Ain Shams Journal of Forensic Medicine and Clinical Toxicology
Jan 2016, 26: 115- 128

The immunotoxic effects of short term chronic exposure to Titanium Dioxide Nanoparticles on spleen of adult albino rats and the role of after toxic effect follow up

Arwa A. El-Sheikh, Shimaa Hamed Ameen[1], Hanaa M. Ibrahim[2] and Samaa Salah AbdEl-Fatah[3]

[1] Departments of Forensic Medicine and Clinical Toxicology
[2] Departments of Pathology
[3]Departments of Anatomy

Faculty of Medicine, Zagazig University, Alsharqia, Egypt.

All rights reserved.

Abstract **Objective**: The usage of Titanium Dioxide NanoParticles (TiO2NPs) on a large scale of applications was a reason of a variety of many health problems. The aim of the current study was to evaluate the immune toxic effects of short term administration of TiO2NPs on spleen. **Material and Methods:** Forty Adult male Rats were equally divided into four groups as follows: Group I: negative control, Group II: positive control, Group III: received TiO2NPs (1200 mg/kg) orally daily for 12 weeks. Group IV: follow up group received TiO2NPs by the same dose, route and for the same duration as TiO2NPs group and then they were left untreated for another 8 weeks. Total leukocytes, differential leukocytic counts, Interleukins IL-2 and IL-10 levels were measured, the spleen sections were examined immunohistochemically for the detection of CD4+ and iNOS expressing cells. Histopathological alterations in the spleen were also evaluated. Moreover, DNA damage was evaluated by comet assay. **The results:** TiO2NPs exposure for 12 weeks resulted in significant decreased in the total and deferential leukocytic counts, serum interleukins IL-2 as well as IL-10. It caused marked decrease in CD4+T-lymphocytes and increase in iNOS expressing cells indicating oxidative stress in spleen tissues. It also caused histopathological disruption of spleen architecture and produced DNA damage in splenocytes. Discontinuation of TiO2NPs administration for 8 weeks resulted in significant improvement of leukocytes, interleukins, increase in CD4+ T-lymphocytes and decrease in iNOS expressing cells in spleen tissues. Moreover, there was moderate improvement in histopathological alterations and DNA damage.
Conclusion: TiO2NPs consumption have immunotoxic effects which may resulted from genetic damage and oxidative stress in the spleen of adult male albino rats which could be improved by its discontinuation for a period of time and it was recommended to increase the period of discontinuation as complete improvement may occur

Keywords TiO2NPs, spleen, immune toxic, interleukin, iNOS, CD4+, DNA, Rats.

Introduction

Nanomaterials are new engineered structures with one dimension of 100 nanometers or less (Nel et al., 2006). Titanium dioxide NanoParticles (TIO2NPs) that have specific properties such as higher stability, anti-corrosive and photo-catalytic effects make them have large scale of applications in several areas (Riu et al., 2006). TiO2NPs are used in a variety of consumer products, as surface coatings, paints, toothpastes, sunscreens, cosmetics, food products (Gurr et al., 2005) and in the environmental decontamination of air, soil, and water (Choi et al., 2006). In medical treatment TiO2NPs also have been used in photodynamic therapy (Szacilowski et al., 2005) and antibacterial drugs (Montazer et al., 2011).

TiO2NPs are small sized particles with large surface area and have surface reactive chemical agents which facilitates their endocytosis into different cells (Robertson et al.,2010) providing opportunity for accumulation in several organs such as the liver, kidneys, spleen, lungs, and heart of animals (Liu et al.,2009). Previous researches have proved the toxic effects of TiO2NPs on various organs by different mechanisms as DNA damage, apoptosis with formation of apoptotic bodies and mitochondrial abnormalities (Wang et al.,2007a), oxidative stress (Park et al.,2008),

enzymatic activity changes followed by cell apoptosis or necrosis (Zhao et al.,2009) and penetration of the blood–brain barriers and blood placenta barriers (Sang et al.,2012).

Recently, the studies have been reported that exposure to TiO2NPs produced immune system affection (Li et al.,2010) by changing cytokine production and decrease immune function (Liu et al.,2011). Also, the systemic immune response associated with inhalable TiO2NP provided new strategy for risk assessment of TiO2NP exposure (Yanyun et al.,2014).

Recent *in* vitro studies have demonstrated the genotoxic potential of higher concentration of TiO2NPs as shown in root meristem cells of *Allium cepa* (Demir et al.,2014).Also, another study have been reported that there is significant DNA damage in TiO2NPs exposed cultured WISH cells (Saquib et al.,2012).

The spleen is the largest lymphoid organ in animals, involved in host immune response against blood borne antigens, producing lymphocytes and reflecting the histopathology of the immune system (Mebius and Kraal,2005). Xenobiotics that produced immunotoxicity affect lymphocytes population that is represented in the spleen (Elmore, 2006). T lymphocyte, is a type of leukocytes that play a central role in cell-mediated immunity. It has several subsets each have a distinct function, from these subsets T helper cells (T_h cells) that is also known as $CD4^+$ T cells because they express the CD4 glycoprotein on their surfaces. T_h cells divide rapidly and secrete small proteins called cytokines that regulate or assist in the active immune response. If CD4 cells become depleted following immune suppression, the body is left vulnerable to a wide range of infections (McClory et al.,2012).

Interleukin-2 (IL-2) is a type of cytokine that secreted in the activation program of CD4+T cells while Interleukin-10 (IL-10) is primarily produced by monocytes and to a lesser extent, T_h lymphocytes and mast cells (Sojka et al.,2004).

The immune system may be affected by oxidative stress that contributes to several organs damage and cell death as disturbed balance between oxidants and antioxidants leading to depression of immune organ function (Parkash and Nagarkatti, 2014). Inducible nitric oxide synthase (iNOS) described as the immunological NOS and expressed by macrophages and characterized by increase production of nitric oxide (NO) in pathological conditions. NO generated enzymatically by synthase [nitric oxide synthase (NOS)] which oxidize L-arginine to L-citrulline (Gnarro,2002), having the chance to react with superoxide and formation of peroxynitrite inducing cell toxicity (Mungrue et al.,2002). NO from iNOS-expressing cells suppresses mouse T cell proliferation. Also, in case of deficient iNOS, NO did not produced and this may attributed to increase the infiltration and expansion of CD4 cells (Aheng et al.,2011).The spleen was approved to be important organ for iNOS/NO responses and highly expressed iNOS in the splenocytes may regulate T_h response negatively (Qing Shen et al.,2015).

The comet assay or single cell gel electrophoresis (SCGE) provides a simple and effective method for evaluating DNA damage and breakage in individual cells (Demir et al.,2011) so, it can be used to test if TiO2NPs produce DNA damage.

On that basis, The aim of the current work was to study immunetoxic effects of short term chronic exposure to TiO2NPs and to investigate the possible underlying molecular mechanisms of such toxicity and role of follow up in adult albino rats.

Materials and Methods

1. Chemicals and preparation

Titanium dioxide nanoparticles (TiO2NPs) was white odorless fine powder (21 nm particle size, surface area of 35-65 m^2/g, purity ≥99.5% trace metals basis and Its CAS No is 13463-67-7), manufactured by Sigma - Aldrich Chemical Company, Germany and purchased from Sigma –Egypt. It was dissolved in 5% gum acacia solution which prepared by dissolving 10 gm of powder in 100 ml boiled distilled water. It was obtained from El- Nasr Pharmaceutical Chemicals Company, Egypt.

2. Experimental Animals and Design

Forty adult albino rats were purchased from the animal breeding house of Faculty of Medicine Zagazig university weighing 200-210 gm. The animals were housed in stainless steel cages and provided with commercial laboratory animal food and water ad libitum. All ethically approved conditions used for animal housing &handling were considered. Standards for animal care and administration met those acquired by applicable international laws and regulations (ILAR ,1996). The animals were equally divided into 4 groups :

- **Group I (negative control group)**: 10 rats received only regular diet and water to determine the basic values of performed tests for 12 weeks.
- **Group II (positive control group)** (**gum acacia**)**:** 10 rats each received 1 ml of 5% gum acacia solution (the solvent of titanium dioxide) by oral gavage once daily for 12 weeks.
- **Group III (titanium dioxide treated group) :** 10 rats gavaged orally with 1200 mg/kg body weight titanium dioxide nanoparticles (1/10 LD_{50}) in 1ml of 5% gum acacia solution as a solvent once daily for 12 weeks. The LD_{50} of TiO_2 for rats is more than 12,000 mg/kg body weight after oral administration (Wang et al., 2007b**)**.
- **Group IV (follow up group)**: 10 rats received Titanium dioxide nanoparticles by the same route, at the same dose and for the same duration as group III ,then they were left without treatment for another 8 weeks.

