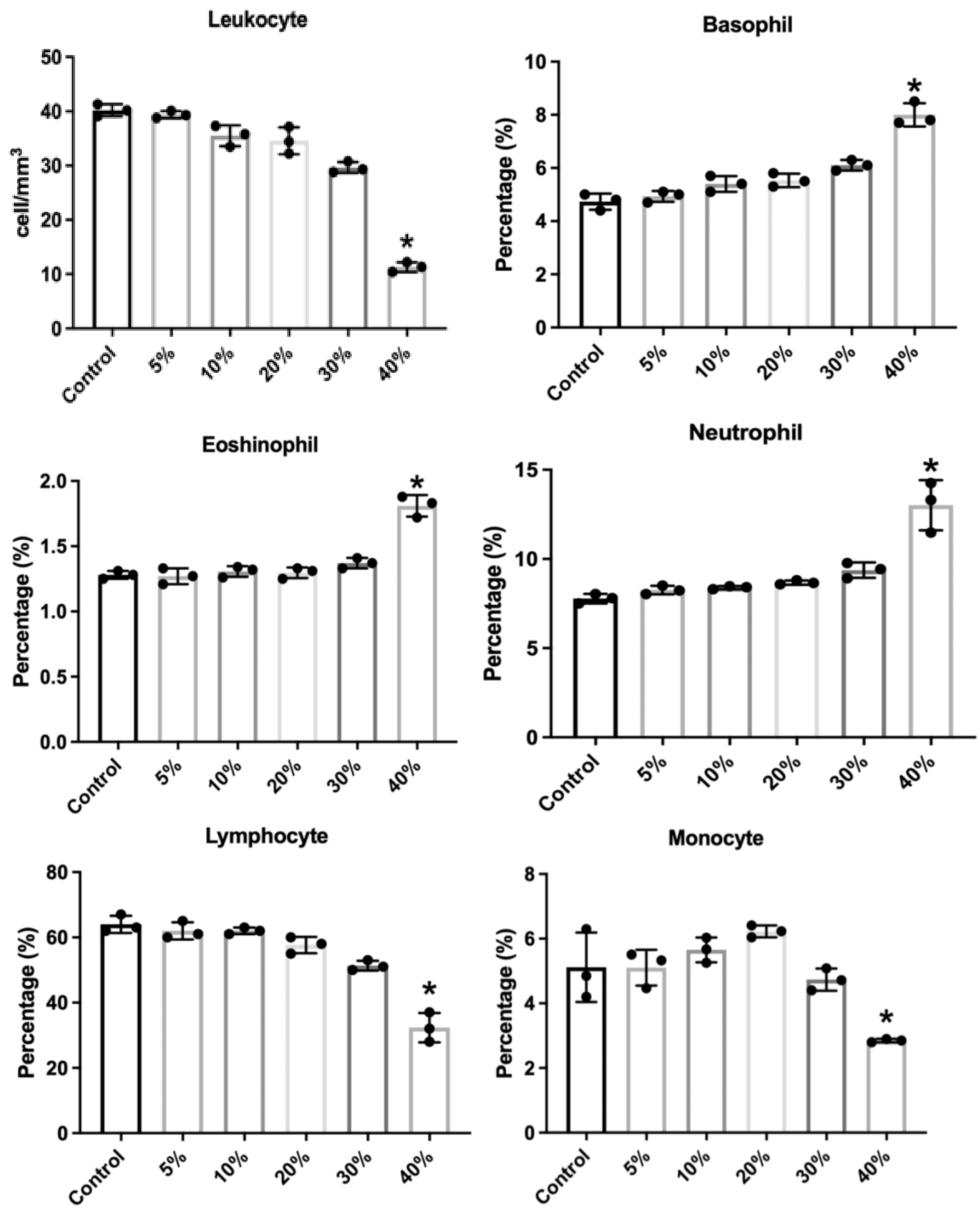

Fig. 1. Analysis of the effect of HEPA filter toxicity on the hematological profile of *Cyprinus carpio*. Hematological test was done to assess total leukocytes and differential leukocyte (basophils, eosinophils, neutrophils, lymphocytes, and monocytes). The results are presented as the mean ± SD. **P*< 0.05 indicates significance.

