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Histo-immunological Aspects of ZnO Nanoparticles in Mice (*Mus musculus*)

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Abstract:

This study aimed to investigate the histo-immunological aspects of zinc oxide nanoparticles (ZnO-NPs) in mice (*Mus musculus*), focusing on the thymus and spleen as the two main target organs involved in the immune system. A group of 75 BALB/c male mice of ca. 6 weeks of age were placed in glass cages held at a room temperature of 20±2^{0}C, a 12:12 light/dark cycle and 50–70% relative humidity. After a 2-week acclimation period, the mice were randomly divided into two groups (n=25): a control group and a ZnO-NPs orally-administered group. The animals were fasted overnight before treatments were offered. Sampling was performed on days 1, 7 and 14 of the experiment. An effect of ZnO-NP exposure was reflected in the lymphocyte count, where the total number of lymphocytes decreased, suggesting inflammatory reactions. The ZnO-NPs were also associated with increased TGF-β levels. Histological assessment of thymus and spleen used to verify the toxicity of ZnO-NPs demonstrated that ZnO-NPs induce diverse pathological lesions in both organs. This study suggests that exposure to ZnO-NPs can induce inflammation in the thymus and spleen tissues, as two main target organs involved in the immune system.

Keywords: ZnO-nanoparticles, TGF-β factor, thymus, spleen, lymphocyte, mice

INTRODUCTION

The molecular events that regulate bioaccumulation and toxicity of nanoparticles (NPs) are increasing. Nanoparticles are particles with lengths that range from 1-100 nm in two or three dimensions[1]. As NPs are of a similar size to typical cellular components and proteins, they may bypass natural mechanical barriers, possibly leading to adverse tissue reaction[2]. The behavior of NPs inside the cells is still unclear and no metabolic and immunological responses induced by these particles are understood thus far[3]. The immunotoxic potential and ability of various NPs including poorly soluble NPs of low toxicity, such as nano-sized titanium dioxide (TiO2) and carbon black (CB)[5-7] to alter immune responses has been documented[4]. NP-induced oxidative damage could be one of the leading factors causing an immune imbalance, because oxidative stress increases in pathological situations and there is a relationship between oxidative stress and inflammation.

Zinc (Zn) is an essential trace element usually found in dietary supplements. Its deficiency results in a variety of disorders including growth retardation, infections, cancer, skin diseases, and slow wound healing[8]. Because of their large surface area, ultra-high reactive surface sites and quantum effects, nanoscale metal powders are being widely used in the traditional industries such as nano-electronics, opto-electronics, nano-generators, sensors, light-emitting diodes, field emission, photocatalysis and nanopiezotronics[9-11].

ZnO-NPs are utilized in various applications including cosmetics, textiles, food additives, and personal hygiene products[12] and in spite of their extensive usage, very few reports on the toxicity of these NPs are available. Health effects of nanoparticle penetration of the human body are still largely unknown. Numerous studies have focused on the inflammatory responses to nanoparticles [13-16]. For instance, it has been demonstrated that inhalation of Carbon Nanotubes (CNTs) suppresses B cell function and that the TGF-β produced by alveolar macrophages is a key factor in the mechanism of the observed immunosuppression[17].

Transforming growth factor (TGF) is a protein secreted by 'transformed' cells that can stimulate the growth of normal cells[18,19]. The TGF-β is known to increase in response to injury and during inflammation [20] and acts as a potent chemo-attractant for monocytes as well as macrophages, neutrophils, lymphocytes, and fibroblasts. It induces release of other growth factors and stimulates its own autoexpression [21].

ZnO-NPs are usually absorbed and transported via the blood stream reaching body organs such as the thymus, spleen, and kidney[22]. The ZnO-NPs accumulate in the body and induce the generation of intracellular reactive oxygen species (ROS) which trigger a decrease in mitochondrial membrane potential (MMP) with a simultaneous increase in the ratio of Bax/Bcl2 leading to mitochondrial-mediated apoptosis.

In particular, increases in apoptosis and the expression of tumor necrosis factor-a (TNF-a) have been observed in the thymus[22]. Recent studies suggest that increased activities in transforming growth factor β and glucocorticoids are involved in the regulation of a variety of genes in the thymus [23,24].

The thymus is a specialized organ of the immune system in which lymphocytes mature. It is located anatomically in the anterior superior mediastinum and composed of two identical lobes. Histologically, each lobe of the thymus can be divided into a central medulla and a peripheral cortex which is surrounded by an outer capsule. Another important organ involved in the immune system is the spleen, the largest secondary lymphoid organ which initiates immune responses to blood-borne antigens [25-27]. Studying the thymus and spleen organs may provide insight into the mechanisms governing the effects of nanoparticles on the body. This study aimed to investigate the histo-immunological aspects of ZnO-NPs in mice

(*Mus musculus*), focusing on the thymus and spleen as the two main target organs involved in the immune system.