Twenty-four hour after the final dosing/non-dosing day, the rats (had been fasted over-night) were weighed and venous blood samples were collected from retro-orbital plexus of each rat while the animal was anesthetized with ether. The animals were then euthanized by cervical dislocation and the spleens were excised and weighed accurately.

3. Body weight and Coefficient of spleen

after weighing the body and spleens, the coefficient of spleen to body weight was calculated as the ratio of spleen (wet weight, mg) to body weight (g).

4. Leucocytes Counts

2.5 ml of whole blood samples from each group were collected in tubes containing EDTA as anti-coagulant. Total (WBCs) and deferential leukocytes counts (lymphocytes, monocytes and granulocytes) were measured using a hematology cell counter.

5. Cytokines Assay

2.5 ml of serum from each group were harvested by centrifuging blood at 2500 rpm for 10 min at 4 °C and immediately frozen at −80 °C for further detection of levels of interleukin IL-2 and IL-10.Serum IL-2 and IL-10 levels were assayed for 4.5hours using Rat Quantikine® ELISA kits (R&D Systems Inc., Minneapolis, MN). Sensitivity of the kits was natural and recombinant rat IL-2 and IL-10.Manufacturer's instruction was followed. The absorbance was measured on a microplate reader at 450 nm then IL-2 IL-10 concentration of samples were calculated from a standard curve.

6. Immunohistochemical Staining of spleen

Immunohistochemistry was performed using primary antibodies against CD4+ (ready to use; Dako Carpinteria, California, USA)and anti-iNOS (isoform Nitros Oxide) (dilution 1 : 50; Santa Cruz Biotechnology) using streptavidin–biotin immunoperoxidase technique (DakoCytomation, California, USA). Formalin-fixed, paraffin-embedded tissues (FFPE) tissues were cut into (3- 4-µm) thick sections and transferred to 3-aminopropyltriethoxysilane (APTS) coated glass slides. Then, sections were subjected to dewaxing, rehydration, blocking with hydrogen peroxide, and antigen retrieval that was performed by heating specimens at 100°C for 20 min in citrate buffer (pH 6.0) within microwave. One to two drops of the primary ready-to-use monoclonal antibody, anti-CD4 and anti-iNOS were then placed on the sections on separate slides. Slides were incubated at room temperature for 60 min. Incubation with secondary antibody and product visualization (Dako) was performed with DAB chromogen (3, 3-diaminobenzidine tetrahydrochloride).Sections were counter-stained with hematoxylin, dehydrated with ethanol and xylene and mounted permanently with Di-n-butylPhthalate in Xylene (DPX).

Assessment of Immunohistochemical Staining Results

Microscopic evaluation of CD4+

Assessment of membranous immunostaining were scored by counting the number of CD4 marker expression over each lymphocyte in five randomly selected high power fields at 40X magnification and the sections were graded as follow: + (1-25 cells), ++ (26-50 cells), +++ (≥51 cells) (Guo et al.,2008).

Microscopic evaluation of inducible nitric oxide synthase (iNOS)

Assessment of Cytoplasmic immunostaining for iNOS was semiquantitatively scored as follows: 0 (negative), 1(weak i.e. <10% expression of cells), 2 (moderate i.e. 10–20% expression of cells) and 3 strong i.e. expression in >50%). A score of 2 or higher was considered positive (Ross et al.,2005).

7. Histopathological Examination of spleen

All tissue samples were fixed with 10%formalin. Consecutive 5-µm thick sections from formalin-fixed, paraffin-embedded tissue blocks were prepared and stained with hematoxylin and eosin (H&E) for histopathological classification (Frank et al., 2005).

8. Comet Assay of spleen

The principle of the method (Singh et al.,1988) under highly alkaline conditions there is denaturation, unwinding of the duplex DNA and expression of alkali labile sites as single strand breaks. The parameters measured to analyze the electrophoretic patterns were: Tail length were measured from the middle of the nucleus to the end of the tail and relative DNA content in the tail. with 40x objective fluorescence microscope (With excitation filter 420-490nm [issue 510nm]). a Komet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpool, UK) linked to a charge-coupled device (CCD) camera to assess the quantitative and qualitative extent of DNA damage in the cells by measuring the length of DNA migration and the percentage of migrated DNA. Finally, the program calculates tail moment. Generally, 50 to 100 randomly selected cells are analyzed per sample.

9. Statisticalanalysis

Results were expressed as mean ± standard deviation (SD). Multigroup comparisons of the means were carried out by one way analysis of variance (ANOVA) test. Least significant difference (LSD) test was used to compare the difference between the experimental groups and the control group. Descriptive data were compared by (chi-square test). The statistical significance difference for all tests was set at $P< 0.05$. Correlation coefficient (r) was used for testing the association between two continuous variables using SPSS software (v.16; SPSS).

Results

1. Body weight and Coefficient of spleen

Results of this study revealed that TiO2NPs treatment for 12 weeks induced highly significant increase in the coefficients of the spleen ($P<0.0001$) when compared to control group. Stopping of administration of TiO2NPs for 8 weeks in the Follow up group significantly reduced these values compared to TiO2NPs group ($P<0.0001$) (Table 1).

2. Leucocytes Counts

Rats of TiO2NPs treated group showed highly significant decrease of total White blood cells (WBC) and differential counts($P<0.0001$). Stopping of administration of TiO2NPs significantly increased total WBC and differential counts ($P<0.0001$) when compared to TiO2NPs treated group (Table 2).

3.Cytokines Assay

Titanium dioxide NanoParticles (TiO2NPs) treated group showed significant decrease in both IL-2 and IL-10 as compared to control ($P<0.0001$)while cessation of treatment showed significant increase of both

Interleukins as compared with TiO2NPs group (P<0.0001) (Table 3 Bar charts 1 and 2).

4.Immunohistochemical Staining of spleen

CD4 and iNOS expression was examined in spleen tissues of studied groups and results were showed in (Table 4) and (Table 5).

In terms of CD4 marker expressions, there was an highly significant reduction in TiO2NPs treated group as compared to control groups and were graded (+) P<0.0001 (Table 4). InTiO2NPs treated group all splenic sections showed low level expression of CD4+ T-lymphocytes (Plates1 Cand F) as compared to control tissues that showed numerous brown positive membranous immunoreactions around white pulp (Plates 1 A, B and E).

While the splenic sections of rats of Follow up group showed highly significant increase in CD4 marker expressions as compared to TiO2NPs treated group and were graded (++) P<0.0001 (Table 4) and(Plates 1D).

According to inducible nitric oxide synthase (iNOS) expressions, there was highly significant difference between TiO2NPs treated group and control groups where all splenic sections were positive and 60% of them had scored (3) or strong expressions P<0.0001 (Table 5), also TiO2NPs treated group showed brown cytoplasmic immune reactions staining the red pulp of spleen (Plates 2 C and E) compared to control sections (negative iNOS immunostained) (Plates 2 A, B).

While when rats of Follow up group were left without treatment for 8 weeks, their splenic sections showed highly significant difference as compared to that of TiO2NPs group where most of splenic sections were negative and 60% of them had scored (0) P<0.0001 (Table 5) and showed weaker immunoractivity within red pulp and in sinus lining cells (Plates2 D and F).

From the Immunohistochemical staining results, a significant negative correlation between CD4 and iNOS expression was found in both TiO2NPs treated group (r= -0.97) and follow up group (r= -0.56) (Table 6) (Figure 1 A and B).

