# Effect of titanium dioxide nanoparticles on the spleen of adult male albino rats: Histological and immunohistochemical study

Original Article

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## ABSTRACT

**Introduction:** Titanium dioxide nanoparticles ($TiO_2$ NPs) are commonly used in industry (e.g. cosmetics, sunscreens, food products, paints and drugs). But, they have different adverse cellular effects including oxidative stress and cellular damage.
**Aim of the work:** To evaluate the possible toxic effect of titanium dioxide nanoparticles on the structure of the spleen of rats.
**Materials and Methods:** Forty-five adult male rats were divided into three groups. Group I served as the control group. Group II (low-dose group) rats were given oral dose of $TiO_2$ (600 mg/kg/day) daily for 8 weeks. Group III (high-dose group) rats were given a daily oral dose of $TiO_2$ (1200 mg/kg/day) for 8 weeks. Spleen specimens were taken after 4 weeks, 8 weeks and 12 weeks from the start of the experiment. Thereafter, spleen specimens were processed for histological and immunohistochemical examinations.
**Results:** The low-dose group showed slight disturbance in architecture of white pulp, slight congestion in red pulp and significant increase ($P< 0.01$) in CD4 and CD68 immuno-expression only after 8 week specimens compared with control group. The high-dose group showed marked disturbance in architecture of white pulp and significant increase ($P< 0.01$) in CD4 and CD68 immuno-expression after 4 week specimens, CD4 and CD68 immuno-expression significantly decreased ($P< 0.01$) in the 8 and in the 12 week specimens. No obvious improvement occurred after 12 weeks (withdrawal specimens).
**Conclusion:** Prolonged exposure to daily high oral dose of $TiO_2$ NPs has deleterious and toxic effects on the spleen of rat, besides disruption of its structure.

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**Key Words:** Spleen, titanium dioxide nanoparticles.

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## INTRODUCTION

In the last three decades, nanotechnology had flourished significantly and has transitioned from bench top science to applied technology. Whereas, nanomaterials (NMs) have been exclusively developed as well as widely applied in a broad variety of products including medicine, industry, personal care products and cosmetics[1, 2]. The wide use of nanotechnology raised concern about their adverse effects. There is increasing need to evaluate risk associated with their use, as humans potentially exposed to nanoparticles (NPs) due to their wide use in diagnosis or therapy[3,4].

Titanium dioxide ($TiO_2$) nanoparticles are noncombustible odorless powder that have been widely used in various industries such as paints, printing, food, toothpaste, medicine and cosmetics due to their high stability, anticorrosion, and efficient photo catalysis[5,6]. Moreover, $TiO_2$ has been proved to be efficient in killing antibiotic resistant bacteria by destroying bacterial spores[7].

The advantageous combination of its biological and physico-chemical properties, resulted in that Titanium was extensively used for a broad range of implanted medical devices, for example, dental implants, joint replacements, cardiovascular stents, and spinal fixation devices. However, any change in the physiological conditions like decreasing pH or under mechanical stress, titanium-based implants can release huge amounts of particle debris, in the nano and micro size range[8].

The tiny size of NPs has the ability to penetrate basic biological structures, which may in turn, disturb their normal function[9, 10]. Tissue deposition of NPs and their toxicity are mostly related to the route of exposure. Titanium dioxide nanoparticles could penetrate the skin inducing oxidative damage of DNA, enter systemic circulation and migrate to various organs as brain where they remain trapped promoting varying degrees of tissue damage[11, 12].

Spleen is a large, encapsulated, hemo-lymphatic complex structure[13]. It looks like a large lymph node; however, the spleen sinuses are filled with blood[14, 15]. Its

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stroma is composed of capsule, trabeculae, reticular fibers, and fibroblasts. Its parenchyma is composed of white pulp and red pulp form[16, 17].

The splenic artery branched into the parenchyma of the spleen from the hilum, branching within trabeculae. In their terminal few millimeters, their connective tissue adventitia is replaced by a sheath of T-lymphocytes, the peri-arteriolar lymphatic sheath (PALS). Frequently, enclosed within the PALS are lymphoid follicles (LFs) which are composed of B cells and displace the central arteriole to a peripheral position. Primary and secondary LFs together display an outer marginal zone, composed of lymphocytes with abundant cytoplasm[13, 18, 19]. Red pulp is composed of venous sinuses filled with blood and cords of splenic tissue called splenic (Billroth's) cords. Splenic cords consist of lymphocytes, red blood cells, macrophages, plasma cells, and granulocytes[17].

Spleen performs both immune and hematopoietic functions. The white pulp is responsible for immune action as it has phagocytic activity, while the red pulp is responsible for filtration[20].

## AIM OF THE WORK

The present study aimed to evaluate the possible toxic effect of titanium dioxide nanoparticles on the structure of the spleen of adult male Albino rats.

## MATERIALS AND METHODS

### *Drugs*

Titanium dioxide (Ex-Pure): Cas No. 13463-67-7, molecular weight: 79.87, powder particle size:80 nm, purity 99-100% (Neminath Industrial Estate No. 6 Navghar, Vasai (E), Dist. Thane, Maharashtra, India.) was used. One gram of the powder was dissolved in one mL of 5% gum acacia solution as a solvent and was given orally by gastric gavage in one of two doses. On a daily basis, the low-dose (600 mg/Kg/day) was given for eight weeks also the high-dose (1200 mg/ Kg/day) was given for eight weeks [21,22].

### *Animals*

After permission from the Institutional Animal Care Committee of Benha Faculty of Medicine , 45 adult male albino rats (150-200) g were obtained from the animal house, Moshtohor Faculty of Veterinary Medicine, Benha University. The animals were served in animal cages under the exiting atmospheric conditions as strict cleaning measures and care to keep them in a normal healthy state with normal equitable diet and tap water.

### *Experimental procedure*

The rats were divided into three equal groups; 15 animals each. Group I (control group): rats were given oral gum acacia solution daily for 8 weeks, then left without any treatment until the12th week.

Group II (low dose group): rats were given nanosized $TiO_2$ (600 mg/ Kg/day) orally by gastric gavage daily for 8 weeks, then left without any treatment until the 12th week.

Group III (high dose group): rats were given nanosized $TiO_2$ (1200 mg/ Kg/day) orally by gastric gavage daily for 8 weeks, then left without any treatment until the 12th week.

The fasted rats were anesthetized by ether and sacrificed by cervical decapitation. Spleen specimens were taken after 4, 8 and 12 weeks (withdrawal specimens) in all groups from the start of experiment. After taking the specimens, the animals (5 rats at each time) were eliminated by incineration in Benha University Hospital incinerator.

### *Light microscopic studies*

The specimens were fixed in 10% buffered formol saline and processed to prepare paraffin sections of 5-7 µm thickness, mounted on glass slides for H & E stain[23]. Other sections were mounted on +ve charged slides for immunohistochemical study[24] using avidin–biotin immunohistochemical staining for detection of CD4 (marker for T helper cells) and CD68 (marker for white pulp macrophages).

### *Electron microscopic studies*

Specimens from the spleen were fixed in 3% glutaraldehyde and post-fixed in 1% osmium tetraoxide. Semithin sections (0.5-1 um thick) were prepared and stained with toluidine blue. Ultrathin sections (500-800A) were cut from selected areas in semithin sections, mounted on cupper grids and counterstained with uranyl acetate and led citrate. Specimens were processed[25] and examined using the transmission electron microscope JEOL (JEM-100 SX Akishima, Tokyo, Japan) in Electron Microscope Unit, Faculty of Medicine, Tanta University.

### *Immunohistochemical staining for CD4 and CD68*

Immunohistochemistry was performed according to standard protocol for the used kits. Paraffin sections were deparaffinized then hydrated. After blocking the endogenous activity of peroxidase using 10% hydrogen peroxide, the sections were incubated with primary antibodies; Rabbit Polycolonal CD4 and CD68 antibody (Lab Vision Corporation, Neomarkers Laboratories, Westinghouse, Thurmont, California, USA). The specimens was washed with phosphate buffer then, the secondary antibody was applied (biotinylated goat anti rabbit). The slides were incubated with labeled avidin–biotin peroxidase, to enable binding of biotin to the secondary antibody. The site of antibody binding appeared as a brown precipitate that was visualized after adding (diaminobenzidine) chromogen, by peroxidase. Sections were counterstained with Meyer's hematoxylin. Phosphate-buffered saline (PBS) was used instead of the primary antibody in the negative control.

Positive immunohistochemical staining of CD4 (marker for T helper cells) and CD68 (marker for white pulp macrophages) was demonstrated as brown cytoplasmic staining in a specimen of human tonsil.

### *Morphometric examination*

The mean area percentage of CD4 and CD68 immuno-expression was quantified in five images from five non-overlapping fields of each rat in all groups using Image-Pro Plus program version 6.0 (Media Cybernetics Inc., Bethesda, Maryland, USA). The digital photos were captured in the Microscopic Photography Unit (Histology Department, Faculty of Medicine, Benha University) using a Leica DM2500 optical microscopes.

### *Statistical analysis*

All the data collected from the morphometric study were recorded and analyzed using IBM SPSS Statistics software for Windows, Version 20 (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) with Post Hoc LSD test was used to compare differences among the groups of morphometric results at the same time samples. In each test, the data was expressed as the mean (M) value, standard deviation (SD) and differences were considered to be significant at $P < 0.01$.