HEPA filter toxicity induced a significant increase of basophils in *Cyprinus carpio* blood at a concentration of 40%, with about 8.5%. Eosinophils in *Cyprinus carpio* was also significantly increased at the highest concentration of 40%, with about 1.88%. Eosinophils are effective in controlling parasitic infections in the body. The amount of neutrophils were significantly increased, with about 7.8, 8.03, 8.3, 8.65, 9.77, and 11.48%, respectively. Interestingly, the number of lymphocyte significantly decreased after all the exposures, reaching its lowest value at around 32%. The percentage of monocytes in *Cyprinus caripo*, the subject fish, fluctuated as follows: 4.47, 4.85, 5.67, 6.23, 4.2, and 2.9%, respectively.

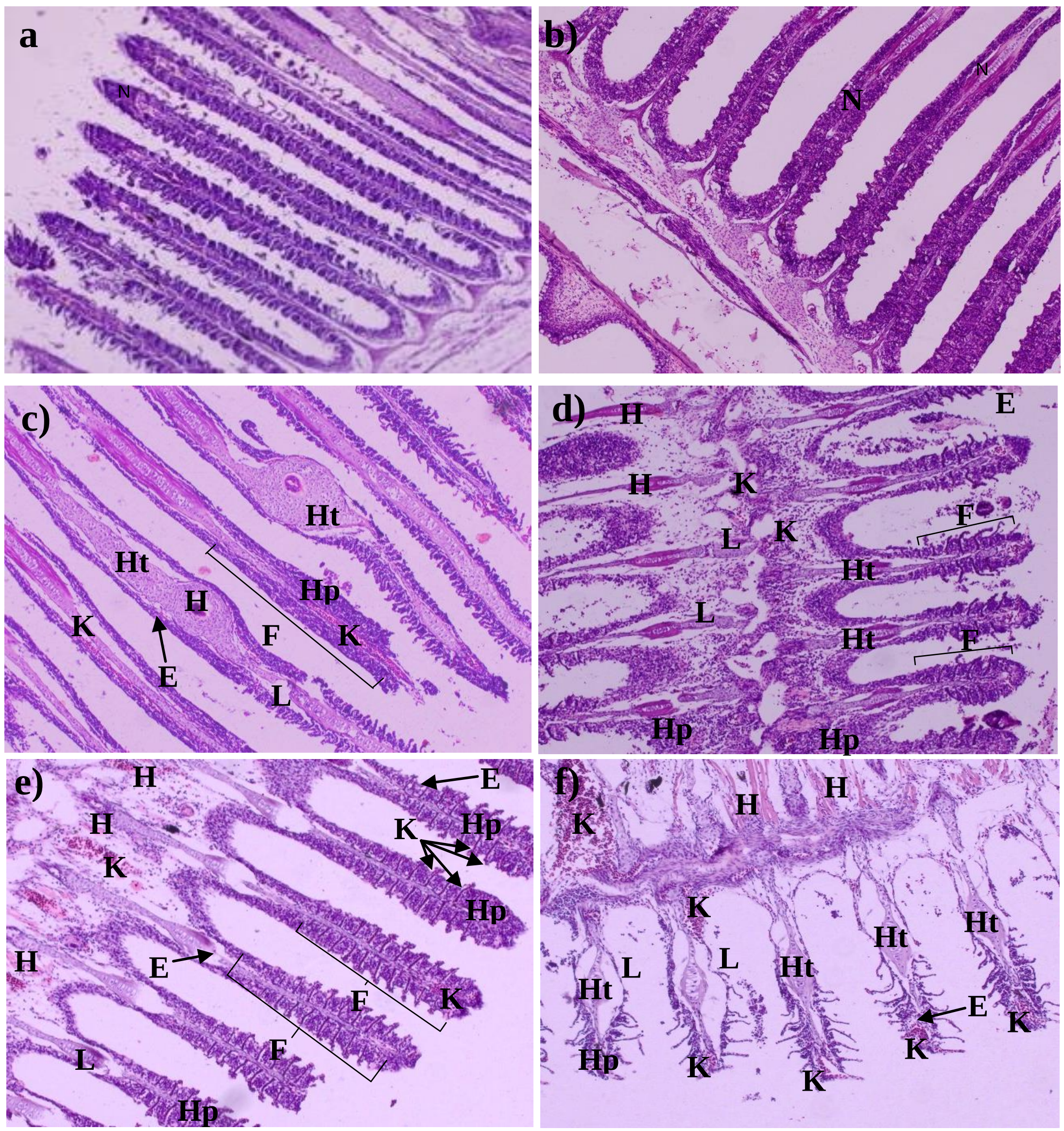

Fig. 2. Analysis of histology in the structure of gill tissue in common carp. **a)** Control; **b)** 5%; **c)** 10%; **d)** 20%; **e)** 30%; **f)** 40% concentration, respectively. (N) Normal, (Ht) Hypertrophy, (E) Oedema, (F) Fusion, (L) Lysis, (Hp) Hyperplasia, (Hg) Haemorrhagic, (K) Congestion (400× magnification, Olympus BX 41 microscope, Olympus DP 20 camera)

2. Histology of gill and stomach of *Cyprinus carpio*

HEPA filter has a toxic effect on the structure of the gill tissue so that there is an abnormality of respiratory activity through the opening speed of the operculum, then an analysis was carried out through changes in the structure of the tissue in the gills. The results of the histology of gills are shown in Fig. (2). It was noticed that, at the concentration control of 5%, the gills tissue structure of common carp fish exhibited normal gills lamellae. The issue of alteration was observed in the structure of common carp gills tissue at concentrations of 10 to 40%. At the concentration of 10% in the gill lamellae, minor oedema, hyperplasia, hypertrophy, lysis, and congestion began to appear. The higher concentration which was 20% showed more damage to the gills lamellae. The damage found were