5.Histopathology Examination of spleen

Examination of H&E stained spleen sections of control –ve and control +ve showed normal spleen consisted of white and red pulps. The white pulp was composed of well-circumscribed lymphoid follicles, a peri-arterial lymphoid sheath and marginal zone which is clearly differentiated between the non-lymphoid red pulp and the lymphoid white-pulp. The lymphoid follicles consisted of a large number of lymphocytes, most of them appeared to have condensed darkly stained nuclei. The red pulp was composed of branching and anastomosing splenic cords and blood sinusoids in between (Plates 3 A and B). Treatment with TiO2NPs for12 weeks caused disruption of splenic architecture, apparent white pulp atrophy in the form of reduction of both size and cellular component of lymphatic follicles, differentiation between red and white pulp is indistinct and congested sinusoids (the sinusoids were packed with RBCs) (Plates 3 C and E).While in follow up group that left without treatment for 8 weeks splenic tissues showed an improvement of the white pulp architectures as increase in the size and cellular components of the lymphatic follicles and marginal zone is differentiated from the red pulp which still showed congested sinusoids (Plates 3D and F).

6.Comet Assay of spleen

Splenocytes exposed to TiO2NPs treatment for 12 weeks have exhibited DNA damage in the form of increased tail moment percentage and tail length as compared to control group that showed un-damaged nuclei. Tail moment reveal (the product of tail length and the fraction of total DNA in the tail) where the tail length represent the smallest detectable size of migrating DNA and the fraction of total DNA or the intensity of DNA represent the number of relaxed/broken pieces in the tail. On the contrary, on stopping administration of TiO2NPs, moderate improvement was noticed in the form of reduction of the damaged cells in Follow up group as compared to TiO2NPs group (Table7) and (Figure 2) .

Table 1: ANOVA (F-test) statistical analysis for comparison of Body weight and spleen Coefficient of rats from different studied groups.

Index	Group I (-ve Control)	Group II (+veControl)	Group III (TiO2NPs)	Group IV (Follow up)	F test	P values
	mean ± SD	mean ± SD	mean ± SD	mean ± SD		
Body weight (g)	205.50 ± 3.43	204.4 ±3.40	*178 ±14.94	**202.5 ±2.63	27.96	0.000
Spleen coefficient (g)	2. 905 ± 0.09	2.87 ± 0.11	*4.82 ± 0.28	**3.016 ± 0.031	333.19	0.000

Results are expressed as mean ± SD (Number = 10 rats/group), P value of > 0.05 = Non-Significant
*Least significant difference (LSD) (P=0.05) = 11.56, 191.17 respectively,Least significant difference(LSD) (P=0.01) = 14.33, 236.91 respectively, * Highly significantly different compared to control (P < 0.0001), **Highly significantly different compared to TiO2NPs (P<0.0001).*

Table 2: ANOVA (F-test) statistical analysis for comparison of Total WBC and Differential WBCs counts in different studied groups.

Group	Group I (-veControl)	Group II (+veControl)	GroupIII (TiO2NPs)	Group IV (Follow up)	F test	P value
parameter	mean ± SD	mean ± SD	mean ± SD	mean ± SD		
WBCs(10^3/cc)	6.03 ± 0.048	6.02 ± 0.049	*5.05 ± 0.052	**5.90 ± 0. 059	807.468	0.000
Lymphocyte (%)	86.88 ± 0.103	86.89 ± 0.087	*83.13 ± 0.115	**85.30 ± 0.209	1.7623	0.000
Monocyte (%)	6.89 ± 0.137	6.87 ± 0.141	*5.93 ± 0.105	**6.11 ± 0.082	113.522	0.000
Granulocyte (%)	8.75 ± 0.108	8.77 ± 0.094	*7.84 ± 0.060	**8.59 ± 0.875	189.662	0.000

Results are expressed as mean ± SD (Number = 10 rats/group), P value of > 0.05 = Non-Significant
Least significant difference (LSD) (P=0.05) = 5.73, 21.41, 5.70, 4.82 respectively, Least significant difference (LSD) (P=0.01) = 7,11, 26.53, 7.06, 5.97 respectively.
** Highly significantly different compared to control (p < 0.0001), ** Highly significantly different compared to TiO2NPs (p < 0.0001).*

Table 3: ANOVA (F-test) statistical analysis for comparison of serum levels of IL-2 and IL-10 in different studied groups.

Group	Group I (-veControl)	Group II (+veControl)	GroupIII (TiO2NPs)	Group IV (Follow up)	F test	P value
parameter	mean ± SD	mean ± SD	mean ± SD	mean ± SD		
IL-2 (Pg/ml)	134.2 ± 0.29	134.07 ± 0.19	*129.2 ± 0.49	**133.4 ± 0.39	421.964	0.000
IL-10 (Pg/ml)	62.64 ±0.43	62.68 ±0.44	60.76 ±0.17	61.92 ±0.26	66.802	0.000

Results are expressed as mean ± SD (Number = 10 rats/group), P value of > 0.05 = Non-Significant
Least significant difference (LSD) (P=0.05) = 28.57, 11.69 respectively, Least significant difference (LSD) (P=0.01) = 35.41, 14.50 respectively.
** Highly significantly different compared to control (p < 0.0001), ** Highly significantly different compared to TiO2NPs (p < 0.0001).*

Table 4: Chi-square test statistical analysis of CD4 expressions in different studied groups.

CD4 expressions	Groups								Total		P value
	Control -ve		Control +ve		TiO2NPs		Follow up				
	No	%	No	%	No	%	No	%	No	%	
+(1-25 cell)	0	00.0	0	00.0	10	100	0	00.0	10	25	0.000
++(26-50 cell)	2	20	1	10	0	00.0	9	90	12	30	0.000
+++(≥51 cells)	8	80	9	90	0	00.0	1	10	18	45	0.000
Total	10	100	10	100	10	100	10	100	40	100	0.000

P value = **0.0001** Highly Significant, $\mathbf{X^2 = 61.111,}$ (Number = 10 rats/group).

Table 5: Chi-square test statistical analysis of iNOS expressions in different studied groups.

iNOS expressions	Groups								Total		P value
	Control -ve		Control +ve		TiO2NPs		Follow up				
	No	%	No	%	No	%	No	%	No	%	
Negative(0)	8	80	9	90	0	00.0	6	60	23	57.5	0.000
Weak(1)	2	20	1	10	0	00.0	4	40	7	17.5	0.000
Moderate(2)	0	00.0	0	00.0	4	40	0	00.0	4	10	0.000
Strong(3)	0	00.0	0	00.0	6	60	0	00.0	6	15	0.000
Total	10	100	10	100	10	100	10	100	40	100	0.000

***P value= 0.0001** Highly Significant. X^2 =43.4783, (Number = 10 rats/group).*

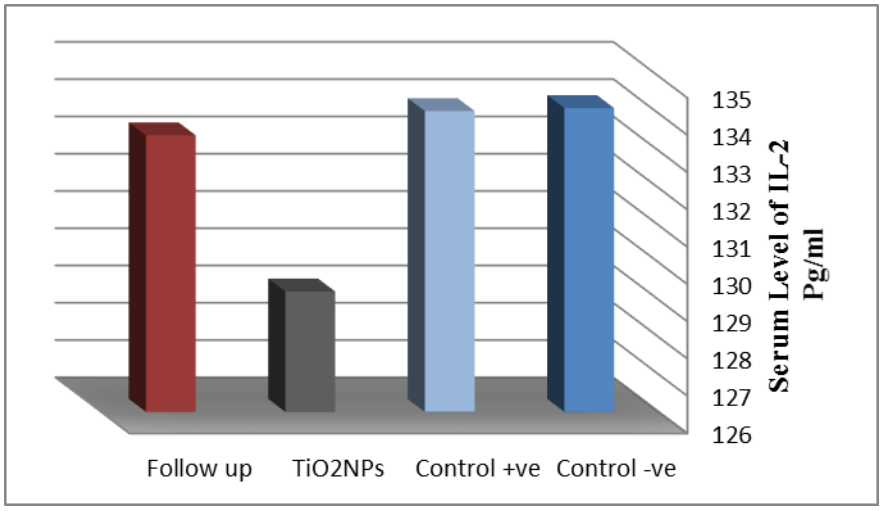
Table 6: Pearson Correlation statistical analysis showing negative correlation between iNOS expressing cells and the CD4⁺ T cell count in (A) TiO2NPs treated group and (B)Follow up group.