## RESULTS

### *H&E stained sections*

Group I (Control Group) showed that the histological structure of spleen at the three time specimens consists of, the parenchyma of the spleen consisted of white and red pulps (Fig. 1). The white pulp was formed of lymphoid follicles with an eccentrically located central arteriole which surrounded by a periarterial lymphatic sheath (PALS). The lymphoid follicles appeared to compose of a germinal center (only found in secondary follicles) which surrounded by a ring of lymphocytes (mantle zone), which was surrounded by the marginal zone that contains lymphocytes. A marginal zone (MZ) demarcated the splenic lymphoid follicle from the red pulp. The red pulp appeared to be formed of branching splenic cords (Billroth cords) and blood sinusoids in between (Fig. 2). Group II (low dose $TiO_2$ group) showed slight disturbance in architecture of white pulp after the 4th week specimens (Fig. 3). The 8th week specimens showed dilatation in central arteriole, enlargement and disturbance in architecture of white pulp and slight congestion in red pulp (Fig. 4). The 12th week showed slight dilatation in central arteriole and disturbance in architecture of white pulp (Fig. 5). Group III (high dose $TiO_2$ group) showed dilatation and distortion of central arteriole with marked disturbance in architecture of white pulp after 4th week specimens (Fig. 6). The 8th week specimens showed contracted central arteriole with shrinkage white pulp and congested red pulp (Fig. 7). The 12th week specimens showed contracted central arteriole with marked disturbance in architecture of white pulp and congested red pulp (Fig. 8).

### *Immunohistochemical results*

#### *CD4*

Group I showed cytoplasmic immuno-expression for CD4 mainly in the germinal center of lymphoid follicle and in marginal zone of white pulp (Fig. 9). In the 4th week specimens of Group II, immuno-expression of CD4 was apparently similar to that seen in control sections (Fig. 10). An apparent increase of the reaction was observed in the 8th week specimens (Fig. 11). In the 12th week specimens, reduction of the immuno-expression of CD4 was noticed (Fig. 12). In the 4th week specimens of Group III, immuno-expression of CD4 was apparently increased compared with that of control group (Fig. 13). An apparent decrease of the reaction was seen in the 8th week specimens (Fig. 14) and in the 12th week specimens (Fig. 15).

#### *CD68*

Group I showed cytoplasmic immuno-expression for CD68 in white pulp mainly in the germinal center of lymphoid follicle and in marginal zone (Fig. 16). In the 4th week specimens of Group II, immuno-expression of CD68 was apparently similar to that seen in control sections (Fig. 17). An apparent increase of the reaction was detected in the 8th week specimens (Fig. 18), whereas in the 12th week specimens, reduction of the immuno-expression of CD68 was noticed (Fig. 19). In the 4th week specimens of Group III, immuno-expression of CD4 was apparently increased compared with that of control group (Fig. 20). An apparent decrease of the reaction was observed in the 8th week specimens (Fig. 21) and in the 12th week specimens (Fig. 22).

### *Morphometric results*

The mean area %, standard deviation (SD) of CD4 and CD68 immuno-expression for all groups were represented in Tables 1, 2 and Histograms 1, 2. Compared with control group, there was a significant increase in the mean area % of CD4 and CD68 immuno-expression in 4th week specimens of group III (high dose group) and insignificant increase in low dose group. In addition, there was a significant decrease in the mean area % of CD4 and CD68 immuno-expression in 8th week and 12th week specimens of group III while in group II, the 8th week sample showed a significant increase, insignificant increase was also detected in the 12th week specimens.

### *EM results*

Group I showed multiple plasma cells, neutrophils, lymphocytes and RBCs. Lymphocytes had a condensed chromatin pattern in their nuclei that were surrounded by a thin rim of cytoplasm (Fig. 23). The plasma cell appeared normal with large spherical nucleus, well-developed rough endoplasmic reticulum which extends throughout the

cytoplasm and multiple mitochondria (Fig. 24). Group II showed the plasma cells with hyperchromatic nuclei in the 4th week specimens (Fig. 25). The 8th week specimens showed hyperchromatic nuclei, slight dilatation in the cisterna of ER and mild vacuolations in some mitochondria of plasma cells (Fig. 26). The 12th week showed nearly the same picture as that seen in control sections (Fig. 27). Group III showed hyperchromatic nucleui and slight dilatation in the cisterna of endoplasmic reticulum of the plasma cells in the 4th week sample (Fig. 28). Some cisterna of ER appeared widely separated, some mitochondria showed destruction in their cristae and there were multiple vacuoles in the cytoplasm of the plasma cells in the 8th week samples (Fig. 29). By the 12th week, samples showed wide separation of some cisterna of ER, and some mitochondria showed destruction in their cristae in addition to multiple vacuoles in the cytoplasm of the plasma cells (Fig. 30).

**Table 1:** Showing the mean (M), SD of the CD4 immuno-expression at 4th, 8th, and 12th week for all groups with comparison between all groups by Post Hoc LCD test. Significance (Sig.) at $P < 0.01$

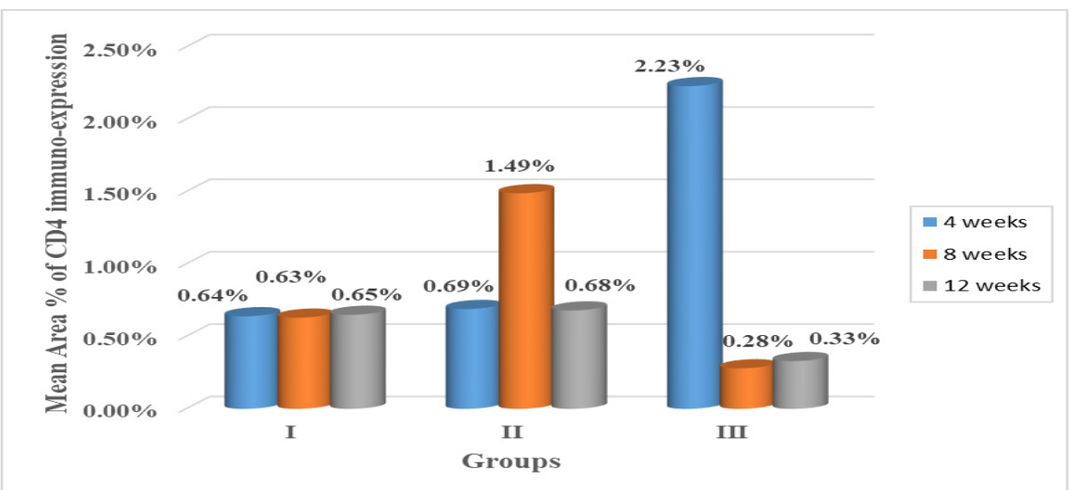
| Week | Group I | | | Group II | | | Group III | | |
|---|---|---|---|---|---|---|---|---|---|
| | M | SD | Sig. | M | SD | Sig. | M | SD | Sig. |
| 4 | 0.64 | 0.0381 | c | 0.69 | 0.0230 | c | 2.23 | 0.2971 | a,b |
| 8 | 0.63 | 0.0339 | b, c | 1.49 | 0.1534 | a,c | 0.28 | 0.0468 | a,b |
| 12 | 0.65 | 0.0381 | c | 0.68 | 0.0590 | c | 0.33 | 0.0327 | a,b |

a= Sig. with group I, b= Sig. with group II, c= Sig. with group III

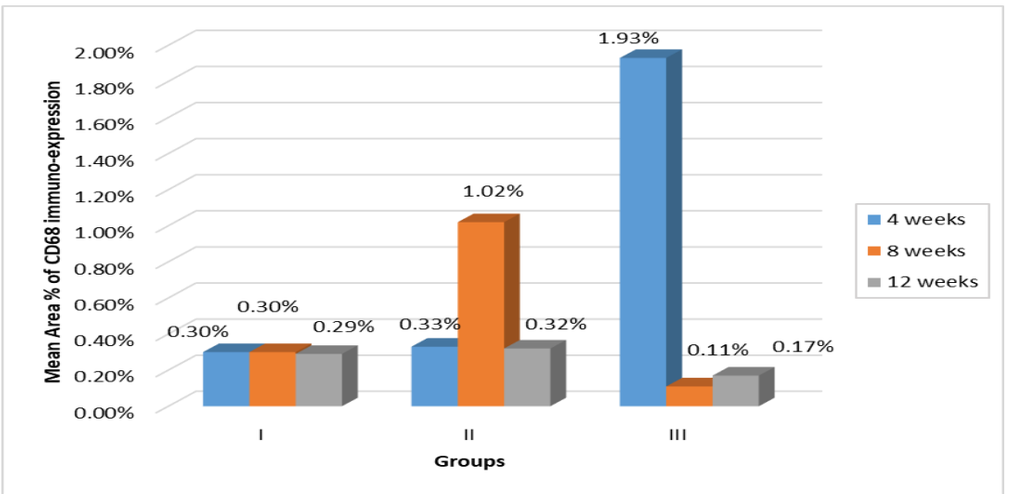
**Table 2:** Showing the mean (M), SD of the CD68 immuno-expression at 4th, 8th, and 12th week for all groups with comparison between all groups by Post Hoc LCD test. Significance (Sig.) at $P < 0.01$

| Week | Group I | | | Group II | | | Group III | | |
|---|---|---|---|---|---|---|---|---|---|
| | M | SD | Sig. | M | SD | Sig. | M | SD | Sig. |
| 4 | 0.30 | 0.0365 | c | 0.33 | 0.0308 | c | 1.93 | 0.0644 | a,b |
| 8 | 0.30 | 0.0339 | b,c | 1.02 | 0.0858 | a,c | 0.11 | 0.0218 | a,b |
| 12 | 0.29 | 0.0247 | c | 0.32 | 0.0357 | c | 0.17 | 0.0302 | a,b |

a= Sig. with group I, b= Sig. with group II, c= Sig. with group III




**Histogram 1:** Showing the mean of the CD4 immuno-expression at 4th, 8th, and 12th week in groups I, II and III.




**Histogram 2:** Showing the mean of the CD68 immuno-expression at 4th, 8th, and 12th week in groups I, II and III.

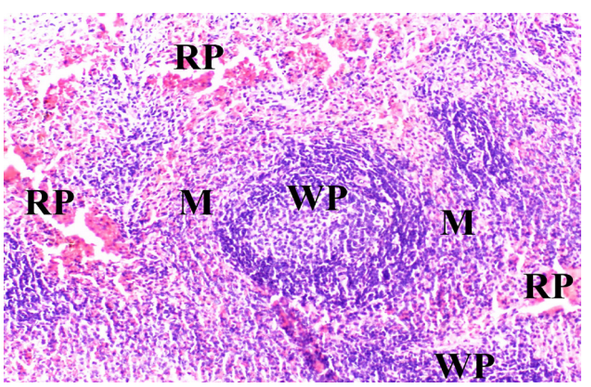



**Fig. 1:** A photomicrograph of a section in spleen of group I (control group) showing the white pulp (WP), red pulp (RP) and the marginal zone (M) in between. [H&E, X200]

