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ORIGINAL RESEARCH ARTICLE

Silver Nanoparticle-Induced Lymphocyte Activation in Zebrafish

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ABSTRACT

Silver nanoparticles (Ag-NPs) are among the most widely used nanomaterials in the world. Nanotoxicological data on these additives remain unclear. The aim of this study is to evaluate the *in vivo* effects of Ag-NPs bioaccumulation on the hematological cells of adult zebrafish *Danio rerio* after three weeks of oral exposure. Four concentrations of Ag-NPs (65 nm nanosphere), are used: control group 1 (0.00 g/kg of food), group 2 (0.05 g/kg of food), group 3 (5 g/kg of food) and group 4 (50 g/kg of food). The experiment is conducted in triplicate. Cytological and histological results show, a significant increase in neutrophils associated with a significant decrease in lymphocytes in both group 3 and 4 compared to the control group ($p<0,05$ and $p<0,005$, respectively). This reflects an elevated neutrophil/lymphocyte ratio (NLR), suggesting a biased immune response associated with hyperplasia of the hematopoietic tissue and histopathological images of nephrological lesions, following medium to high dose of Ag-NPs intake. Besides, microscopic observation of cell morphology revealed lymphocyte activation only in groups 2 and 3 ($p<0,005$ and $p<0,05$, respectively). These results suggest that Ag-NPs induce a dose-dependent bioeffects on the immune system in zebrafish. This study reports that at low doses, Ag-NPs may activate lymphocytes but do not alter the NLR, thus demonstrating an immunostimulatory effect. The present investigation is the first study, as far as we know, reporting that Ag-NPs induce a dose-dependent immunomodulation in zebrafish.

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INTRODUCTION

Silver nanoparticles (Ag-NPs) are widely used in industry [1] with over 113 800 patents records up to early 2022 [2]. This is explained by the interesting physico-chemical properties of this compound which increases the resistance of materials, their conductivity or their reactivity with other substances [3]. These advantages lead to an ever-increasing interest for these nanoparticles [4]. In addition, Ag-NPs are used in the composition of the packages for their anti-microbial properties,

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allowing a longer shelf life [5], [6]. However, these nanoparticles migrate from the packaging to the food and are then consumed by Humans [7]. In addition, Ag-NPs are authorized by the European Food Safety Authority (EFSA) as an additive food (E 174) in chewing gums, chocolates and spirit drinks [8]. However, these NPs present real dangers as they have a median L (E) C50 value (mg/L) of 0.01, 0.36 and 1.36 for crustaceans, algae and fish, respectively [9].

The assessment of element-induced effects in organisms is a major concern in modern medicine and even in ecotoxicology because of the critical consequences they can have in the environment. The effects of chronic exposure are usually subtle and require careful biological testing. Cortisol levels remain a classic biomarker for stress assessment in vertebrates [10]. New approaches could elucidate dose-response relationships for chronic exposure to different toxic elements. Thus, perturbations in leukocyte morphology and formula may provide a tool for early biomonitoring of disturbances by exogenous contaminants. Indeed, alternative methodologies for assessing stress, such as leukocyte profiles, are becoming increasingly popular [11], [12]. It was demonstrated that stress induces changes in leukocyte formula resulting in an increase in circulating neutrophils (N) versus a decrease in circulating lymphocytes (L) [13]. In addition, the relative increase in the neutrophil/ lymphocyte ratio (NLR) is considered a secondary response to the increase in cortisol levels, which acts as a suppressor of the immune response [12]. Furthermore, several studies report that Ag-NPs can activate immune cells and thus participate in the modulation of the immune response. This modulation can be stimulating or suppressive since an over-stimulation or an over-suppression must be avoided to preserve an effective immune response. *In vivo,* experiments in mice show that injections with high concentrations of Ag-NPs lead after 2 days, in a dose-dependent immunomodulation of monocytes and lymphocytes [14].

In addition, Ag-NPs interact with primary peripheral blood mononuclear cells by increasing the oxidative stress of a cell culture of Human neutrophils [15]. Other works showed that the incubation of Ag-NPs with Human mononuclear cell for 24 hours resulted in a time dependent cytotoxicity [16].

Data on the *in vivo* impact of Ag-NPs on vertebrates are limited. Furthermore, there are serious concerns about the oral exposure of Ag-NPs because of the long persistence and accumulation of these nanomaterials in the organism, particularly in the brain and the testicles [17].