A		CD4	iNOS	**B**		CD4	iNOS
CD4	Pearson Correlation	1	-.97	CD4	Pearson Correlation	1	-.556
	Sig. (2-tailed)		.000		Sig. (2-tailed)		.00
	N	10	10		N	10	10
iNOS	Pearson Correlation	-.97	1	iNOS	Pearson Correlation	-.556	1
	Sig. (2-tailed)	.000			Sig. (2-tailed)	.00	
	N	10	10			10	10

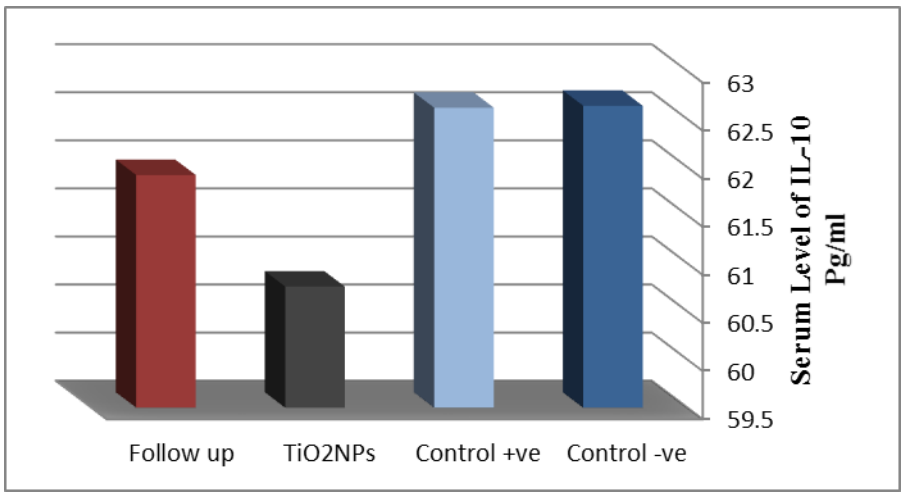
r= - 0.97. P=0.0001. *Highly Significant,* ***r= - 0.56. P=0.001.*** *Highly significant .(Number = 10 rats/group).*

Table 7: Comet Assay showing distribution of changes in DNA parameters in different studied groups.

UNIT	**%**	**%**	**µm**	**%**	**%**
DNA parameter	Tailed	Head	Tail length	Tail DNA	Tail moment
Control -ve	3	97	1.80	1.60	0.028
Control +ve	3.2	96.8	1.82	1.70	0.030
TiO2NPs	14.7	85.3	4.95	5.04	0.249
Follow up	5	95	2.80	2.42	0.067



Bar chart 1: Serum Interleukin (IL-2). Values obtained from rats treated for 12 weeks and others untreated for 8 weeks.
Data expressed as Mean ± SD (Number = 10 rats/group).



Bar chart 2: Serum Interleukin (IL-10). Values obtained from rats treated for 12 weeks and others untreated for 8 weeks.
Data expressed as Mean ± SD (Number = 10 rats/group).

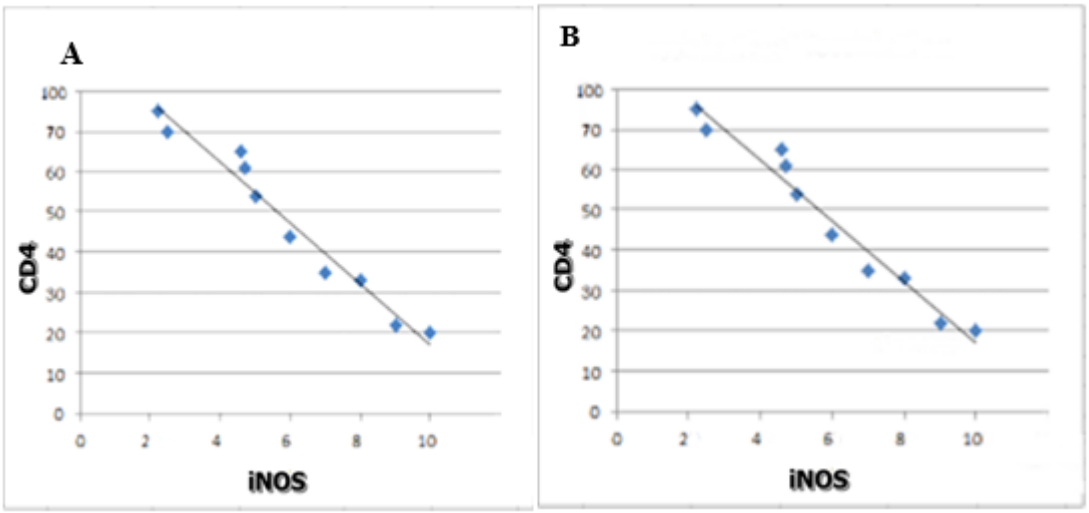


Figure 1: Pearson Correlation statistical analysis showing negative correlation between iNOS expressing cells and the CD4⁺ T cell count in (A) TiO2NPs treated group and (B) Follow up group. r= -0.99. P=0.0001.

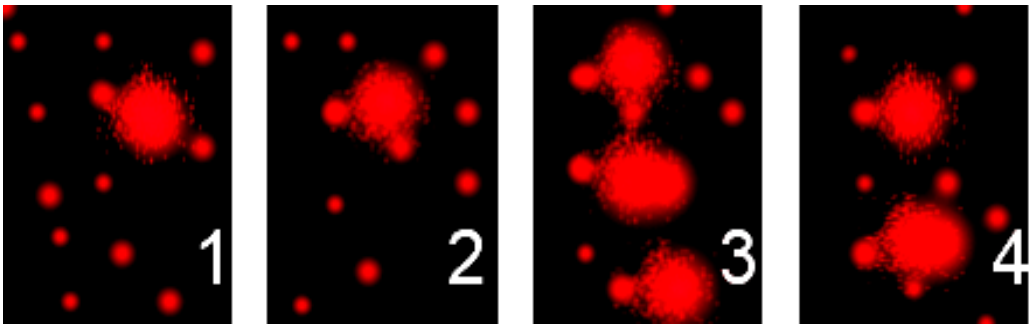


Figure 2: Epi-fluorescence images of DNA damage in Comet Assay.
(1) Control–ve and (2)Control + ve showing nuclei of un damaged cells (3)TiO2NPs group showing induced various degree of damage in DNA of splenocytes (4) Follow up group after stopping of TiO2NPs showing decreased the damaged cells.