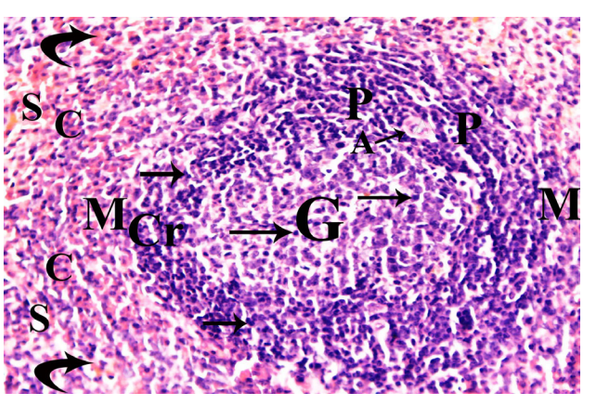



**Fig. 2:** A higher magnification of the previous photomicrograph showing the white pulp containing the central arteriole (A), peri-arterial lymphatic sheath (P) and lymphatic follicle that is composed of germinal center (G) and peripheral (coronal) zone (Cr) with lymphocytes (arrow). The red pulp contains cords of lymphocytes (C) and blood sinusoids (S) with few RBCS inside (curved arrow). Note the well-defined marginal zone (M) [H&E, X400]

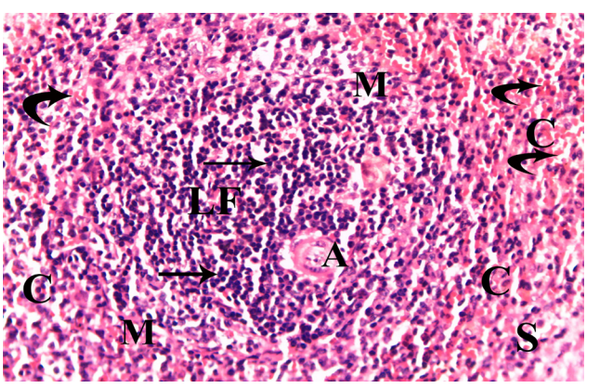



**Fig. 3:** A photomicrograph of a section in spleen of group II (4th week specimens) showing the white pulp containing the central arteriole (A), slightly disturbed lymphatic follicle (LF) with lymphocytes (arrow). The red pulp contains cords of lymphocytes (C) and blood sinusoids (S) with few RBCS inside (curved arrow). Note the well-defined marginal zone (M). [H&E, X 400]

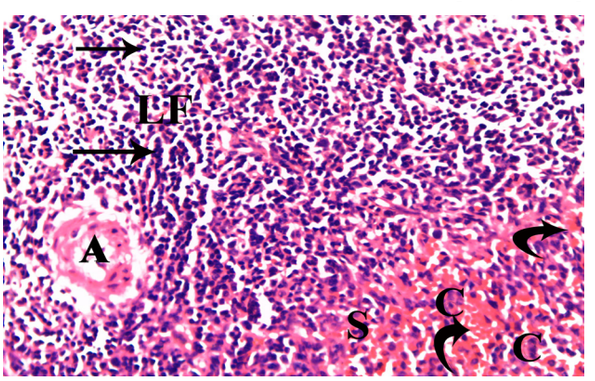



**Fig. 4:** A photomicrograph of a section in spleen of group II (8th week specimens) showing the white pulp with slightly dilated central arteriole (A), disturbed and enlarged lymphatic follicle (LF) with lymphocytes (arrow). The red pulp contains cords of lymphocytes (C) and blood sinusoids (S) with excess RBCS inside (curved arrow). [H&E, X 400]

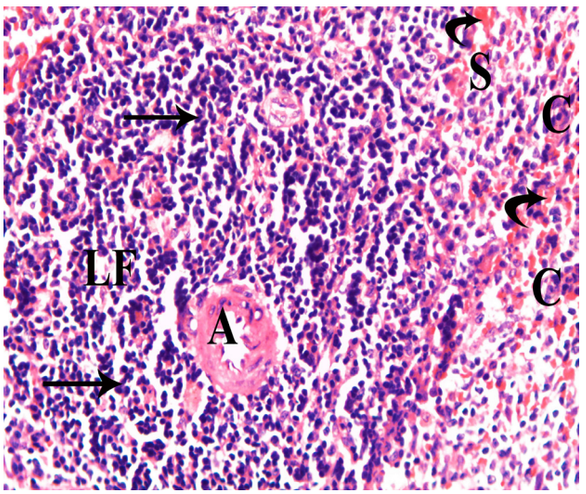



**Fig. 5:** A photomicrograph of a section in spleen of group II (12th week specimens) showing the white pulp with slightly dilated central arteriole (A), disturbed lymphatic follicle (LF) with lymphocytes (arrow). The red pulp contains cords of lymphocytes (C) and blood sinusoids (S) with few RBCS inside (curved arrow). [H&E, X 400]

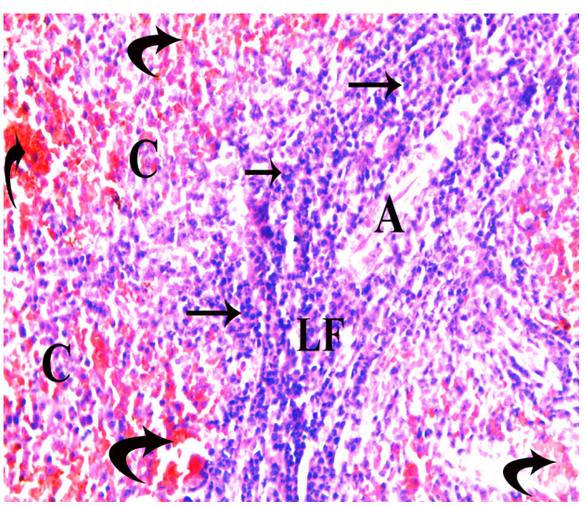



**Fig. 6:** A photomicrograph of a section in spleen of group III (4th week specimens) showing dilatation and distortion of central arteriole (A), marked disturbance in architecture of lymphoid follicle (LF) with lymphocytes (arrow) and congested blood sinusoids (curved arrow). Note the cords of lymphocytes (C) in red pulp. [H&E, X400]

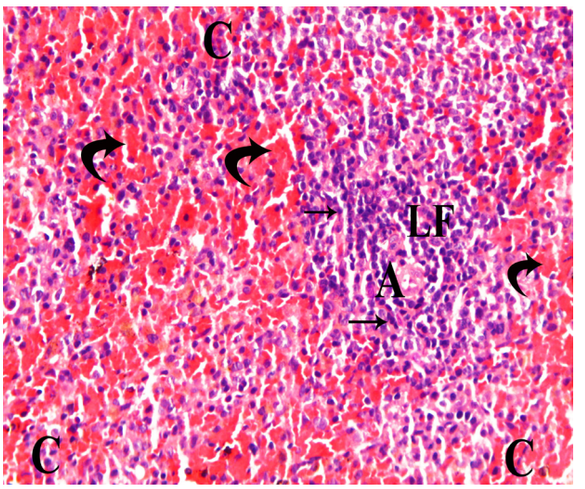



**Fig. 7:** A photomicrograph of a section in spleen of group III (8th week specimens) showing contracted central arteriole (A), shrinkage of lymphoid follicle (LF) with lymphocytes (arrow). Cords of lymphocytes (C) and congested blood sinusoids (curved arrow) are seen in the red pulp. [H&E, X400]

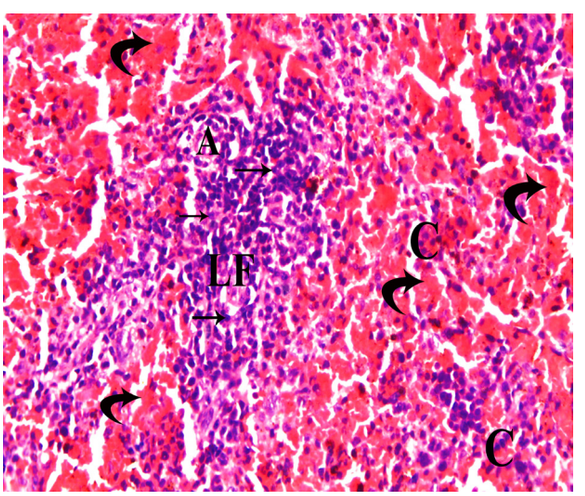



**Fig. 8:** A photomicrograph of a section in spleen of group III (12th week specimens) showing contracted central arteriole (A) marked disturbance in architecture of lymphoid follicle (LF), with lymphocytes (arrow). Cords of lymphocytes (C) and congested blood sinusoids (curved arrow) are seen in the red pulp. [H&E, X400]

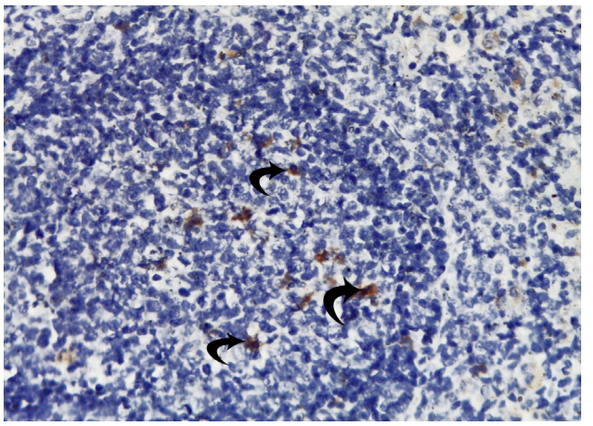


**Fig. 9:** A photomicrograph of a section in spleen of group I (control group) showing positive (+ve) immuno-expression for CD4 mainly in the germinal center of lymphoid follicle and in marginal zone of white pulp (curved arrow).