hypertrophy, hyperplasia, haemorrhagic, fusion to the lamellae, lysis, oedema, and congestion. Furthermore, a lot of hyperplasia and haemorrhagic happened at the concentration of 30%. While, at the concentration of 40%, HEPA filter induced greater oedema, followed by changes in the tissue structure of hyperplasia, and a shrinkage of the structure, also known as hypertrophy, on the gill lamellae. The common carp gills lamellae were further destroyed, worsening the structure and causing it to appear shortened. The total damage produced by HEPA filter at the concentration of 40% was about 66.3% (Table 2).

Table 1. Histology of gill tissue structure of common carp fish induced by HEPA filter

Concentration (ppm)	Replication	Hp	Oedema	Ht	K	L	Hg	F	Total (%)
	1	0	0	0	0	0	0	0	0
control	2	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0
	1	0	0	0	0	0	0	0	0
5%	2	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0
	1	0	0	0	1	0	1	0	2
10%	2	0	0	1	1	0	1	0	3
	3	0	1	0	1	0	0	0	2
	1	1	1	2	3	1	3	0	11
20%	2	2	1	2	3	2	3	0	13
	3	1	1	3	1	1	4	1	12
	1	3	3	5	4	5	4	3	25
30%	2	4	4	5	3	3	6	3	28
	3	5	5	4	3	4	5	7	33
	1	12	10	8	11	6	8	10	65
40%	2	10	8	10	12	8	10	8	66
	3	9	11	9	9	7	12	11	68

* (Hp) Hyperplasia, (Ht) Hypertrophy, (K) Congestion, (L) Lysis, (Hg) Haemorrhagic, (F) Fusion.

Alteration in the histological structure of common carp stomach can also be seen after being exposed to HEPA filter. This is caused by the presence of heavy metals, Cu and Ag, in the HEPA. Heavy metal Cu is one of the elements needed by fish for metabolic functions. However, it is potentially toxic when the internal available concentration exceeds the capacity of physiological detoxification processes. Excessive Cu induces histological alterations, oxidative stress, influencing immunity and lipid metabolism, gut microbiota dysbiosis, and growth retardation. The HEPA filter induced some tissue damage in the stomach of common carp fish. The results of the damages in the stomach structure of common carp are shown in Fig. (3).