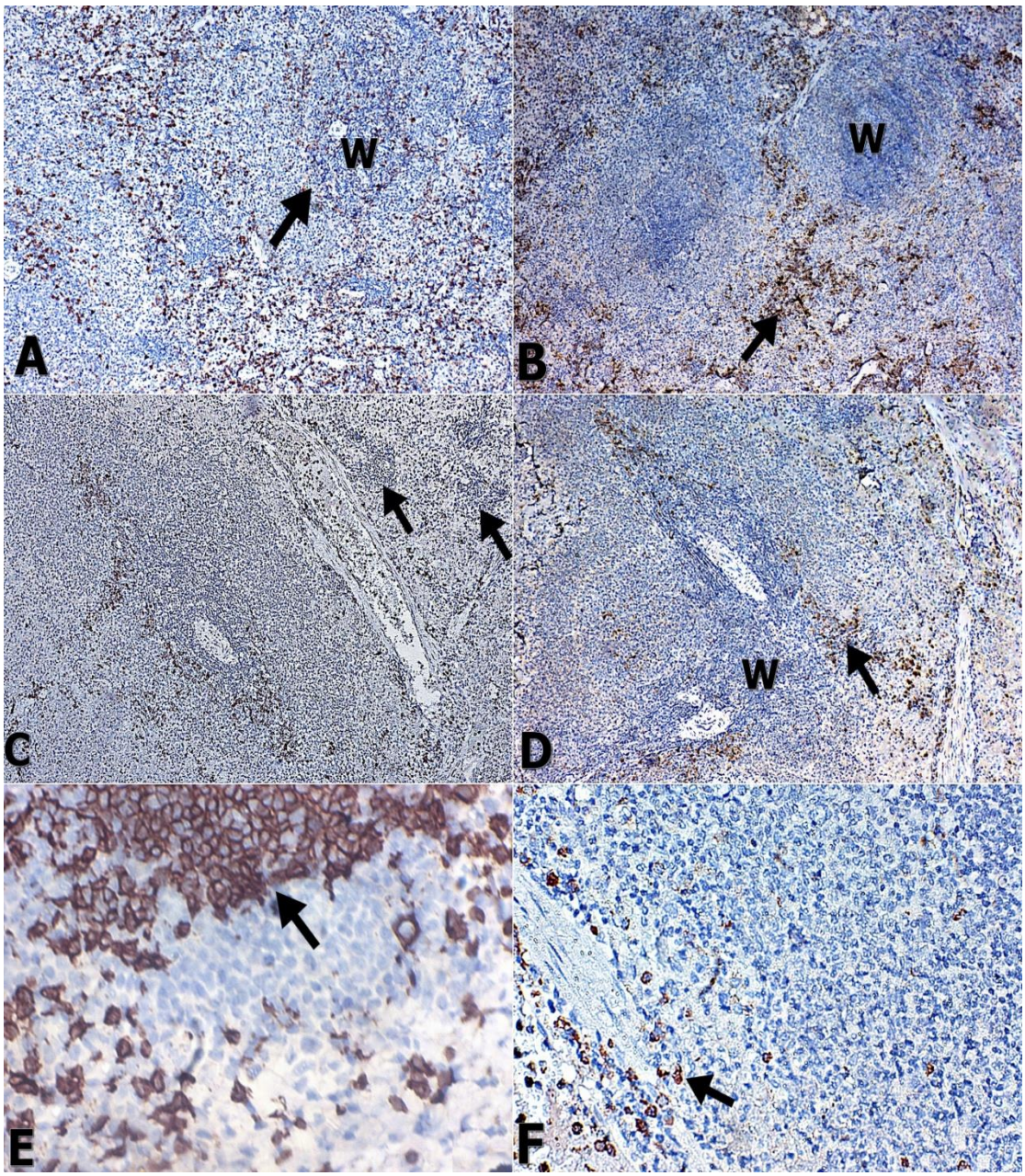


Plate 1: Immunohistochemical staining micrographs of Spleen for CD4. (A) - ve Control group showing numerous brown positive immunoreactions for T-lymphocytes (arrow) grade ++ arranged around white pulp (W). (B)+ ve Control group showing numerous brown positive immunoreactions for T-lymphocytes (arrow) grade +++.(C)TiO2NPs treated group showing decreased level of CD4 grade + and three small size follicles were seen in the upper right area (arrows).(D)Follow up group showing brown positive immunoreactions of CD4 grade ++ around normal sized follicles (arrow).(E)Control group showing positive membranous staining for CD4 (arrow).(F) TiO2NPs treated group showing few number of cells with positive membranous stain (arrow). (Images magnification, A,B,C,D, 100 X& E, F, 400X).

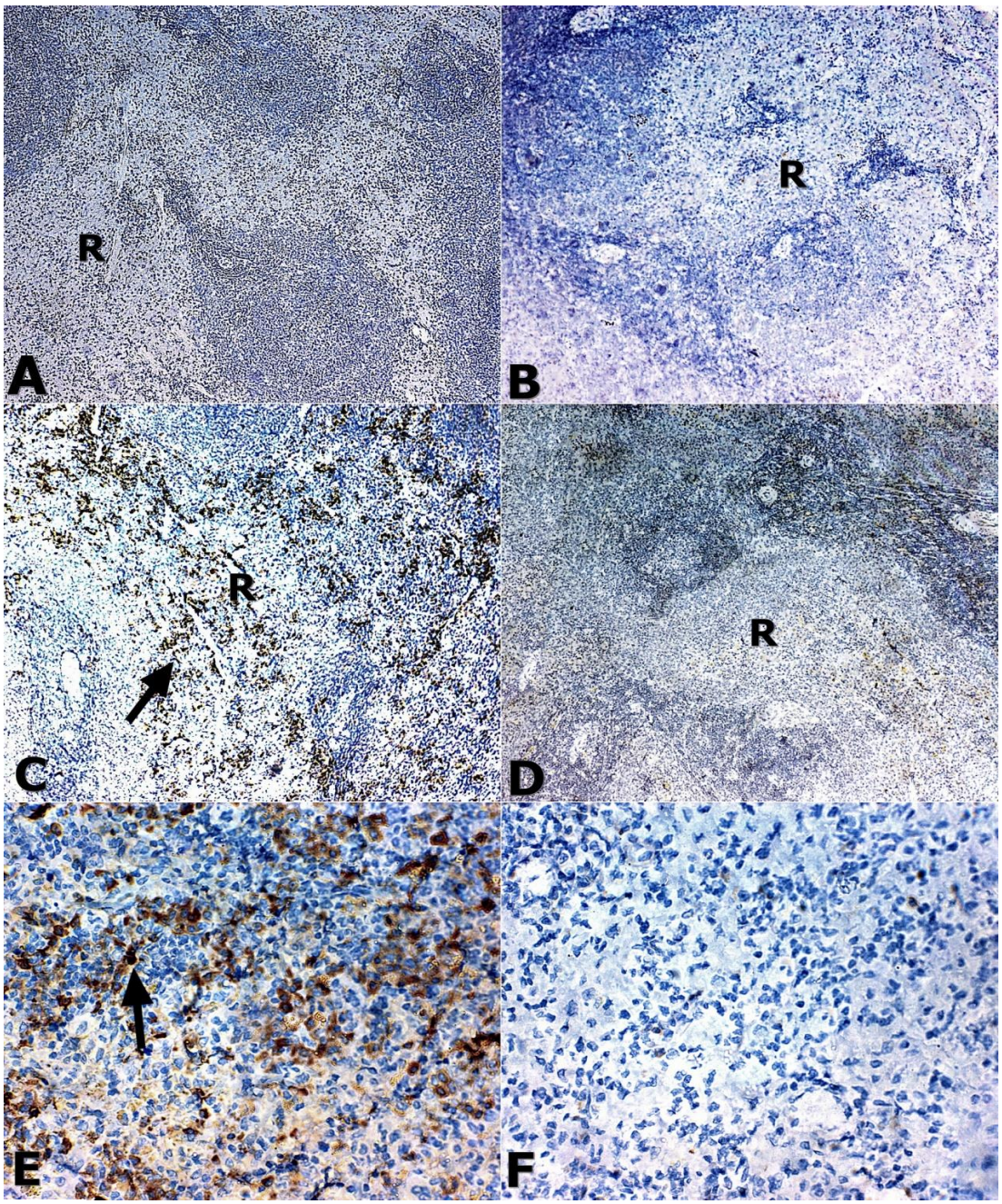


Plate 2: Immunohistochemical staining micrographs of Spleen tissues for iNOS. (A,B) Control group showing red pulp (R) with negative staining for iNOs. (C) TiO2NPs treated group showing positive brown staining for iNOS (arrow) in the red pulp (R). (D) Follow up group showing weak immunohistochemical staining for iNOS. (E) TiO2NPs treated group showing positive strong cytoplasmic staining for iNOS (arrow). (F) Follow up group showing negative staining for iNOS. (Images magnification, A,B,C,D, 100 X & E, F, 400X).