[Immunohistochemistry stain for CD4 X400]

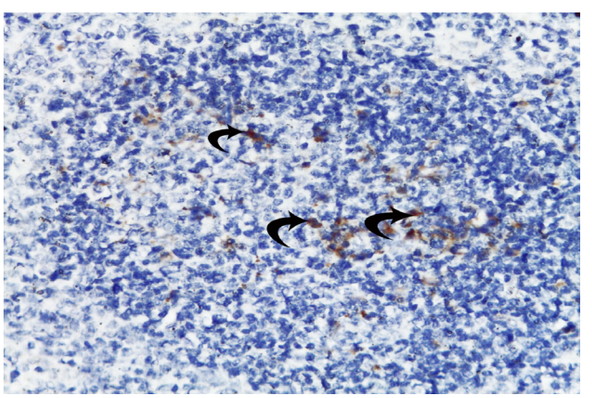


**Fig. 10:** A photomicrograph of a section in spleen of group II (4th week specimens) showing positive (+ve) immuno-expression for CD4 (curved arrow).

[Immunohistochemistry stain for CD4 X400]

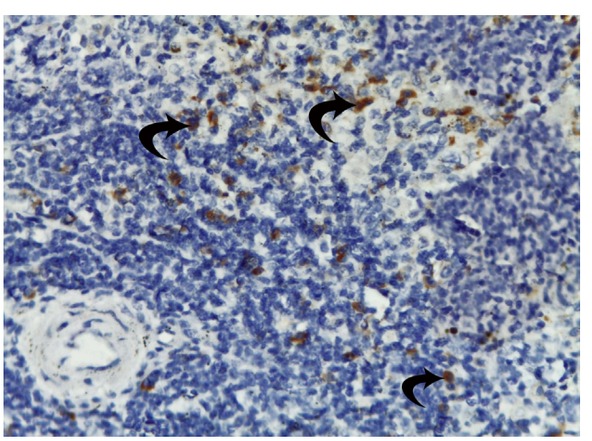


**Fig. 11:** A photomicrograph of a section in spleen of group II (8th week specimens) showing an apparent increased immuno-expression for CD4 (curved arrow).

[Immunohistochemistry stain for CD4 X400]

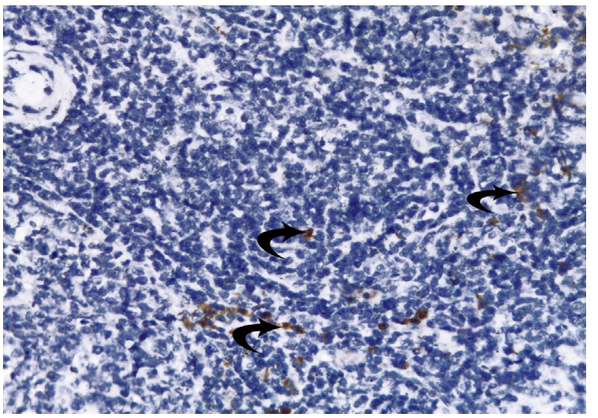


**Fig. 12:** A photomicrograph of a section in spleen of group II (12th week specimens) showing an apparent decreased immuno-expression for CD4 (curved arrow).

[Immunohistochemistry stain for CD4 X400]

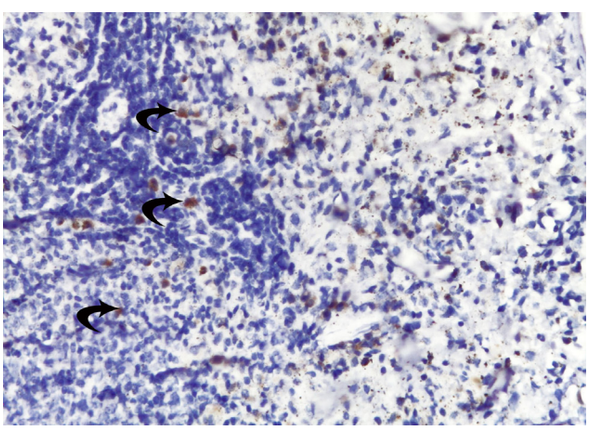


**Fig. 13:** A photomicrograph of a section in spleen of group III (4th week specimens) showing (+ve) strong reaction for CD4 (curved arrow).

[Immunohistochemistry stain for CD4 X400]

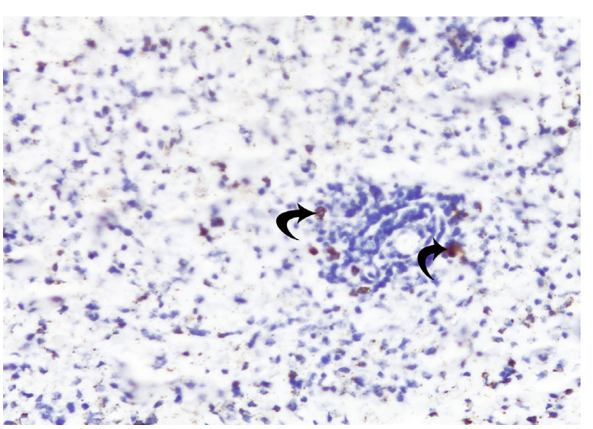


**Fig. 14:** A photomicrograph of a section in spleen of group III (8th week specimens) showing an apparent decreased immuno-expression for CD4 (curved arrow).

[Immunohistochemistry stain for CD4 X400]

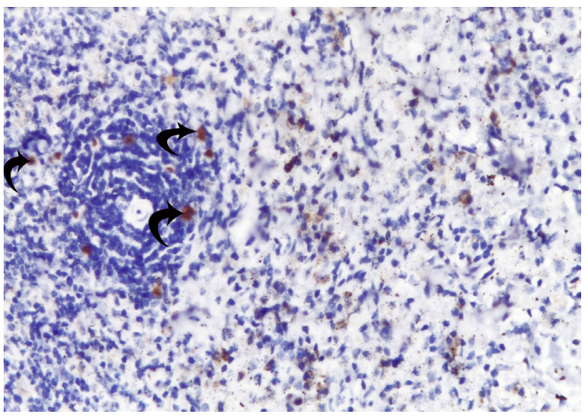


**Fig. 15:** A photomicrograph of a section in spleen of group III (12th week specimens) showing an apparent decreased immuno-expression for CD4 (curved arrow).
[Immunohistochemistry stain for CD4 X400]

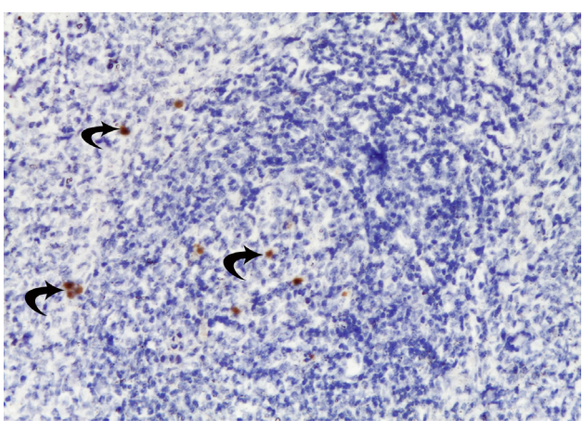


**Fig. 16:** A photomicrograph of a section in spleen of group I (control group) showing positive (+ve) immuno-expression for CD68 (curved arrow).
[Immunohistochemistry stain for CD68 X400]

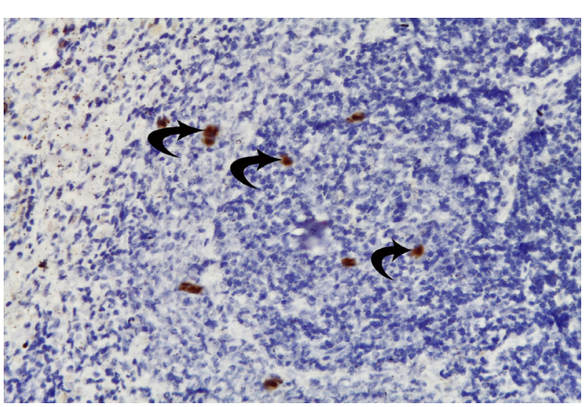


**Fig. 17:** A photomicrograph of a section in spleen of group II (4th week specimens) showing an apparent increased immuno-expression for CD68 (curved arrow).
[Immunohistochemistry stain for CD68 X400]