In addition, the *in vivo* impact of Ag-NPs on immune cells and hematopoietic tissues are poorly documented. In order to have a better understanding, we chose to use the zebrafish as an experimental animal. Indeed, zebrafish is a powerful model to investigate the toxicity of chemical compounds in vertebrates beside a high genetic homology with humans[18]–[20].

The aim of the present study is to evaluate the *in vivo* effects of subacute to chronic oral exposure of Ag-NPs on zebrafish immune cells through cytological and histological assessment.

MATERIAL AND METHODS

Zebrafish husbandry and breeding

Experiments were performed on 6 - 12-month-old out bred wild-type zebrafish. Animals originated from our fish facility based on the biochemistry department of the National School of Veterinary Medicine of Sidi Thabet in Tunisia. Zebrafish were housed and reared according to Aleström and *al.* 2020 [21]. Four groups of six adult zebrafish each were randomly constituted; they were maintained in four aquariums in triplicate, for a total of twelve experimental tanks. An acclimation period of 15 days was respected before the experiment. Each group was kept in 5.50 L tank under a photoperiod of 14 hours light: 10 hours dark and sex-ratio (SR) 1 male: 2 females, SR = 0.50. Tap water was used and changed daily after passing through two filters, the first one with 5µm mesh diameter and the second with activated carbon. Water tank was calibrated at 27 ± 2°C. Zebrafish were fed twice a day at 5% of body weight with a commercial pellet food of 1 mm of diameter (Skretting, France).This work followed the guidelines of Committee for the Update of the Guide for the Care and Use of Laboratory Animals [22]. Ethical approval for this study was obtained from the Animal Ethics Committee - National School of Veterinary Medicine, ENMV- Sidi Thabet, Tunisia- and registered under Number: CEEA-ENMV 32/21.

Preparation of contaminated food with Ag-NPs

The Ag-NPs used in this experiment were purchased (Sigma Aldrich, nano powder, Size ≈ 65nm particle size, contains PVP as dispersant, 99.5% trace metals basis, reference: 576832-5G, lot:

MMKBX3387V, USA). Analyses of the Ag NPs have been carried out by the manufacturer which states that these particles were analyzed *via* TEM with an Average Particle Size (APS) for batch MKBX3387V, of 65nm, with spherical morphology. Furthermore, the purity was analyzed by trace metals and was found to be 99.50%. All relevant certificates are available on the manufacturer's website [23].

Three concentrations of Ag-NPs were incorporated to the fish diet at the following concentrations: 0.05 g/kg of food (group 2), 5 g/kg (group 3), and 50 g/kg (group 4). The Ag-NPs were dissolved in distilled water and set to 30 minutes of sonication. The food pellets then were mixed with the water containing Ag-NPs. Control group 1 received the same diet without Ag-NPs. The same procedure was respected for the control group by using distilled water without Ag-NPs. A drying step of 24 hours at room temperature under a hood was carried out. Food was distributed twice daily at 5% of body weight for three weeks.

Cytology and histology

At day 21, one fish from each group, collected in triplicate, was randomly selected, and euthanized in an ice water bath. Immediately after the fish death, an incision in the caudal peduncle was made to collect a blood sample. Although, the fish was wiped before blood collection, residual water could cause blood cells to burst due to an osmotic imbalance, making it difficult to analyze. To ovoid this, the euthanasia was performed in ice-cold isotonic water. Blood smears were made directly after blood collection on a microscope slide.

The smears were stained with a modified Giemsa stain (HiMedia Laboratories, India).

Each slide was carefully examined under microscope at 1000x magnification with immersion oil. The leukocyte formula was established following 100 White blood cells (WBC) differential count (Fig. 1).

To determine the percentage of lymphocyte activation, an additional reading of the slides was taken and a count of 100 lymphocytes for each blood sample was performed.

For the histological study, the whole fish, after euthanasia, was fixed in 10% neutral buffered formalin and embedded in paraffin. The samples were sectioned on a microtome and placed on slides for hematoxylin staining. The tissues were examined using a light microscopy coupled to a camera.

Statistical analyses

The differential count of lymphocytes and neutrophils was assessed with a one-way ANOVA test at 5% threshold. Pairwise comparisons of each sample against the control were then evaluated using a two-sided t-test post-hoc [24].