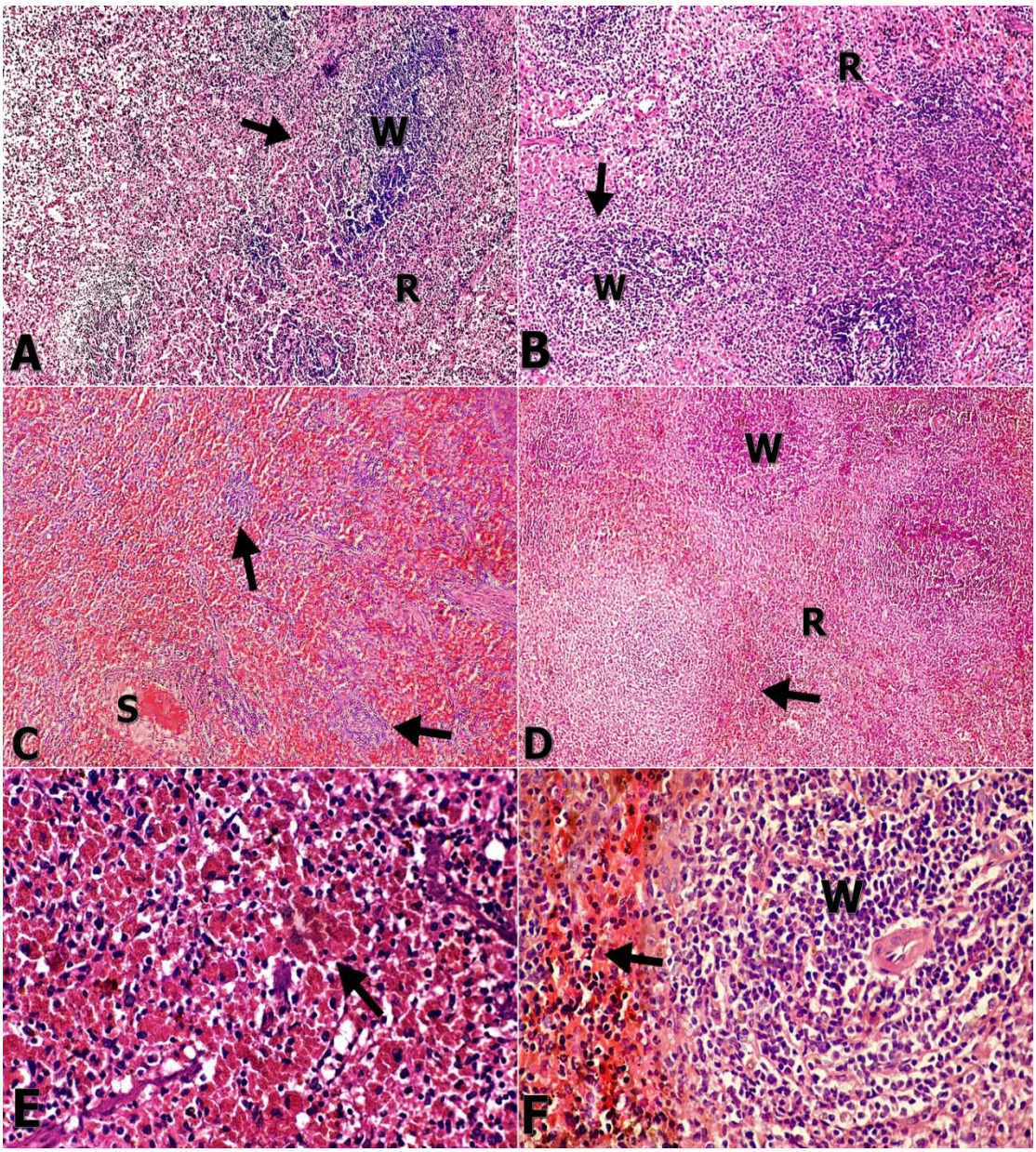


Plate 3: H&E staining micrographs of spleen tissues. (A,B) Control group showing normal spleen architecture with intact white pulp (W),clear red pulp (R) and distinct marginal zone (arrow). (C) TiO2NPs treated group showing spleen architecture disruption, decreased in both size and cellular component of the spleen follicle (arrows) and congested sinusoids (S).(D) Follow up group showing reconstruction of the spleen architecture, increased lymphocytes, follicle size improvement in white pulp (W) and the marginal zone become distinct between white and red pulp (R)(arrow).(E) TiO2NPs treated group showing sinusoids were packed with RBCs. (F) Follow up group showing areas of congestion (arrow). (Images magnification, A,B,C,D, 100 X& E, F, 400X).

Discussion

Toxicologists and regulatory scientists become interested with nanotoxicology and nano-risk particularly regarding to the fine-sized nanoparticles that may render them potentially toxic (Li and Nel, 2011).

TiO2NPs is a versatile compound that has broadly been used in modern cosmetics. The spleen play an important role in immune response (Mebius and Kraal,2005), thus, any alteration of T lymphocytes population or its cytokines and any affection of DNA after consumption of TiO2NPs may reflect its immunotoxicity.

In the current study, oral gavages of TiO2NPs for 12 weeks resulted in reduction of the body weight of TiO2NPs treated rats and increase the coefficients of the spleens. These are in consistent with Li et al.,2010 who observed significant increase in the coefficients of the spleens after intra-peritoneal injection of TiO2NPs at doses of 50 and 150 mg/kg BW for 45days and attributed the cause to TiO2NPs significant accumulation in the mouse spleen.

Moreover, Xu et al.,2013 found increase in the organ coefficients of the spleens after intravenous injection of 140, 300, 645, and 1387 mg/kg BW of TiO2NPs and both suggested increase spleen coefficient to TiO2NPs accumulation in the organs. On the other hand, after stopping of oral treatment of TiO2NPs for 8 weeks, there was reduction in the splenic coefficient compared to that of TiO2NPs treated group, although not reaching to the spleen coefficients of control. This may be attributed to weight regain after its reduction during TiO2NPs administration and attributed also to decreased the congestion of splenic tissues.

The immunological function of each of the WBC types has been reviewed extensively elsewhere (Thrall,2004) where neutrophils /heterophils and lymphocytes make up the majority of WBCs in mammals (Jain, 1993). T-lymphocytes have an important role in maintaining of immune response (Ayuob,2013) and CD4 + T cells can produce IL-2 and to lesser extent IL-10 which primarily produced by monocytes (Sojka et al.,2004). Therefore, examination of WBCs and differential leukocytes related to CD4+T cells and also examination of CD4+T cells in splenocytes could determine the immunological state.

The results of the present study showed deficiency of total and differential leukocytes counts (lymphocytes, monocytes and granulocytes) in rats that treated with oral TiO2NPs for 12 weeks. These results are supported by that of Duan et al., 2010 who reported significant decrease in WBC, RBC, HGB, MCHC, and PCT blood levels after intragastric administration of TiO2NPs at doses of 125, 250 mg/kg BW for 30 consecutive days and attributed the deficient metabolism and immune response of mice to marked decrease in O2 content in the blood by the effect of the higher dose of TiO2NPs.

Hamrahi-michak et al., 2012 explained the decreased level of leucocytes after its pervious increase because of the high concentrations of nanoparticles can enter into the lymphatic system, producing inflammation and enlargement in lymph nodes leading to increase the number of WBCs. But, after a period of time, the activity of these glands decreased and lymph node atrophy has been noted.

leukocytes and differential leukocytic counts compared toTiO2NPs treated group. No previous studies on the improvement potentials after stopping of TiO2NPs administration were found.

Cytokines are cells expressing proteins that are considered important mediators to regulate immune response (Szelényi,2001), also any changes in these proteins levels could be considered an indirect index to assess immune function status (Liu et al.,2014). Therefore, cytokines levels were examined to evaluate the immune response to TiO2NPs administration.

In the present study, TiO2NPs treatment for 12 weeks showed significant decrease in both IL-2 and IL-10 as compared to those of control group while cessation of treatment for 8 weeks showed significant increase of both interleukins as compared to TiO2NPs group. These results are consistent with those of Duan et al.,2010 who reported significant decrease in IL-2 activity of the mouse serum after intragastric administration of TiO2NPs at doses of 125, 250 mg/kg BW for 30days and explained this to the decreased proliferation of CD4+ T cells. Also, Sojka et al., 2004 observed a decrease in IL-10 activity in TiO2NPs treated rats,which may be caused by decreased lymphocytes population. The potential improvement after cessation of TiO2NPs consumption for a period of time could be attributed to moderate restoration of the activity of lymphocytes after a period of complete discontinuity.

The immunohistochemical staining of splenic tissue sections of TiO2NPs group treated for 12 weeks showed decreased expression of CD4+ marker indicating reduced T lymphocyte cells in white pulp as compared to that of control. While, stopping of TiO2NPs administration for 8 weeks allowed moderate restoration of T lymphocyte cells guided by significant increase of CD4+ marker expression when compared to TiO2NPs treated group.