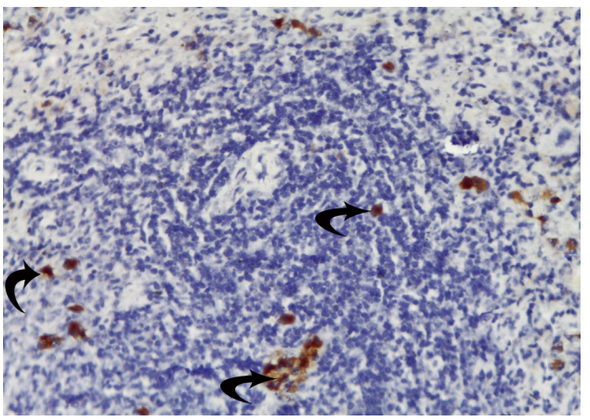


**Fig. 18:** A photomicrograph of a section in spleen of group II (8th week specimens) showing an apparent increased immuno-expression for CD68 (curved arrow).
[Immunohistochemistry stain for CD68 X400]

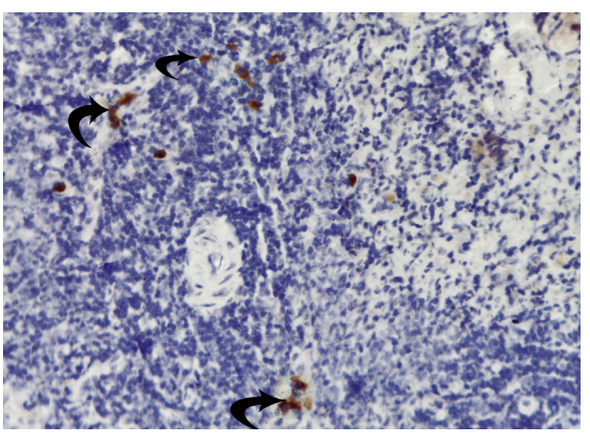


**Fig. 19:** A photomicrograph of a section in spleen of group II (12th week specimens) showing an apparent decreased immuno-expression for CD68 (curved arrow).
[Immunohistochemistry stain for CD68 X400]

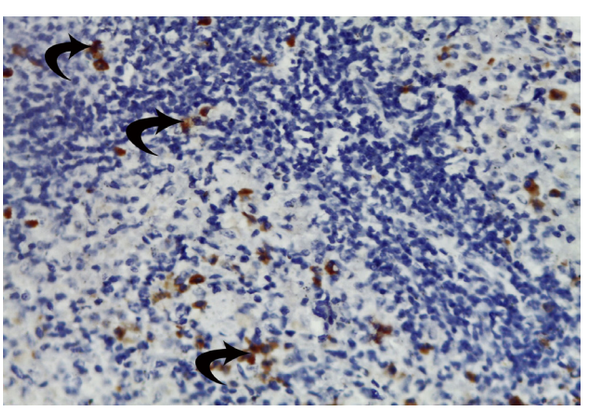


**Fig. 20:** A photomicrograph of a section in spleen of group III (4th week specimens) showing positive (+ve) strong reaction for CD68 (curved arrow).
[Immunohistochemistry stain for CD68 X400]

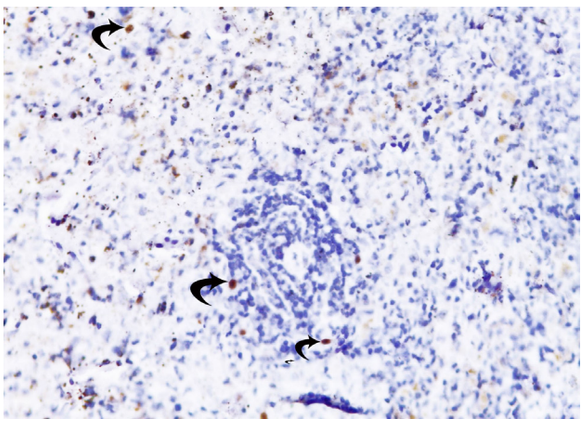


**Fig. 21:** A photomicrograph of a section in spleen of group III (8th week specimens) showing an apparent decreased immuno-expression for CD68 (curved arrow).
[Immunohistochemistry stain for CD68 X400]

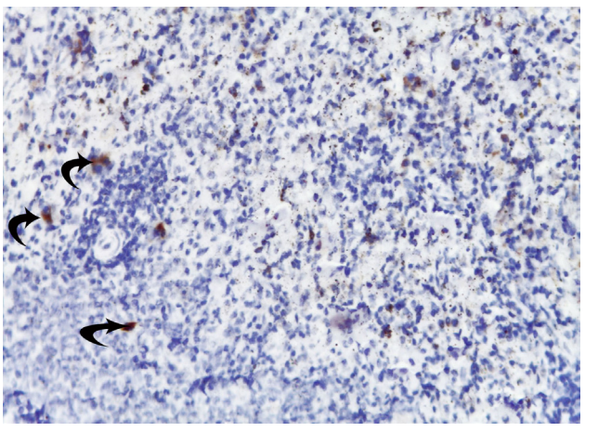


**Fig. 22:** A photomicrograph of a section in spleen of group III (12th week specimens) showing an apparent decreased immuno-expression for CD68 (curved arrow).
[Immunohistochemistry stain for CD68 X400]

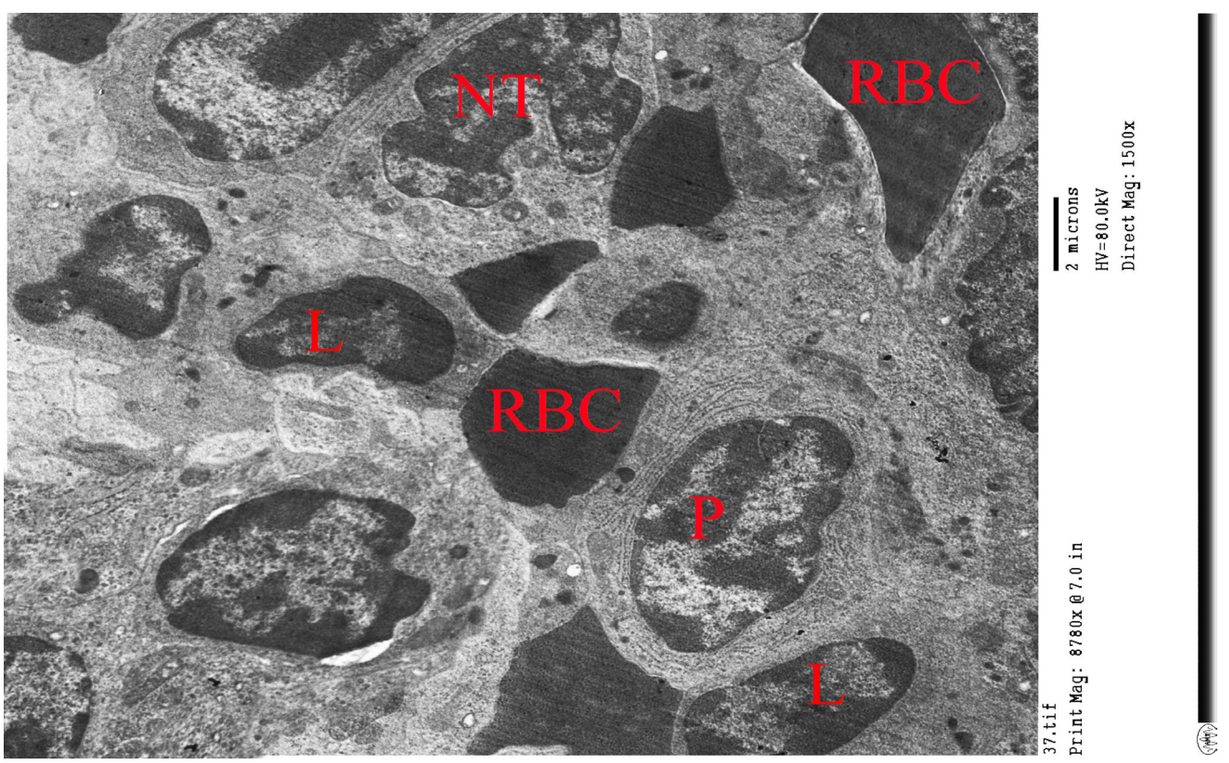



**Fig. 23:** An electron micrograph of spleen in group I (control group) showing multiple lymphocytes (L), plasma cells (P), neutrophil (NT) and RBCs.
[EM X1500]

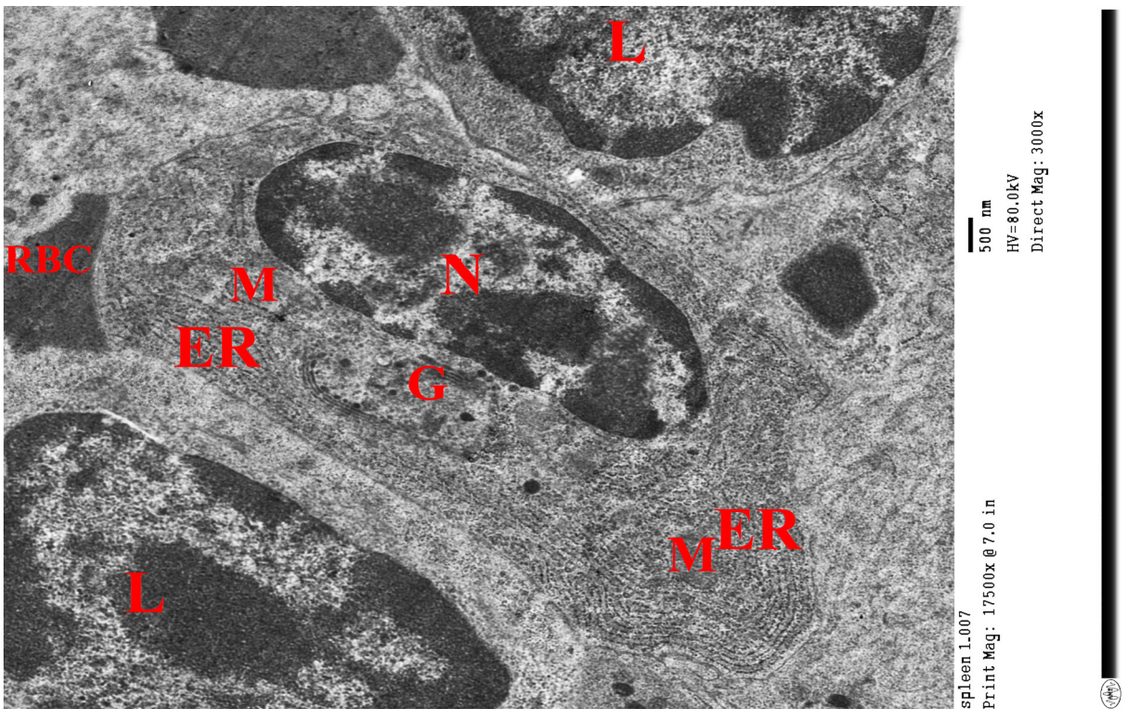



**Fig. 24:** An electron micrograph of spleen in group I showing plasma cell with large spherical nucleus (N) with clear nucleolus (Nu) and peripheral heterochromatin (HC), well-developed rough endoplasmic reticulum (ER) and multiple mitochondria (M). RBCs and a part of lymphocyte (L) are observed. [EM X 3000]