MATERIALS & METHODS

Mice holding

Animal experimentation was performed in compliance with the ethics committee of the Shahrekord University. A group of 75 BALB/c male mice of ca. 6 weeks (weighting 20.2±3.0 g; mean weight±SD) were purchased from the Medical Faculty of Shahrekord University and then transferred into the University laboratory. The mice were held in a single group and fed with a commercial pellet diet and offered deionized water *ad libitum*. The animals were placed in glass cages at a room temperature of 20±2 ◦C, a 12:12 light/dark cycle and 50–70% relative humidity. After a 2-week acclimation period, the mice were randomly divided into two groups (n=25 per group): a control group and a ZnO-NPs (2.5 mg/kg) orally-administered group The animals were fasted overnight before being offered the treatment.

Preparation of Zno-NPs:

Zinc oxide nanoparticles (ZnO-NPs) were purchased from the Iranian Nanomaterial Pioneers Co. with a purity of 99%. The final concentration of the solution was 0.05g/ml. According to the manufacturer, the average diameter of ZnO-NPs was 20 nm (in the 10-30 nm range). Table 1 shows more detailed information provided by the company.

Table 1: Physicochemical properties of ZnO-NPs provided by the company

Chemical formula	Color	morphology	APS[a] (nm)	SSA[b] (m2/g)	TD[c] (g/cm3)	Purity[d] (%)
ZnO	White	Elongated	10–30	200-600	5.606	+99

a- Average particle size measured by high resolution SEM with charge compensation system.
b- Specific surface area measured by Brunauer, Emmett and Teler (BET) technique.
c- True density.
d- Purity level measured by inductive coupled plasma-mass spectroscopy (ICP-MS) technique.

A solution of ZnO-NPs in distilled water at a concentration of 50 mg/ml was prepared, treated by ultrasound for 15-20 min and mechanically vibrated for 3 min to prepare nanoparticle suspensions. The mice received a single dose of ZnO-NPs suspension at 2.5 mg/kg by oral gavage.

Serum preparation &biochemical analysis of blood serum

The mice were anesthesized by intramuscular injection of 10 ml ketamine, 0.5 ml acepromazine, 2 ml diazepam and about 0.5 ml xylazine solution at a dose of 50 mg/Kg.Blood was taken directly from the heart of each mouse on days 1, 7 and 14 of the experiment.

Blood samples were centrifuged for 15 min at 3000 rpm and lymphocyte counts noted. The level of TGF- β was measured with a commercial ELISA kit (Boster mouse TGF-β ELISA, from R&D Systems, China).

Statistical analysis

The results were presented as mean ± SD. The data were checked for normality and homogeneity of variance. Statistical analysis was conducted by one-way ANOVA using SPSS (version 21). A p-value less than 0.05 was considered to be statistically significant.

RESULTS

Ann effect of ZnO-NPs exposure was detected on the lymphocyte count, where the total number of lymphocytes decreased, although this reduction was not significant.

Serum level of the TGF-β was measured. One day following exposure, no changes were found in the level of TGF-β in ZnO-NPs-treated groups of mice, as compared to the control. At day 7 of the exposure however, the level of TGF-β increased in mice treated with ZnO-NPs, after treatment with ZnO-NPs, TGF-β levels increased from 0.391 to 0.416.

Thymus and spleen were congested (abnormal accumulation of blood in them), suggesting toxicity of these organs with ZnO-NPs. Significant histopathological lesions were noted in the sampled organs among the control and ZnO-NPs oral exposure, which suggests that the oral administration of ZnO-NPs in mice caused obvious adverse effects. Intense vacuoles were observed in the cytoplasm of thymus cells (Figure 1).

In addition to lymphocytes, many neutrophils were found in the spleen of ZnO-NPs-treated mice. In some parts of the spleen, penetration and accumulation of blood cells into the interstitial tissues were observed. The histopathologic findings of ZnO-NP-induced inflammation in these two organs are shown Figures 1 and 2. The treated animals showed distinct morphological changes on microscopic observation, indicating unhealthy cells.