RESULTS

Ag-NPs characterization

The Ag-NPs used in this experiment were purchased (Sigma Aldrich, nano powder, Size ≈ 65nm nm particle size, contains PVP as dispersant, 99.5% trace metals basis, reference: 576832-5G, lot: MMKBX3387V, USA). Ag NPs nanoparticles were analyzed via TEM with an Average Particle Size (APS) for batch MKBX3387V, of 65nm, with nanospherical morphology.

Cytological study

The hematocytological results were obtained following microscopic examination of zebrafish blood smears, performed following three weeks of oral exposure with different doses of Ag NPs: 0.05 g/kg of food (group 2), 5 g/kg (group 3), and 50 g/kg (group 4). Group 1 did not receive Ag-NPs and was considered as control group. A careful blood cell characterization, based on the information available on this species, was then performed [25].

Identification of blood cells in zebrafish

Unlike mammals, fishes present thrombocytes instead of platelets and nucleated erythrocytes [26]. Small lymphocytes contain lightly dark nucleus with a very high nucleoplasmic ratio (N/C) (Fig. 1.A). Activated lymphocytes show dispersed chromatin pattern and more abundant cytoplasm with lower N/C compared to the inactivated state (Fig. 1.B). Neutrophils contain a light pink to light blue cytoplasm and generally a lobed or banded nucleus, although some neutrophils had a rounded nucleus shape (Fig. 1.E). Monocytes are large cells with a greyish sky colored cytoplasm riddled with vacuoles, and a round or irregular nucleus (Fig. 1.G). Thrombocytes, which has a light bluish grey cytoplasm, homogeneous dense chromatin, and an oval-shaped nucleus. These cells are frequently observed as an aggregate (Fig. 1.D)

Leukogram determination

After differentiation of blood cell lineage, we were able to perform the leukogram differential count in the four experimental groups (Fig. 2).

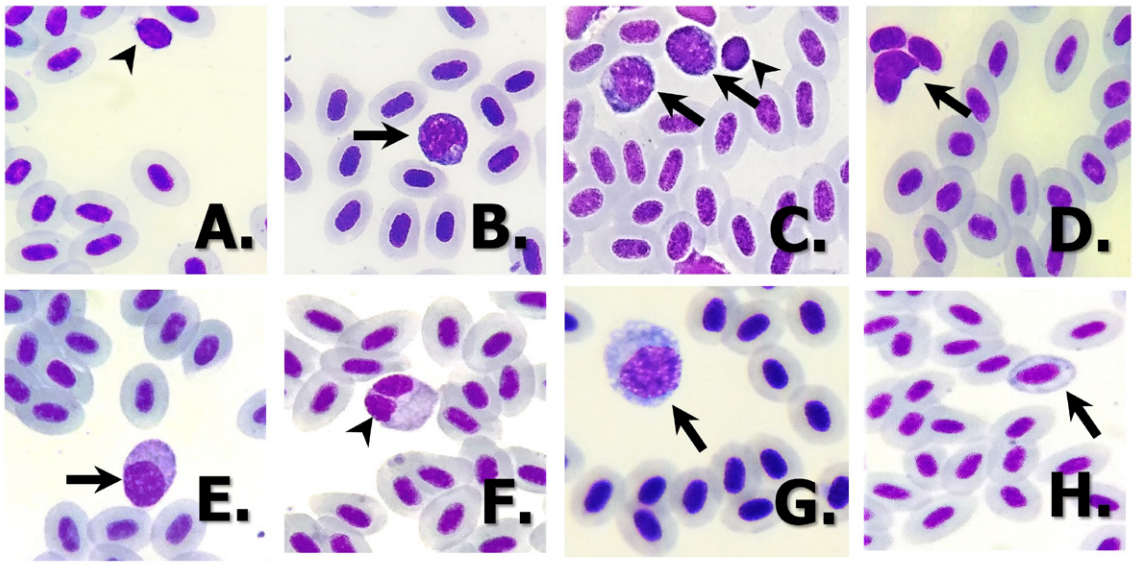

Fig. 1. Zebrafish blood cells. Modified-Giemsa-Stained zebrafish blood smear.

(A - C) arrowhead: Lymphocyte core chromatin nucleus with a very high nucleoplasmic ratio. arrow: Activated lymphocyte, increased size with very basophilic cytoplasm.