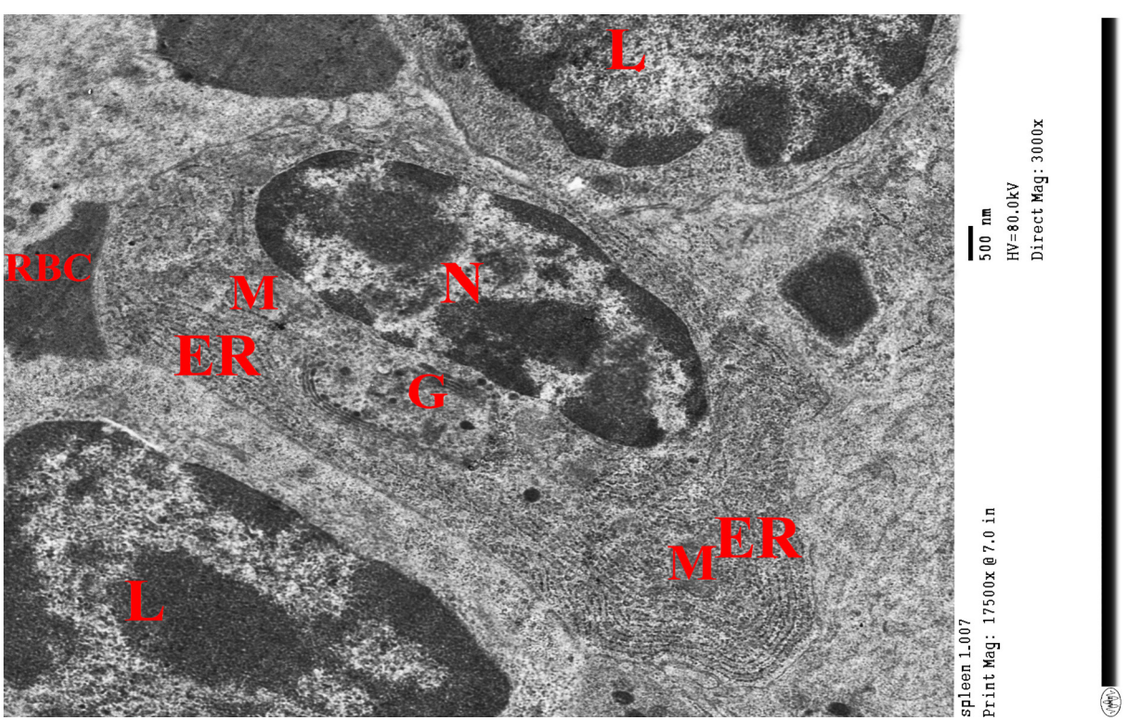



**Fig. 25:** An electron micrograph of spleen in group II (4th week specimens) showing plasma cell with hyperchromatic (N), well-developed rough endoplasmic reticulum (ER), Golgi complex (G) and multiple mitochondria (M). RBCs and parts of lymphocytes (L) are observed. [EM X 3000]

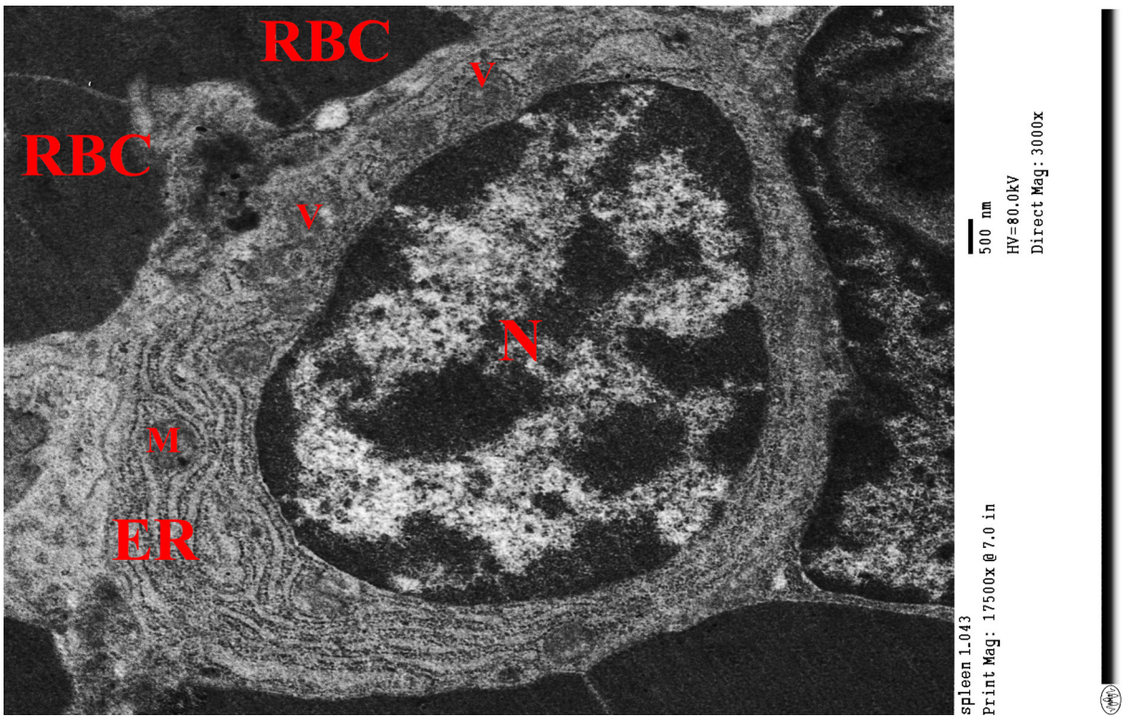



**Fig. 26:** An electron micrograph of spleen in group II (8th week specimens) showing plasma cell with hyperchromatic (N), slight dilatation in the cisterna of rough endoplasmic reticulum (ER) and multiple mitochondria (M) with mild vacuolation in some of them (V). Parts of RBCs are observed. [EM X 3000]

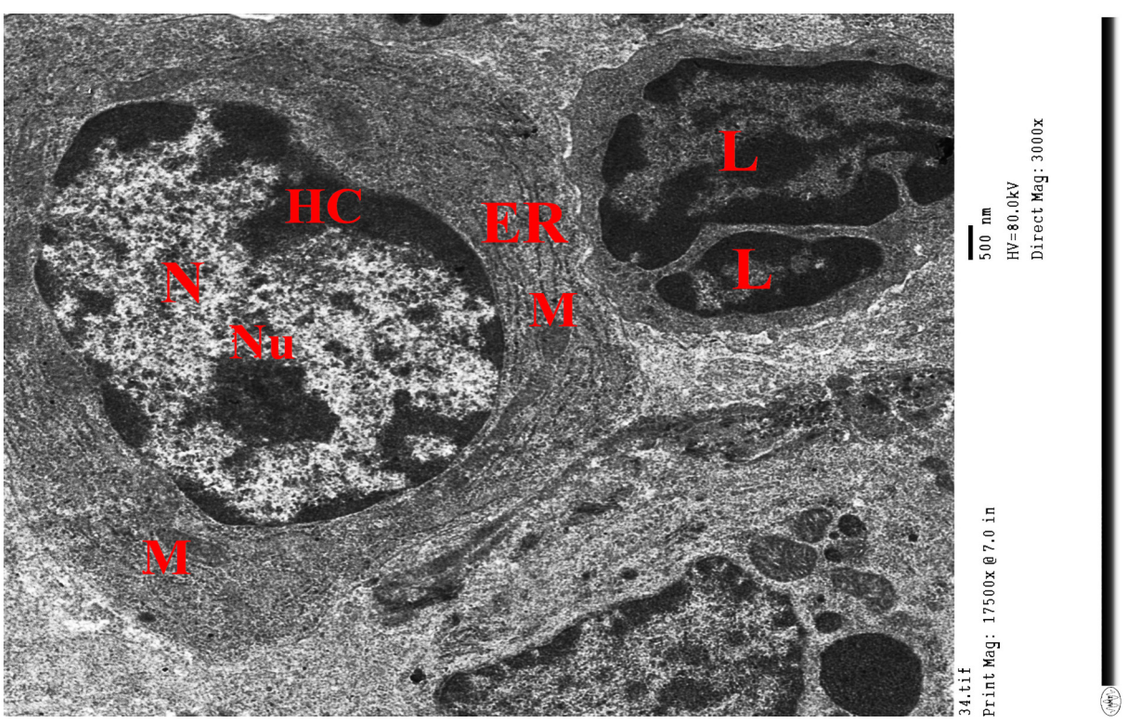



**Fig. 27:** An electron micrograph of spleen in group II (12th week specimens) showing plasma cell with large spherical nucleus (N) with clear nucleolus (Nu) and peripheral heterochromatin (HC), well-developed rough endoplasmic reticulum (ER) and multiple mitochondria (M). Two lymphocytes (L) are noticed. [EM X 3000]