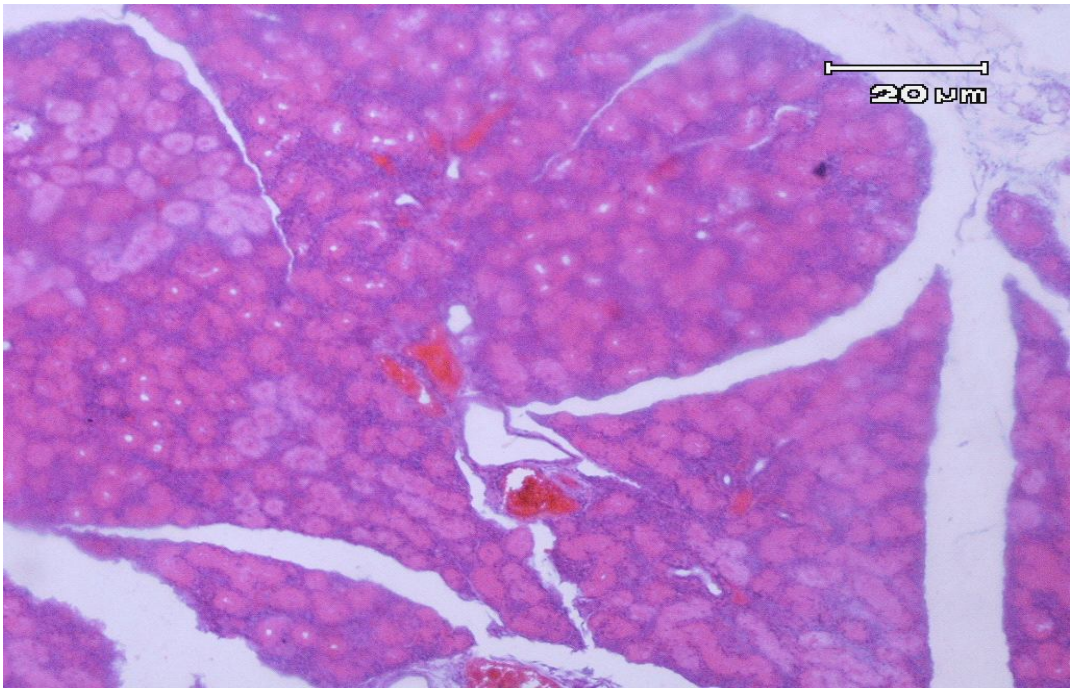

Figure 1: A histological examination results of orally administered ZnO-NPs mice thymus stained with hematoxylin and eosin (×200). Arrow shows congestion of thymus which indicates toxicity of this organ with ZnO-NPs.

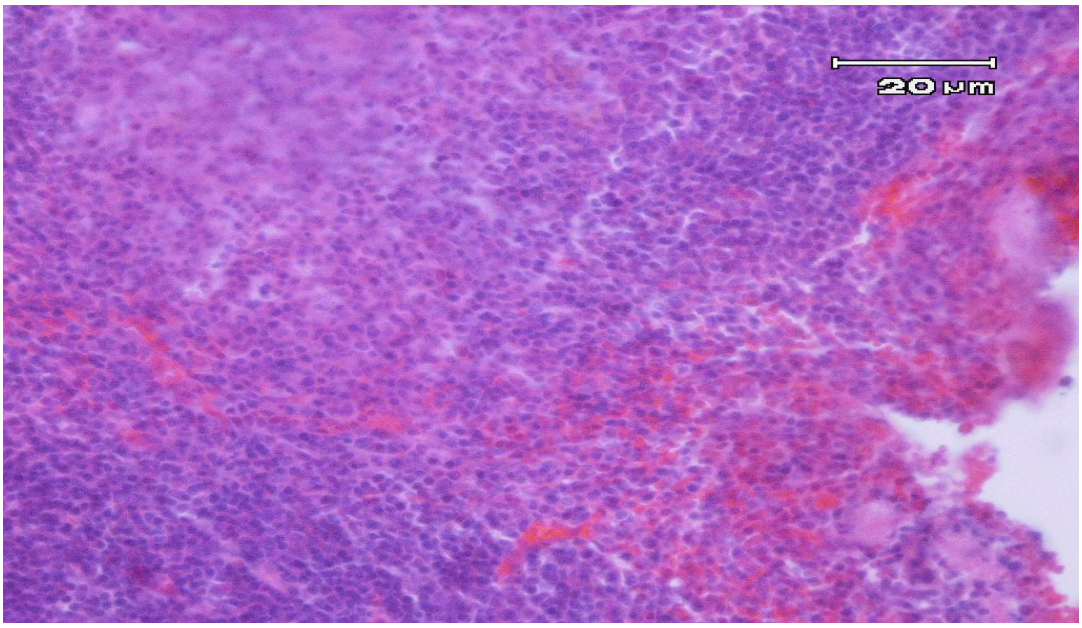

Figure 2: A histological examination results of orally administered ZnO-NPs mice spleen stained with hematoxylin and eosin (×200). Congestion can be seen in some parts of the white pulp. An obvious necrosis with neutrophil and lymphocyte infiltration indicate toxicity of this organ with ZnO-NPs.