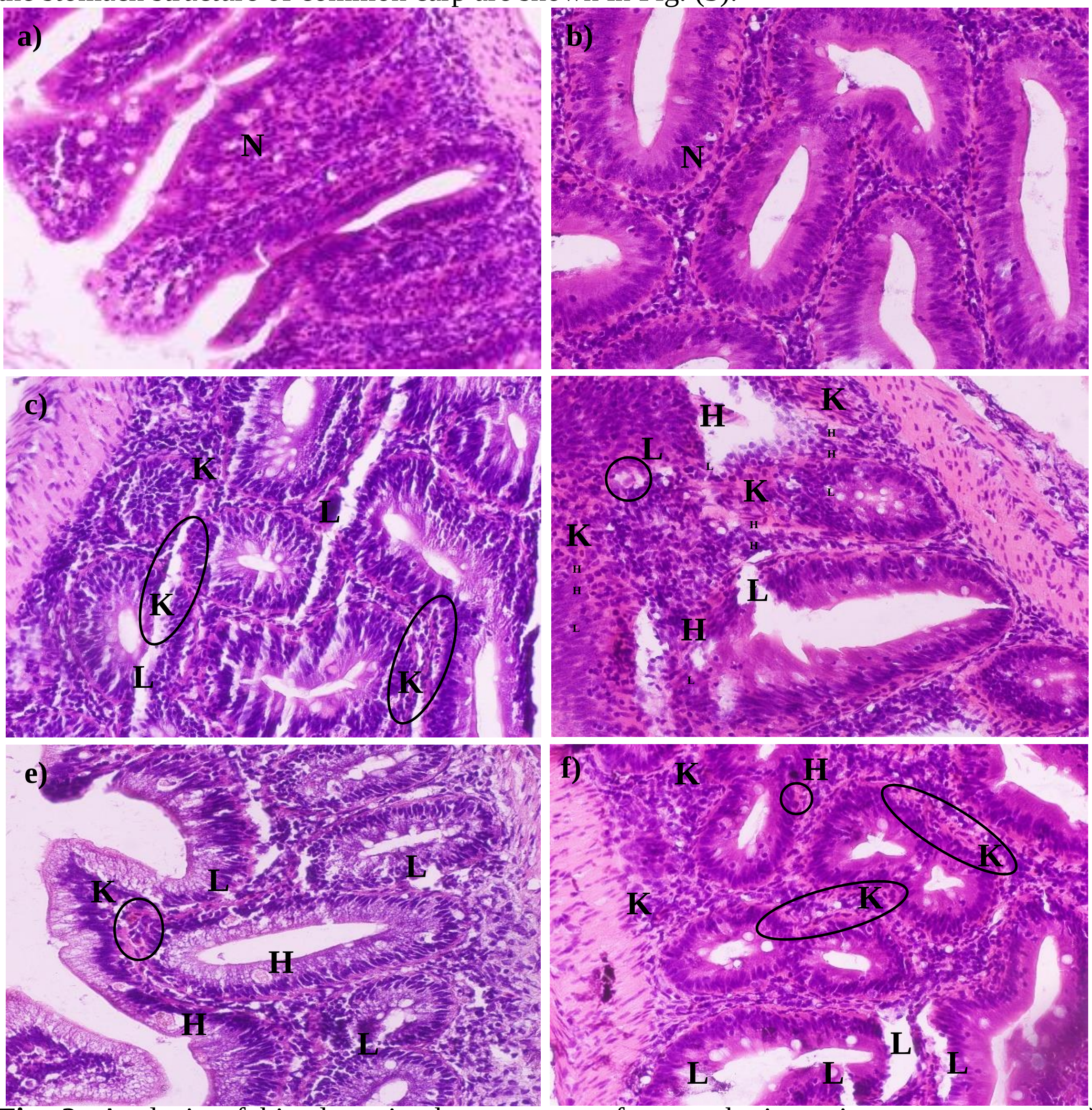

Fig. 3. Analysis of histology in the structure of stomach tissue in common carp. **a)** Control; **b)** 5%; **c)** 10%; **d)** 20%; **e)** 30%; f) 40% concentration, respectively. (N) Normal, (K) Congestion, (L) Lysis, (H) Haemorrhagic (400× magnification, Olympus BX 41 microscope, Olympus DP 20 camera)