(D) arrow: Platelet aggregates, notice that platelets are like lymphocyte, however the nucleus is oval, flattened with a very dense chromatin.

(E - F) arrow: Neutrophil with a peripheral nucleus with abundant cytoplasm. arrowhead: Neutrophil with segmented nucleus.

(G) arrow: Monocyte with large cell, abundant gray-sky cytoplasm, and several vacuoles.

(H) arrow: Polychromatophil, notice the large cell with a heterogeneous basophilic cytoplasm compared to mature red blood cells.

Blood smear images were photographed under 1000 x magnification with immersion oil.

		Neutrophils			Lymphocytes			
Group	**n***	**Counts**	**Mean**	**SD**	**Counts**	**Mean**	**SD**	**Neutrophil / Lymphocyte Ratio**
1	3	33, 23,22	26	6.1	67.75,78	73.3	5.7	0.35
2	3	15, 20,40	25	13.2	85.60,80	75	13.2	0.33
3	3	62, 63,38	54.3	14.2	38.37,62	45.7	14.2	1.19
4	3	55, 59, 64	59.3	4.5	45.41,36	40.7	4.5	1.46

***number of observations**

Fig. 2. Leukograms of the four zebrafish groups

In this study, we focused on the differential count of lymphocytes and neutrophils, to establish the NLR.

In all groups, percentage of monocytes did not exceed physiological values [27] and did not present significant changes among individuals.

The control group showed a typical physiological leukocyte count of zebrafish with predominance of lymphocytes (Fig. 3).

Zebrafish exposed to Ag-NPs showed dose-dependent changes in leukocyte formula.

The group 2, exposed to the lowest Ag-NPs concentration of 0.05 g / Kg of food did not show a significant difference in the WBC formula (Fig. 3).

The leukograms of groups 3 (5 g / Kg of food) and group 4 (50 g / Kg of food) indicated significant variations with the control group. Indeed, a significant increase of the neutrophils in group 3 and 4 was observed (p = 0.033 and p =

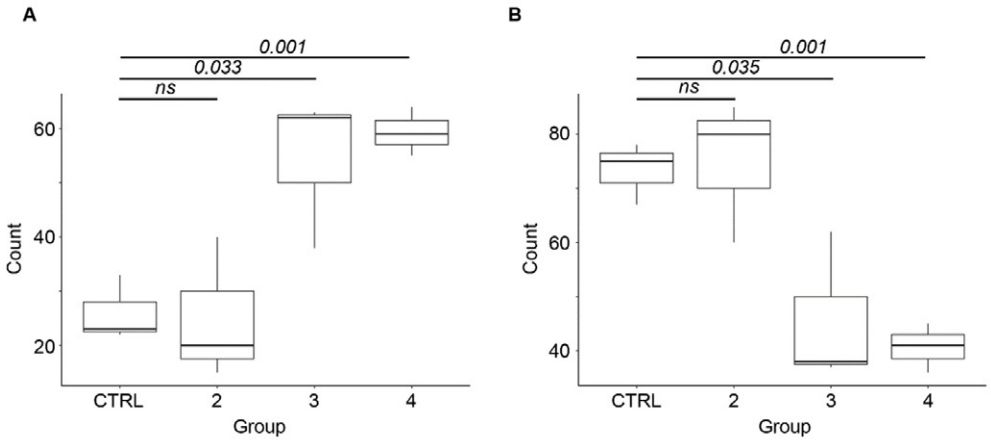

Fig. 3. Blood cell count in the four studied zebrafish groups, control (CTRL), 2 (0.05 g of Ag-NPs/ Kg of food), 3 (5 g of Ag-NPs/ Kg of food) and 4 (50 g of Ag-NPs/ Kg of food). No significant difference was observed between control group and group 2 (ns: not significant). Significant differences ($p<0.05$) were observed between control group and group 3 and 4. (A) Neutrophils counting. (B) Lymphocytes counting.