Also, the results of the present study are consistent with those of Duan et al., 2010 who reported decreased proliferation of T lymphocytes (including CD3, CD4, and CD8), B lymphocyte and natural killer lymphocyte and the ratio of CD4 to CD8 of mice after intragastric administration of TiO2NPs at doses of 125, 250 mg/kg BW for 30days and attributed this to inhibition of IL-2 activity that lead to reduction of the proliferation of T lymphocytes and inhibition of other immunologically competent cell activation. No previous studies discussed the potentialities of regaining T lymphocytes activities after a period of cessation of TiO2NPs consumption.

The structural modifications in T-lymphocytes may be affected by oxidative stress that lead to being hypo-responsive (Cemerski et al.,2003). Nitric oxide production from iNOS-expressing cells suppresses mouse T cell proliferation leading to oxidative cell toxicity(Aheng et al.,2011). Therefore, examination of iNOS expressing cell in splenocytes could determine its role in immunological state.

The immunohistochemical staining of splenic tissue sections of TiO2NPs group treated for 12 weeks showed strong iNOS immunreactivity in the red pulp pointed to increase production of NO inducing oxidative stress and suppressing CD4+T cells.These results are consistent with those of Duan et al., 2010 who reported higher levels of NO in blood serum after intragastric administration of TiO2NPs at doses of 125, 250 mg/kg BW for 30days and also with Ma et al.,2010 who reported excessive release of nitric oxide in brain tissues of mice after injection into abdominal cavity of 50,100, and 150 mg/kg BW of TiO2NPs for 14 days. Where Filep et al.,1996 stated that NO is closely related to cellular immune function by mediating natural killer cells to kill YAC-1 lymphocyte tumor. While, stopping of TiO2NPs administration for 8 weeks showed weak iNOS immunreactivity in the red pulp of splenic tissues giving a thought that the period of discontinuity allowed CD4+T cells to regain its

activity leading to decreasing in the level of NO that indicated by decreasing iNOS expressing cells.

Also, The histopathological examination of splenic sections of rats treated with TiO2NPs for12 weeks caused disruption of spleen architecture, sever congestion of tissues, decreased in both size and cellular component of the lymphatic follicles and absence of demarcation between red pulp and white pulp as compared to spleen architecture of control.These findings are supported by those of Li et al.,2010 who reported congestion of the spleen tissue and proliferation of lymphatic follicles after intra-peritoneal injection of 50mg/kg BW TiO2NPs for 45days suggested that TiO2NPs induced oxidative reaction causing splenic lesion. Chen et al.,2009 found a mass of neutrophilic cells in spleen tissues as a result of accumulated large number of TiO2 particles that induced severe splenic lesion after intra-peritoneal injection of higher doses of TiO2NPs(324–2592 mg/kg) for 7 days.

On the other hand, stopping of TiO2NPs administration for 8 weeks, splenic tissues showed increase in the size and cellular components of the lymphatic follicles and marginal zone is differentiated from the red pulp which still showed congested sinusoids.

The results of the present Comet Assay after treatment with TiO2NPs for12 weeks showed genotoxic effects of TiO2NPs on spleen tissues. These results are in agreement with the review of Landsiedel et al.,2009 where they reported genotoxic effects about nanomaterials including TiO2NPs and describing micronuclei development, as an indicative of chromosomal damage and DNA damage caused byTiO2NPs.These results also are in consistent with those of Trouiller et al., 2009 who reported significant increase in tail moment after oral treatment with 500 mg/kg BW of TiO2 NPs for five consecutive days where they measured DNA stand breaks by the alkaline comet assay in mice peripheral blood and attributed DNA damage to oxidative stress.

Mohamed and Hussien, 2016 also reported the genotoxic effects of TiO2NPs on brain tissue after treatment of mice with TiO2NPsat 500 mg/kg BW for five consecutive days TiO2NPs by using comet assay and referred these effects to oxidative burst that caused by TiO2NPs leading to release superoxide anions ($O2^{-\cdot}$) which convert to multiple reactive oxygen species(ROS).Several previous *in-vivo* studies demonstrated the gentoxic effects of TiO2NPs as Reeves et al., 2008 reported TiO2NPs oxidative-stress–related effects including inflammation, cytotoxicity and genomic instability, either alone or in the presence of UVA irradiation. Gurr et al., 2005 showed that TiO2NPs induced mainly hydrogen peroxide and nitric oxide generation leading to lipid peroxidation and oxidative DNA damage in lung epithelial cells.

On the other hand, the present Comet Assay after cessation of TiO2NPs for 8 weeks showed decreased DNA damaged cells as compared to that of TiO2NPs group. Conclusion:

TiO2NPs consumption have immunotoxic effects which may resulted from genetic damage and oxidative stress in the spleen cells of adult male albino rats which could be moderately improved by its discontinuation for a period of time and it is recommended to increase the period of discontinuation as complete improvement may occur.

References

Aheng C, Ly N, Kelly M et al. (2011): Deletion of UCP2 in iNOS deficient mice reduces the severity of the disease during experimental autoimmune encephalomyelitis. *P.L.o.S. ONE,* 6(8): 22841.

Ayuob NN (2013): Can vitamin E and selenium alleviate the immunologic impact of aluminium on pregnant rats' spleens? *Cell Immunol.,* 284: 104-110.

Cemerski S,Van Meerwijk JP and Romagnoli P (2003): Oxidative-stress-inducedT lymphocyte hyporesponsiveness is caused by structural modification rather than proteasomal degradation of crucial TCR signaling molecules. *Eur. J. Immunol.,* 33: 2178-2185.

Chen JY, Dong X, Zhao J et al.(2009): In vivo acute toxicity of titanium dioxide nanoparticles to mice after intraperitoneal injection. *J. Appl. Toxicol.,* 29, 330–337.

Choi H, Stathatos E and Dionysiou DD (2006): Solgel preparation of mesoporous photocatalytic TiO2 films and TiO2/Al2O3 composite membranes for environmental applications. *Appl. Catal. B-Environ.,* 63: 60–67.

Demir E, Kaya N and Kaya B (2011):Evaluation of DNA damage in *Capsicum annuum* L. exposed to spinosad in the comet assay. *Fresen. Environ. Bull.,* 20: 1926–1930.

Demir E, Kaya N and Kaya B (2014): Genotoxic effects of zinc oxide and titanium dioxide nanoparticles on root meristem cells of *Allium cepa* by comet assay. *Turk. J. Biol.,* 38: 31-39.

Duan Y, Liu J, Ma L et al.(2010): Toxicological characteristics of nanoparticulate anatase titanium dioxide in mice. *Biomaterials,* 31: 894–899.

Elmore SA (2006): Enhanced histopathology of the spleen. *Toxicol. Pathol.,* 34: 648-655.

Filep JG, Baron C, Lachance S et al.(1996): Involvement of nitric oxide in target-cell lysis and DNA fragmentation induced by murine natural killer cells. *Blood,* 87:5136–43.

Frank JMF, Bernd DG and Kuwaki K (2005): Histopathology of spleen allograft rejection in miniature swine. *International Journal of Experimental Pathology*. 86, (1): 57–66.

Gnarro LJ (2002): Nitric oxide as a unique signaling molecule in the vascular system: a historical overview. *J. Physiol. Pharmacol.,* 53:503-514.

Guo X, Fan Y, Lang R et al.(2008): Tumour infiltrating lymphocytes differ in invasive micropapillary carcinoma and medullary carcinoma of breast. *Mod. Pathol.,* 21 :1101-7.

Gurr JR, Wang AS, Chen CH et al.(2005): Ultrafine titanium dioxide particles in the absence of photoactivation can induce oxidative damage to human bronchial epithelial cells. *Toxicology* 213: 66–73.

Hamrahi-michak M, Sadeghi SA, Haghighi H et al.(2012): The toxicity effect of cerium oxide NP on blood cells of male rat. *Ann. Biol. Res.* 3: 2859–2866.

ILAR (Institute of Laboratory Animal Resources) (1996): Laboratory animal management: rodents. Institute of laboratory animal resources, commission on life sciences, national research council, national academy press, Washington, DC.

Jain NC (1993): Essentials of veterinary hematology. *Blackwell Publishing, 1st ed., Philadelphia, Lea & Febiger,* pp: 295–306.