Fig. (3) depicits the stomach tissue structure of common carp appearing normal at the concentration of the control. The HEPA filter induced significant damaging effects on the stomach tissue in all exposured groups compared to the control, particularly from the concentration of 10 to 40%. On the other hand, at the concentration of 5%, a minor congestion began to appear, or the presence of abnormal amount of fluid was detected in the vessels of passages of a part or organ. Furthermore, at the concentration of 10% of HEPA filter induced exposure, enhanced congestion followed by haemorrhagic and lysis were observed. At the concentration of 20% the number of congestion increases and was also followed by haemorrhagic and lysis. Furthermore, at a concentration of 30%, HEPA filter induced greater visible lysis, congestion and haemorrhagic. At the highest concentration which was 40%, the damage became visible, and a lot of lysis was shown at this concentration. The total damage in the stomach tissue of common carp produced by HEPA filter at a concentration of 40% was about 32% (Table 2).

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Concentration (ppm)	Replication	Haemorrhagic	Congestion	Lysis	Total (%)
	1	0	0	0	0
control	2	0	0	0	0
	3	0	0	0	0
	1	0	0	0	0
5%	2	0	0	0	0
	3	0	1	0	1
	1	0	1	0	1
10%	2	1	0	1	2
	3	1	1	0	2
	1	3	3	2	8
20%	2	3	1	2	6
	3	2	4	1	7
	1	4	6	3	13
30%	2	3	5	4	12
	3	5	7	3	15
	1	10	12	9	31
40%	2	14	7	7	28
	3	12	15	10	37

DISCUSSION

The toxicity of HEPA filter still lacked information and needs to be analyzed further. The present investigation involved an assessment of the toxic effects of HEPA filter on test subjects of common carp (*Cyprinus carpio*). Peripheral blood parameters and quantitative evaluation of blood cell shape are often employed and cost-effective techniques in fish toxicology for haematological investigation. Blood indices serve as highly responsive biomarkers for detecting and assessing the effects of different environmental stressors such as water contamination by harmful substances. Blood parameters serve as indicators of many physiological changes, encompassing both beneficial and detrimental modifications **(Witeska *et al.*, 2023)**. Physiological number of differential leukocyte counts are needed to compare and evaluate changes in stressed fish. Stress load can occur due to treatments such as experiments and also naturally. Lymphocytes contribute significantly to total white blood cells. Lymphocytes are usually responsible for specific types of immune responses **(Sanoesi *et al.*, 2020)**. Metal accumulation (e.g. copper and silver) has been previously observed in fish gills and stomach, and was associated to the high concentration of metallothionein proteins, which play essential roles in the regulation and detoxification of intracellular metals **(Hoseini *et al.*, 2022)**. Metals are known to block the active sites of antibody molecules and disturb the metabolism, ionic balance, and cellular division of immunocompetent cells **(Nussey *et al.*, 1955)**. This study found that HEPA filter induced exposure led to a significant decrease on the number of leukocyte, with approximately 11.3×10^4 cells/ mm^3 at a concentration of 40%. Leukocyte count is an indicator of fish health status due to its role in non-specific or innate immune response **(Shahi *et al.*, 2014)**. The decrease in leukocytes number in the blood of common carp after being exposed to Ag and Cu from HEPA filter depends on the concentration and time of exposure **(Ghiasi *et al.*, 2010)**. The decrease in the leukocyte count is attributed to the decrease in small lymphocytes and the increase in the number of neutrophils. The number of lymphocytes and neutrophils was about 32 and 11.48%, respectively. A decrease in the number of lymphocytes usually occurs in fish subjected to stress. Furthermore, the number of monocytes fluctuated, with the lowest value observed at about 2.9%. The decrease in the number of monocytes took place since the WBC leave the circulating blood to protect the body by moving amoeboid to the infected tissue **(Nussey, *et al.*, 1995)**. Monocytes and neutrophils are sensitive to heavy metals **(Thangam *et al.*, 2014)**. The decrease in the white blood cells (WBC) count in HEPA filter exposed common carp suggests an immunological suppression. Phagocytosis is one of the important processes in poikilothermic animals because it is least influenced by temperature. Phagocytic cells are the important cellular components of the innate immune system of fish. Based on the previous study of **Donaldson and Dye (1975)**, exposure to heavy metals in fish causes an increase in cortisol level, resulting in a decrease in phagocytic activity. However, phagocytic activity may increase after a general adaptation to stress.