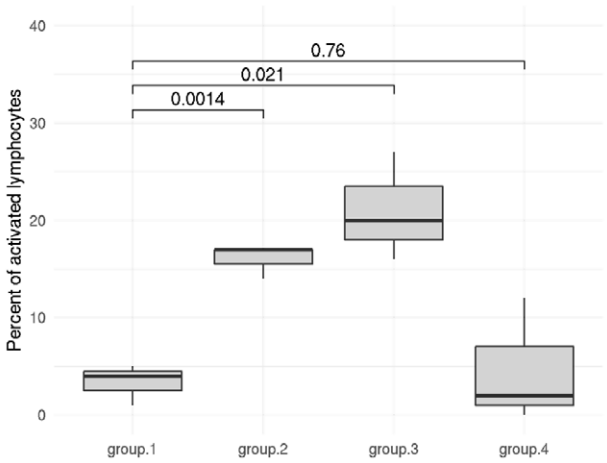

Fig. 4. Percent of activated Lymph cell in the four studied zebrafish groups, control (group 1), group 2 (0.05 g of Ag-NPs/ Kg of food), group 3 (5 g of Ag-NPs/ Kg of food) and group 4 (50 g of Ag-NPs/ Kg of food). No significant difference was observed between control group and group 4. Significant differences ($p<0.05$) were observed between control group and group 2 and 3.

0.001, respectively) (Fig. 3). Similarly, a significant decrease of lymphocytes has been observed in the groups 3 and 4, compared to the control group (p = 0.035 and p = 0.001, respectively) (Fig. 3). This led consequently to an increase in the NLR in the groups 3 and 4, compared to the control group (Fig. 2). Several neutrophils with segmented nucleus were noticed (Fig. 1.F) especially in the group 4, with the highest concentration of Ag-NPs.

Modifications on the lymphocyte activation state

A specific reading of the blood smears was performed to determine the percentage of lymphocyte activation. Indeed, activated lymphocytes show distinct morphological changes that distinguish them from other WBC (Fig. 1.C).

A microscopic count revealed that group 2 and 3 had an increase in the percentage of activated lymphocytes compared to the control group (p=0.0014 and p=0.021, respectively). Whereas in group 4, which received the highest concentration of Ag-NPs, there was no significant lymphocyte activation (Fig. 4).

Modifications on the red blood cells

Several polychromatophils (3% of red blood

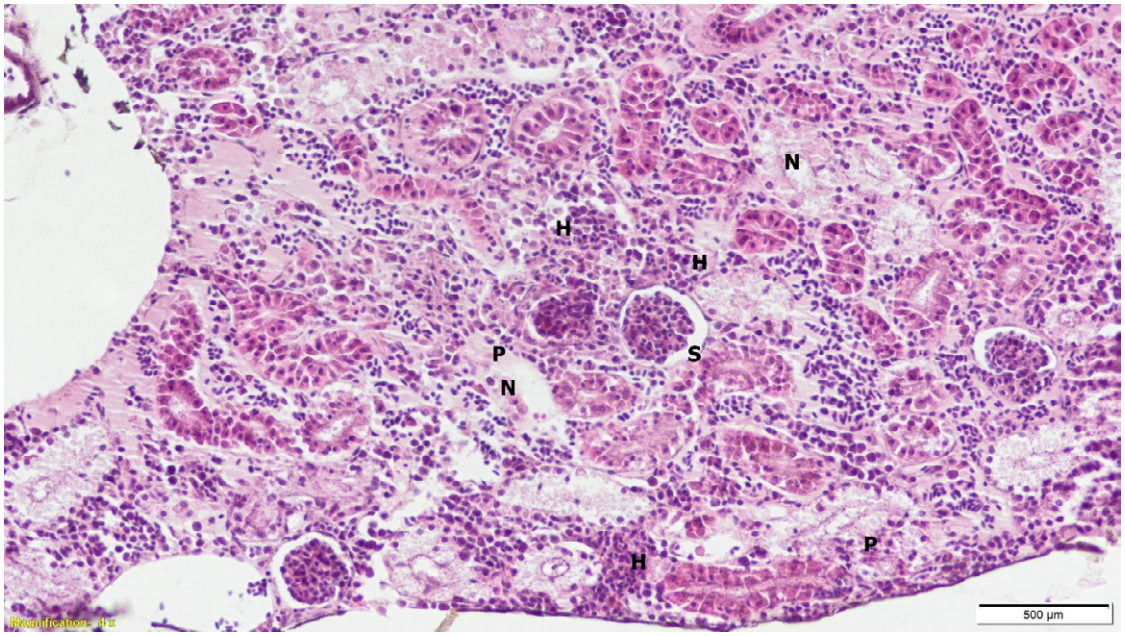

Fig. 5. Kidney histology by light microscopy after 21-day feeding with Ag-NPs (Group 4). Histological section of a zebrafish kidney stained with hematoxylin. (Gx200) :

(H) Hyperplasia in hematopoietic tissues ; (P) Pycnotic nuclei associated with (N) tubular Necrosis ; (S) increase on Bowman´s capsule Space

cells count) were noted in group 4 (Fig. 1.H), while we did not record polychromatophilia in the other fishes' groups.