DISCUSSION

To investigate the immunotoxic effects of ZnO-NPs exposure in mice, we examined the total lymphocyte count and TGF-β level with histology of thymus and spleen. It was we found that exposing mice to ZnO-NPs decreased the total number of lymphocytes but increased expression of TGF-β. This indicates that ZnO-NPs could induce inflammatory reactions in mice. The symptoms of congestion in thymus and spleen with significant histopathological lesions in orally-administered ZnO-NPs mice suggest that these organs must be affected by ZnO-NPs toxicity.

Nowadays, nanotoxicology research is now gaining attention. This is due to many special physicochemical properties of nanoparticles which may yield extraordinary hazards for human health and the environment[28]. With the rapid developments in nanotechnology, ZnO-NPs are increasingly being used in various aspects of our lives. Studies have shown that ZnO-NPs have toxic effects on the mammalian cell and induce inflammation [29]. The toxic effects of ZnO-NPs on many different cell types have been demonstrated [13, 30-34]

However, there are few *in vivo* animal studies providing evidence of their toxicity. Different studies have demonstrated the correlation between NP toxicity and ions released from ZnO-NPs[19,30,35-36]. In fact, Zinc ion, which is present due to the solubility of ZnO-NPs, seems to be responsible for inducing inflammatory responses[37].

For example, an increase in intracellular zinc ion was found in BAL cells and white blood cells from rats after 38 nm ZnO-NP inhalation [38]. Moreover, the capacity of ZnO-NPs to generate reactive oxygen species (ROS) correlates with their potential to induce cellular inflammation[37,39]. Thus, induction of oxidative stress is the most important or most likely mechanism underlying ZnO-NPs toxicity[4].

A reduction in the number of lymphocytes in the thymus, spleen, and blood has been detected in experimental animals with inflammation[40,41]. Similarly, in this study we found that exposing mice to ZnO-NPs decreased the total number of lymphocyte. As low lymphocyte count is a common phenomenon during inflammatory response[42], this finding is consistent with the hypothesis that ZnO-NPs could induce inflammation in mice.

TGF-β is a multifunctional growth factor that can either stimulate or inhibit cell proliferation, mainly depending on cell type and culture conditions[43]. It is the growth factor affecting all cell types that are involved in inflammation[12,20]. It is released by macrophages and acts as a potent chemo attractant for different types of white blood cells including monocytes, macrophages, neutrophils, lymphocytes as well as fibroblasts. In this study, the ZnO-NPs were associated with increased TGF-β levels. Similar results were found in Wistar rats in which the levels of TGF-β were significantly increased at 1 and 4 weeks after instillation of ZnO-NPs[12].

In the current study, histological assessment of thymus and spleen tissue was used to verify the toxicity of ZnO-NPs in mice. It was found that ZnO-NPs induce diverse pathological lesions in both thymus and spleen organs. The representative pathological lesions could be classified as blood infiltration into the interstitial tissue and producing inflammation. In fact, an obvious necrosis with neutrophil and lymphocyte infiltration were observed in the area. These observations are consistent with previous studies regarding ZnO-NPs cytotoxicity [29,30,44] and prove that these NPs induce inflammation.

Since oxidative stress is a critical determinant of ZnO-NPs-induced damage, thus the thymus and spleen damages seen in the current study may be explained through generation of ROS associated with inflammatory, oxidative, genotoxic, and cytotoxic events; and induction of apoptosis[12,44,45]. This is owing to the fact that oxidative stress is an important reason for damage induced ZnO-NPs [12,29,45]. This study reveals the effects of ZnO-NPs on blood lymphocytes, TGF-β and histology of thymus and spleen, providing significant insight into the possible mechanism through which ZnO-NPs exert their toxic effects on healthy cells.

Our results suggest that exposure to ZnO-NPs can induce inflammation in the thymus and spleen tissues, as two main target organs involved in the immune system. This was indicated by dysregulation of lymphocyte populations and expression of TGF-β in orally administered ZnO-NPs mice. Significant histopathological lesions were observed in the aforementioned organs, suggesting ZnO-NPs toxicity with obvious adverse effects. Further works are required to more thoroughly elucidate the mechanisms involved.

CONCLUSIONS

It can be concluded from this study that exposure to ZnO-NPs can induce inflammation in the thymus and spleen tissues, as two main target organs involved in the immune system. This was indicated by dysregulation of lymphocyte populations and expression of TGF-β in orally administered ZnO-NPs mice.

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Conflict of Interest

The authors declare no conflict of interest.

Animal rights

The procedures followed were in accordance with the ethical standards of the responsible committee on animal experimentation of Shahrekord University.

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