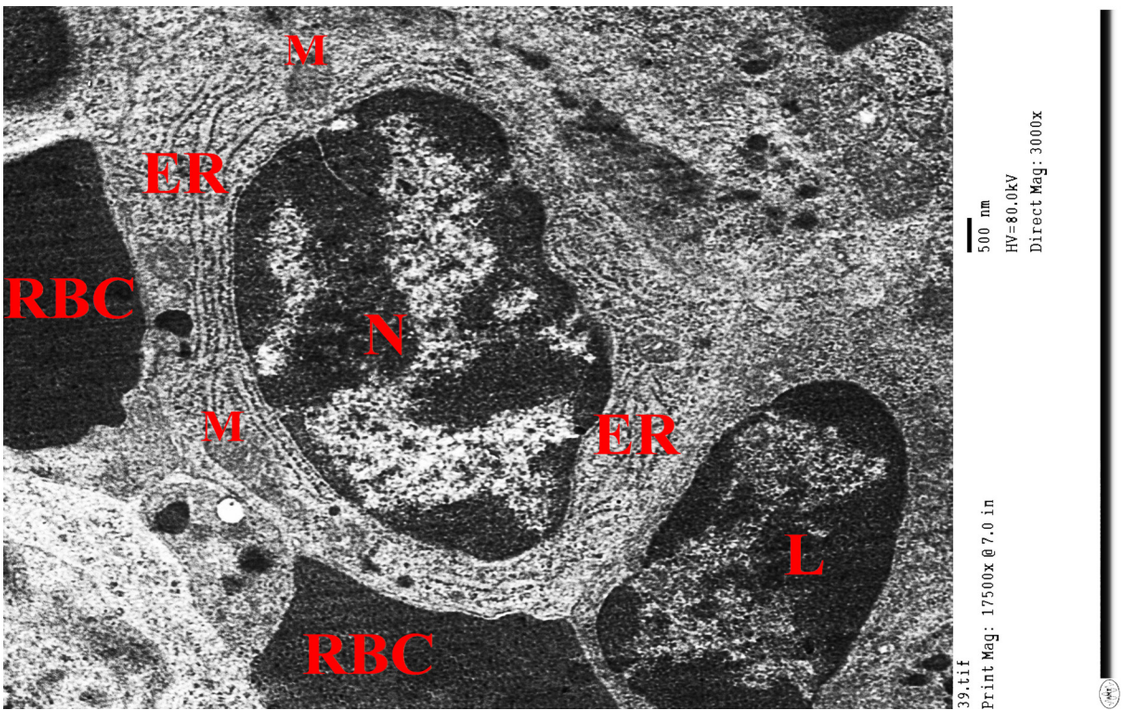



**Fig. 28:** An electron micrograph of spleen in group III (4th week sample) showing plasma cell with hyperchromatic nucleus (N),disturbance in the chromatin distribution, slight dilatation in the cisterna of rough endoplasmic reticulum (ER) and multiple mitochondria (M). Lymphocyte (L) and parts of RBCs are observed. [EM X 3000]

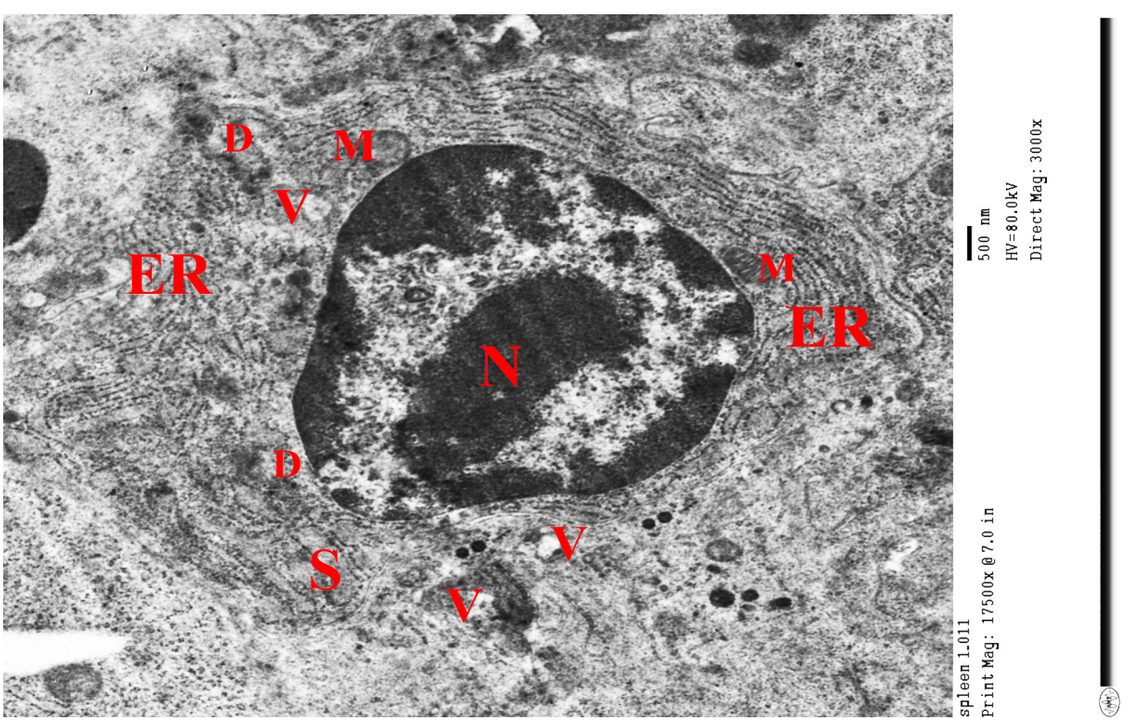



**Fig. 29:** An electron micrograph of spleen in group III (8th week specimens) showing plasma cell with hyperchromatic nucleus (N), slight dilatation in the cisterna of rough endoplasmic reticulum (ER) with wide separation of some cisterna (S) and multiple mitochondria (M) with destruction of cristae in some of them (D). Multiple vacuoles (V) in the cytoplasm are observed. [EM X 3000]

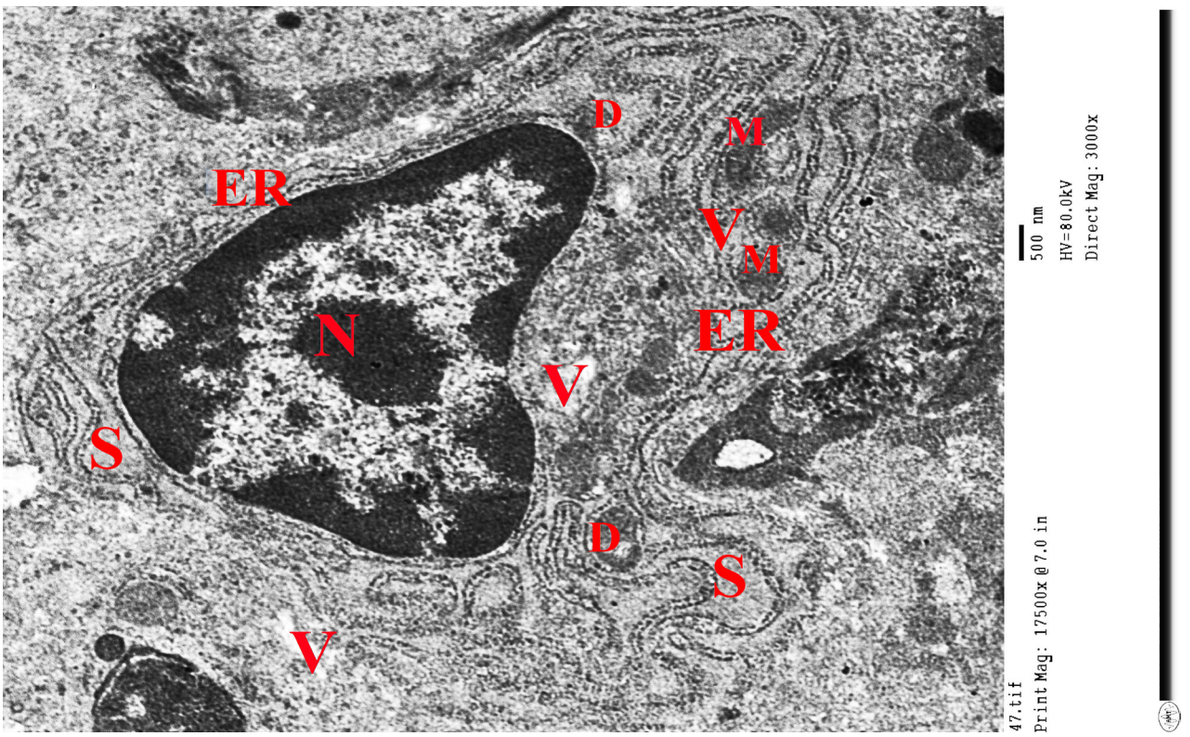



**Fig. 30:** An electron micrograph of spleen in group III (12th week specimens) showing plasma cell with hyperchromatic nucleus (N), slight dilatation in the cisterna of rough endoplasmic reticulum (ER) with wide separation of most cisterna (S) and multiple mitochondria (M) with destruction of cristae in some of them (D). Multiple vacuoles (V) in the cytoplasm are observed. [EM X 3000]

## DISCUSSION

With the increased-application of nanotechnology, there is increasing need to evaluate risk associated with their use[26]. Some investigators have worked on the toxicity of nanoparticles on various organs; however, insufficient data on the toxicity of nanoparticles and their effect on human health[27].