Histological study

In zebrafish, haematopoietic tissue is located in the interstitium of the kidney and the stroma of the spleen. In adult zebrafish, haematopoiesis occurs mainly in the anterior and posterior renal interstitium. [25]. The histological section of the kidney of the individuals who received the highest doses of nanoparticles showed histopathological changes including hyperplasia of the haematopoietic tissue and images of tubular necrosis with increased Bowman's capsule space (Fig. 5).

DISCUSSION

Ag-NPs are extensively used in many fields, from therapeutics to industry to gastronomic decoration. This large use increases the probability of exposure and bioaccumulation of these nanoparticles in human, animals, and the wider environment, which raises legitimate concerns related to nanotoxicology. Our results suggest that the chronic toxicity of Ag-NPs (65 nm, nanosphere) to aquatic species is concentration dependent. This nanotoxicity generates stress to blood cells with immunotoxicity impact in zebrafish. Hence, the immune cell response to stress has been well studied in fish [28]. This trend has apparently continued among biologists. Thus, leukocyte responses to stress hormones can be measured [29] [13] and provide excellent reviews on these answers. Poisoning states also induce stress symptoms in fish WBC formula. Indeed, heavy metals exposure causes leukogram dysplasia [30]. In general, acute and chronic stress both induce neutrophilia and lymphopenia in fish [31]–[33].

The study of the zebrafish leukogram is used as an indicator of metabolic stress [34].

Several studies have highlighted the proinflammatory effects of the Ag-NPs [35].This observation can be explained by the fact that these nano-silver particles increase significantly the biochemical markers of oxidative stress [36].

The results show that for the control group and the group 2 (0.05 g of Ag-NPs/Kg i.e. with the lowest concentration of Ag-NPs), the leukocyte formula is typically physiological with a predominance of lymphocytes compared to the neutrophils [37]. There is no significant change between the

leukogram of control group and group 2. This suggests that this low concentration of Ag-NPs did not cause changes on the WBC formula.

However, as soon as the concentration of Ag-NPs increases, the percentage of the neutrophils rises compared to control group. There is a significant elevation of neutrophils blood count of the groups 3 (5 g/Kg of food) and 4 (50 g/Kg of food). This rise in the percentage of neutrophils is at the expense of lymphocytes. Thus, the NLR ratio is raised up by 240% and 317% compared to the control group for group 3 and group 4 respectively (Fig. 2). In addition, this ratio increased proportionally with the increase in the concentration of Ag-NPs in the diet. It should be noted that an increase in the segmentation of neutrophils was observed especially in the group 4, with the highest concentration of Ag-NPs.

NLR elevation is also observed following chronic stress on fish [38]. In both zebrafish and humans, chronic stress leads to elevated cortisol levels associated with a disorder of the immune defense mechanisms. Indeed, when a high concentration of stress hormones is diagnosed, there is an increase in the segmentation of neutrophil nuclei with a high NLR [34].

The route of administration and the duration of exposure influence the results obtained. *In vivo* experiments on mice show that after two days of parenteral injections of high concentrations of Ag-NPs, a dose-dependent modulating effect on the immune system is observed [14]. Whereas other work shows that oral administration of Ag-NPs in rats for 28 days does not show a significant immunological effect. However, to reach this conclusion, the authors performed *ex vivo* proliferation tests of T and B cells isolated from the spleen and mesenteric lymph nodes [17]. This variation in the results from our findings would be due to the method used.

Besides, microscopic observation of the erythroid lineage did not report any significant modification except for group 4 (50 g/Kg of food) where a polychromatophilia was noted. Polychromatophilia may be the consequence of erythroblastic cell proliferation. A similar result was reported in chickens. Studies established that Ag-NPs stimulate erythropoiesis in chickens [39], this observation is consistent with the images of hematopoietic cell hyperplasia observed (Fig. 5).