The HEPA filter at concentration up to 10% indicates an abnormal issue in the breathing habit of the common carp fish operculum. Gills are the main route of entry of metals into the body of the fish due to the direct exposure to toxins in the aquatic environment, and thus they store largest amounts of metals in the body **(Afaghi & Zare, 2020)**. Based on the histology results, an alteration was detected in the structure of the gills tissue, such as oedema, hyperplasia, and fusion. The occurrence of oedema was due to the entry of heavy metals (Cu & Ag) from the HEPA filter into the gills, which irritated the cells causing a probable cell swelling **(Mulayani & Utami, 2014)**. Oedema is cell swelling caused by the entry of heavy metals into the gills, resulting in anexcess fluid accumulation in the body's tissues. This is characterized by the basement membrane starting to stretch loose, the lacuna cells narrowing, causing the gills to experience a deficiency in function and difficulties in the process of breathing, and the body's metabolism begins to be disrupted **(Fitriawan *et al.*, 2011)**. Another damage that occurs is hyperplasia. The interlamellar space which is the aqueduct and the mucus or mucus production space can be blocked due to hyperplasia of epithelial cells originating from the primary filaments. Eventually, the entire interlamellar space is filled with new cells. Hyperplasia results in thickening of the epithelial tissue at the ends of the filaments, which shows thickening of the epithelial tissue located near the base of the lamella **(Robert 2001)**. Histopathological changes, such as lifting of the epithelium and lamellar fusion, may help fish to overcome pollution effects **(Rajeshkumar *et al.*, 2017)**. These changes however indicate oxygen deficiency, contributing to systemic hypoxia. At the concentration of 40%, gills tissue were badly damage, with a total damage estimated at approximatley 66.3%.

Fig. (2) illustrates an increase in the lamallar fusion originating from the mucus cells located at the base of the lamellae, leading to the merging between secondary lamellae **(Kakade *et al.*, 2020)**. **Robert (2001)** stated that lamella fusion is the attachment of two parts of the secondary lamella. In addition, lamella fusion is caused by excess mucus in the gills so that it will cover the secondary lamellae. Excessive mucus production is a defence mechanism of the mucus glands to protect fish gills from heavy metal ions entering the gills **(Soegianto, 2023)**. Based on the histology results, some minor damages were also detected, including congestion and haemorrhagic that could be caused by the stress experienced by the fish during the exposure time. On the stomach tissue, some damages including congestion, haemorrhagic, and lysis were observed. The total damage produced at the concentration of 40% was about 32%. This finding coincides with what was conveyed by **Garai *et al.* (2021**), who elucidated that the impact of the heavy metal Cu was more severe on the gills when compared to other organs.

CONCLUSION

In conclusion, HEPA filter induced some changes in haematological histopathology and the leukocyte of the common carp. The higher concentration, which was 40% promoted some health issue in *Cyprinus carpio*. The substance in HEPA filter generated immunosuppression by affecting the numbers of leukocytes and differential leukocytes level. Additionally, it caused a significant harm to the gill and stomach tissue structures systems of common carp, resulting in the mortality of the fish species under study.

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