Since many individuals are daily exposed to Tio2 through food additives, pharmaceuticals, or toothpaste and so experimental studies on oral-exposure to $TiO_2$ are needed[28]. Accordingly, this study was performed to assess the histological structure and immunohistochemical changes caused by $TiO_2$ NPs on spleen after prolonged oral exposure. The dose of $TiO_2$ NPs and duration of exposure were depended on the studies of[21, 22, 29].

Spleen was selected for the present study to evaluate the toxicity of $TiO_2$ as some researchers[30] reported that spleen is one of the immune system organs, which has high sensitivity to low-level doses of chemicals, even the other organ systems not affected.

Low dose $TiO_2$ group (group II) in the present study showed slight disturbance in architecture of white pulp with hyper chromatic nuclei and slight dilatation in the cisterna of rough endoplasmic reticulum of plasma cell, dilatation in central arteriole, slight congestion in red pulp and Significant increase ($P< 0.01$) in CD4 and CD68 immuno-expression only in the 8th week specimens which return to near control in the 12th week sample (withdrawal specimens). The aforementioned are consistent with previous researchers who found minimal[22] histological changes on $TiO_2$ low-dose exposure. Others[1, 2] demonstrated that $TiO_2$-NPs explicit dose-effect relationship and it does not show any toxicity until the exposure concentration reaches a certain threshold. Many researchers[32, 33] revealed that the duration and doses directly determine the results of the biological responses. Previous studies[34, 35] showed that low dose of nanomaterials could be successfully adapted by cells through attracting the lipid redistribution and antioxidant defenses processes. Noteworthy, prolonged exposure to low dose $TiO_2$ NPs resulted in spleen injury[30] that may be attributed to fatty degeneration and splenocyte apoptosis. Some researchers[6] stated that exposure of spleen to $TiO_2$ NPs resulted in Reactive oxygen species ROS over production, splenic inflammation and necrosis in a time-dependent manner. On the contrary, others[36, 37] stated that low doses of $TiO_2$ revealed no cytotoxicity.

High-dose $TiO_2$ group (group III) in the present study showed marked disturbance in architecture of white pulp

with hyper chromatic nuclei and slight dilatation in the cisterna of rough endoplasmic reticulum of plasma cell, dilatation and distortion of central arteriole. Significant increase ($P< 0.01$) in CD4 and CD68 immuno-expression in the 4th week specimens was also noticed. The 8th week specimens showed contracted central arteriole, shrinkage white pulp with some cisterna of ER appeared widely separated, some mitochondria showed destruction in their cristae and there were multiple vacuoles in the cytoplasm and congested red pulp. No obvious improvement seen in the 12th week specimens (withdrawal specimens). CD4 and CD68 immuno-expression were significantly decrease ($P< 0.01$) in the 8th and the 12th week samples. In addition, oral administration of TiO2 NPs resulted in structural changes in spleen tissue; congestion, vacuolization, lymph nodule proliferation and splenocyte apoptosis[38-42, 6]. Also, the prolonged exposure of spleen to $TiO_2$NP resulted in a significant histological change; the reduction of white pulp, decrease of T lymphocytes (CD4 cells), B lymphocyte and NK cell proliferation as well as severe macrophage infiltration (CD68 cells)[43, 44]. Interestingly, the phagocytic action of macrophages was increased after exposure to low-doses of $TiO_2$ nanoparticles but decreased after exposure to high-doses[45]. Some researchers[46, 47] reported that $TiO_2$ NPs exposure could lead to immune cell death as it induced a dose dependent decrease in the cell viability. Others[48] postulated that, the function of macrophages impaired by $TiO_2$ NPs, resulting in persistent inflammatory reactions. Moreover, on exposure to NPs the macrophages engulf nanoparticles, secrete cytokines and increase expression of surface receptors resulted in increasing the interaction with other cells. However, the intake of toxic nanoparticles can disturb their normal functioning and lead to undesirable consequences. Two previous studies[29,6] reported that the disturbance of spleen caused by $TiO_2$ NPs were related to enhanced expression of inflammatory cytokines for example, nuclear factor (NF)-κB, tumor necrosis factor (TNF-α), cyclooxygenase (COX)-2, and interleukins (ILs) which resulted in the immunomodulatory impairment in the spleen. In addition, after their entry inside the cell, $TiO_2$ NPs interact with cytoplasmic proteins and induce post translational modifications, such as acetylation, by oxidative stress and other mechanisms[49]. Where they reach the peri region of nucleus, impede the function of endoplasmic reticulum, and block the nuclear pore or enter the nucleus. Inside the nucleus, they interact with DNA and cause the up regulation of cytokines-, oxidative stress, and apoptosis related genes. The adverse health effects induced by nanoparticles may be attributed to oxidative as exposure to nano-$TiO_2$ may increase eradiation of ROS and oxidative products (i.e. lipid peroxidation), deplete cellular antioxidants, such as glutathione, cause mitochondrial damage with prevention of ATP synthesis and induce cell death by lipid peroxidation and DNA fragmentation[50, 51, 26]. In contrast to our findings, a previous study[1] demonstrated that $TiO_2$ is physiologically inactive exhibiting relatively low toxicity and considered unhazardous to humans. Many researchers[52-54] attributed these results to the very low and poor absorption of $TiO_2$ NPs after oral administration to rats so it should not result in excessive accumulation of metal in the organism. However, a previous study[55] stated that the toxicity of $TiO_2$ NPs is still controversial issue; the authors have reported significant adverse effects on in-vitro and in-vivo systems with higher toxicity for size decreasing NPs.

As regards the withdrawal specimens, our findings together with many researchers[56, 33] elucidated that toxicity of nanomaterials leads to irreversible deactivation of proteins and may result in long-term toxicity. The absolute reversibility of the adverse effects of nanomaterials does not exist. On the contrary, Xu *et al.* 2014 and Verneuil *et al.* 2017[57, 58] reported that the toxic effects caused by $TiO_2$ NPs were dose dependent and could be reversed. However, the mechanism of reversibility of adverse effects of $TiO_2$ NPs or even adaptation remains unclear. However, Khatchadourian and Maysinger supposed that exposure to nanoparticles not only lead to disruption of the cellular redox status, but it also leads to increase activity of compensatory mechanisms. Besides, the changes in the lysosomal system have essential role in cellular adaptation process following oxidative stress[60].

## CONCLUSION

Prolonged exposure to large oral doses of $TiO_2$ NPs could result in splenic toxicity and disruption of its structure and immunological functions. Therefore, the application of $TiO_2$ NPs and exposure to its containing products should be limited. Further studies on the possibility of withdrawal of those adverse effects are needed.

## CONFLICT OF INTEREST

There are no conflicts of interest.

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## الملخص العربى

# تأثير جزيئات ثاني أكسيد التيتانيوم النانونية على طحال ذكور الجرذان البالغة البيضاء: دراسة هستولوجية وهستوكيميائية مناعية

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**قسم الانسجة وبيولوجيا الخلية ، كلية الطب ، جامعة بنها ، بنها ، مصر**

**المقدمة:** : تعتبر مادة ثاني أكسيد التيتانيوم النانونية من أكثر المواد النانوية استخداماً فى المنتجات الصناعية مثل مستحضرات التجميل، واقيات الشمس، المنتجات الغذائية، الدهانات والعقاقير. وقد تناولت العديد من الدراسات التأثيرات الخلوية الضارة المختلفة وتشمل الاجهاد التأكسدي وتدمير الخلايا.

**الهدف من البحث:** تقييم التأثير السمي المحتمل لجزيئات ثاني أكسيد التيتانيوم النانونية على تركيب طحال الجرذان.

**مواد وطرق البحث:** تم تقسيم45 من ذكور الجرذان البالغة البيضاء إلى ثلاث مجموعات. المجموعة الاولى: المجموعة الضابطة. المجموعة الثانية (الجرعة الصغيرة): أعطيت ثانى أكيد التيتانيوم بجرعة (600 ملغم/ كغم/ يوم) ثم تركت حتى الاسبوع الثاني عشر. المجموعة الثالثة (الجرعة الكبيرة): أعطيت ثانى أكسيد التيتانيوم بجرعة (1200 ملغم/ كغم/ يوم) لمدة ثمانِ أسابيع ثم تركت حتى الاسبوع الثانى عشر. تمت معالجة عينات الطحال وفحصها باستخدام وسائل هستولوجية وهستوكيميائية مناعية.

**النتائج:** أظهرت مجموعة الجرعة الصغيرة اضطراباً طفيفاً في تركيب اللب الأبيض (النسيج الليمفاوي) واحتقان بسيط في اللب الأحمر وزيادة ذات دلاله احصائيه ($P < 0.01$) في التفاعل المناعي لكل منCD4 و CD68في عينات الاسبوع الثامن فقط مقارنة بالمجموعة الضابطة. كما أظهرت مجموعة الجرعة الكبيرة تغير وتشوه ملحوظ في اللب الأبيض وزيادة ذات دلاله احصائيه ($P < 0.01$) في التفاعل المناعي لكل من CD4 و CD68في عينات الاسبوع الرابع, أما عينات الاسبوع الثامن والثاني عشر فقد أظهرت انخفاض ذو دلاله احصائيه ($P < 0.01$) في التفاعل المناعي لكل من CD4 و CD68مقارنة بالمجموعة الضابطة. كما لم يحدث أى تحسن ملحوظ في عينات الاسبوع الثاني عشر.

**الإستنتاج:** يؤدي تناول جزيئات ثانى أكسيد التيتانيوم النانوية بجرعات كبيرة ولفترات طويلة لتأثيرات سمية على الطحال إضافة إلى تغييرات جوهرية فى تركيبه.