If in this study and others [40] Ag-NPs do not cause a notable modification in monocytes, it is quite different for the lymphocyte lineage. Indeed, a remarkable finding of this study is a change in the lymphocyte profile that occurs in subjects who have consumed low to medium levels of contaminated food with Ag-NPs. An interesting fact in the examination of the blood smears of group 2 is noticed in comparison with the control group, although there are no significant changes in the leukogram, lymphocyte activation is characterized. In contrast to group 2, group 3 with a higher concentration of Ag-NPs, show lymphocyte activation while NLR is elevated, this combination does not allow an optimal immune response.

In vitro, studies confirmed by flow cytometry that nanoparticles cause activation of human and murine lymphocytes after a few hours of exposure [40], [41]. Furthermore, it is reported in Humans that nanoparticle mediated Immunotoxicity [42].

Histological studies of hematopoietic organs such as the kidney in zebrafish reveal pyknosis lesions associated with tubular necrosis and enlarged glomerular chamber (Fig. 5). In addition, images of hematopoietic cell hyperplasia were revealed. Indeed, since the zebrafish kidneys are a filtration organ but also a hematopoietic organ, they are likely to accumulate exogenous nanoparticles, particularly Ag-NPs. This accumulation would have repercussions on both the filtration function and the hematopoietic function and therefore on the immune cells. This would explain the immunological impact of nanoparticles observed in zebrafish. Ag-NPs can act as immunomodulators depending on their characteristics and concentration [40], [43], [44]. Indeed, like most foreign particles, silver nanomaterials could induce an immune response. Blood cells belong to the innate and adaptive immunological systems. Innate immunity, represented by cells of the myeloid lineage (mainly granulocytes and thrombocytes in fish), have an immediate first-line action of defense without creating memory. The adaptive immune system, represented by cells of the lymphoid lineage (mainly T and B lymphocytes), evolve and develop an immune memory against foreign antigens [45], [46]. This memory immune response is particularly important in vaccinology to obtain effective immunity against the vaccine candidate. Immune response modulators are being particularly studied for use as adjuvants. Silver nanomaterials could induce a cascade of inflammatory reactions in the immune system, implying the activation of neutrophils, macrophages and T-helper

lymphocytes, and therefore triggering the release of a multitude of cytokines. These molecules are naturally secreted into the body's normal natural defenses to fight disease. However, in some cases the uncontrolled elevation of cytokine levels in response to nanomaterials can lead to serious side effects, such as excessive systemic inflammation [47]. However, this property of Ag-NPs to activate immune cells might offer exciting future opportunities in vaccine design. Indeed, the action of nanoparticles could be geared to modulate the immune response [48]. Depending on the design of the nanoparticles, they can induce either a suppression or a stimulation of the of the immune system. Ag-NPs could be used as suppressors of the immune response or as anti-inflammatory agents in the management of cancer, autoimmune diseases or inflammatory disorders [49].

In another register, Ag-NPs could be used as immune response stimulators, for example in vaccines based on nanosilver particles that boost immunization. In fact, the use of these Ag-NPs does not present only disadvantages. Ag-NPs are already used in several biomedical antibacterial, antiviral and antitumor applications [50].

Lymphocyte activation following exposure to low concentrations of Ag-NPs observed in this study could explain the adjuvant action of silver nanoparticle-based vaccines. Indeed, these properties of Ag-NPs offer the potential to reduce the number of booster doses needed to get a protective vaccine response, in one oral administration, as reported in a poultry study [51]. This could help optimize the vaccine approach, especially in the post-COVID-19 era.

CONCLUSION

The incorporation of the Ag-NPs at high concentrations into zebrafish feeding revealed a lymphopenia and a neutrophilia. In addition, the NLR increased proportionally with the increase in the concentration of Ag-NPs in the diet. The use of Ag-NPs as additive food (E174) should be subject to stricter control by the competent authorities. The overuse of these fine particles, in packaging and in many food products, leads to a chronic bioaccumulation of these nanoparticles in the body. Microscopic examination of WBC may provide an early warning of adverse effects on the organism and can be used to monitor the effectiveness of exposure mitigation measures.

Another finding of this work reports that at low doses, Ag-NPs can activate lymphocytes but do not alter the NLR, demonstrating an immunostimulatory activity. These results could benefit the design of new innovative nanodrugs.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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