Landsiedel RM D, Kapp M, Schulz K et al.(2009): Genotoxicity investigations on nanomaterials: methods, preparation and characterization of test material, potential artifacts and limitations-many questions, some answers. *Mutation Research/Reviews in Mutation Research*, 681(2-3) :241–258.

Li N, Duan Y, Hong M et al.(2010): Spleen injury and apoptotic pathway in mice caused by titanium dioxide nanoparticules. *Toxicol. Lett., 195*: 161–168.

Li N and Nel AE (2011): Feasibility of biomarker studies for engineered nanoparticles: what can be learned from air pollution research. *Journal of Occupational and Environmental Medicine*, 53 (6): S74–S79.

Liu C, Li M, Cao Y et al. (2014): Effects of avermectin on immune function and oxidative stress in the pigeon spleen. *Chem. Biol. Interact.,* 210: 43-50.

Liu H, Zhang H, Yang D et al. (2011): The effect of nanoparticles on T-lymphocyte populations of peripheral blood in rat. *J.Environ.Occup. Med., 29*: 320–323.

Liu H, Ma L, Zhao J et al.(2009): Biochemical toxicity of nanoanatase TiO2 particles in mice. *Biol. Trace. Elem. Res.,* 129:170-180.

Ma LL, Liu J, Li N et al.(2010): Oxidative stress in the brain of mice caused by translocated nanoparticulate TiO2 delivered to the abdominal cavity. *Biomaterials*, 31(1):99-105.

McClory S, Hughes T, Freud AG et al. (2012):Evidence for a stepwise program of extrathymic T cell development within the human tonsil. *The Journal of Clinical Investigation,* 122 (4): 1403–1415.

Mebius RE and Kraal G (2005): Structure and function of the spleen. *Nat. Rev. Immunol.,* 5: 606-616.

Mohamed HRH and Hussien NA (2016): Genotoxicity studies of titanium dioxide nanoparticles (TIO2NPs) in the brain of mice. *Scientifica, Hindawi Publishing Corporation*, 2016: 7.

Montazer M, Behzadnia A, Pakdel E et al. (2011):Photo induced silver on nano titanium dioxide as an enhanced antimicrobial agent for wool. *J. Photochem. Photobiol. B.,* 103:207-214.

Mungrue IN, Husain M, Stewart DJ (2002): The role of NOS in heart failure: lessons from murine genetic models. *Heart Fail. Rev.,* 7 (4): 407–22.

Nel A, Xia T, Madler L et al.(2006):Toxic potential of materials at the nanolevel. *Science, 311*: 622–627.

Park EJ, Yi J, Chung KH et al.(2008): Oxidative stress and apoptosis induced by titanium dioxide nanoparticles in cultured BEAS-2B cells. *Toxicol. Lett.,* 180, 222–229.

Parkash NS and Nagarkatti M (2014):Immunotoxicology:Modulation of the immune system by xenobiotics. *Defence Science Journal*, 37: 235-244.

Qing Shen J, Yang Q, Xue Y et al.(2015):Inducible nitric oxide synthase response and associated cytokine gene expression in the spleen of mice infected with Clonorchis sinensis. *Parasitol. Res.,* 114:1661–1670

Reeves JF, Davies SJ, Dodd NJF et al. (2008): Hydroxyl radicals (•OH) are associated with titanium dioxide (TiO2) nanoparticle induced cytotoxicity and oxidative DNA damage in fish cells. *Mutat. Res.,* 640: 113–22.

Riu J, Maroto A and Rius FX (2006): Nanosensors in environmental analysis. *Talanta,* 69:288–301.

Robertson TA, Sanchez WY and Roberts MS (2010): Are commercially available nanoparticles safe whenapplied to the skin? J. *Biomed. Nanotechnol.,* 6:452-468.

Ross S, Thomas P, Qian T, et al., (2005): Over expression of cyclooxygenase-2 in nasopharyngeal carcinoma and association with epidermal growth factor receptor expression. *Arch. Otolaryngol. Head Neck Surg.,* 131:147–152.

Sang X, Zheng L, Sun Q et al. (2012): The chronic spleen injury of mice following long-term exposure to titanium dioxide nanoparticles. *J. Biomed. Mater. Res. A.,* 100:894-902.

Saquib Q, Al-Khedhairy AA, Siddiqui MA et al.(2012):Titanium dioxide nanoparticles induced cytotoxicity, oxidative stress and DNA damage in human amnion epithelial (WISH) cells. *Toxicology in Vitro* 26: 351–361

Singh NP, McCoy MT, Tice RR et al. (1988): A simple technique for quantitation of low levels of DNA damage in individual. *cells. Exp. Cell Res.* 175 :184-191.

Sojka DK, Bruniquel D, Schwartz RH et al.(2004): IL-2 secretion by CD4+ T cells in vivo is rapid,

transient, and influenced by TCR-specific competition. *J. Immunol.*,172(10):6136-43.

Szacilowski K, Macyk W, Drzewiecka-Matuszek A et al.(2005): Bioinorganic photochemistry: frontiers and mechanisms. *Chem. Rev.*, 105:2647-2694.

Szelényi J (2001): Cytokines and the central nervous system. *Brain Res. Bull.*, 54: 329-338.

Thrall MA (2004): Hematology of amphibians. In: Veterinary hematology and clinical chemistry:Text and clinical case presentations. *Lippincott Williams & Wilkins, 2nd ed.,Philadelphia PA*, pp:277-289.

Trouiller B, Reliene R, Westbrook A et al.(2009): Titanium dioxide nanoparticles induce DNA damage and genetic instability in vivo in mice. *Cancer Res.*,69(22): 8784–8789.

Wang JJ, Sanderson BJ and Wang H (2007a): Cyto- and genotoxicity of ultrafine TiO2 particles in cultured human lymphoblastoid cells. *Mutat. Res.*, 628:99–106.

Wang JJ, Zhou G, Chen C, et al.(2007b): Acute toxicity and biodistribution of different sized titanium dioxide particles in mice after oral administration. *Toxicol. Lett.*, 168:176-185.

Xu J, Shi H, Ruth M et al. (2013): Acute toxicity of intravenously administered titanium dioxide nanoparticles in mice. *PLoS ONE*, 8(8): e70618.

Yanyun Fu, Zhang Y, Chang X et al.(2014): Systemic immune effects of titanium dioxide nanoparticles after repeated intratracheal instillation in rat. *Int. J. Mol. Sci., 15*: 6961-6973.

Zhao J, Bowman L, Zhang X et al.(2009): Titanium dioxide (TiO2) nanoparticles induce JB6 cell apoptosis through activation of the Caspase-8/Bid and mitochondrial pathways. *J. Toxicol. Environ. Health A.*, 72:1141-1149.

الملخص العربى

الاثار السمية المناعية للتعرض المزمن قصير المدى للجسيمات النانوية لثانى اوكسيد التيتانيوم على طحال الجرذان البيضاء البالغة دور المتابعة

اروى أحمد الشيخ و شيماء حامد آمين[١] وهناء محمد غتور[٢] و سماء صلاح عبد الفتاح[٣]

المقدمة: لقد ادى استخدام الجسيمات النانونية لثانى اوكسيد التيتانيوم فى عدة مجالات الى العديد من المشاكل الصحية.

الهدف : تقييم الاثار المناعية بعد التناول المزمن قصير المدى للجسيمات النانوية لثانى اوكسيد التيتانيوم على الطحال و تقييم دور المتابعة بعد التوقف عن التناول.

المواد والطرق المستخدمة: :لقد تم تقسيم اربعين جرذا أبيض من الفئران البيضاء البالغة إلى اربع مجموعات متساوية كالأتي : المجموعة الأولى (مجموعة ضابطة سالبة) و المجموعة الثانية (مجموعة ضابطة موجبة) تم اعطاء الجرذان صمغ السنط المذيب للتيتانيوم والمجموعة الثالثة: تم اعطاء الفئران ١٢٠٠مجم /كجم من الجسيمات النانونية لثانى اوكسيد التيتانيوم مرو واحدة يوميا عن طريق الفم لمدة ١٢ اسبوع اما المجموعة الرابعة فقد تم اعطاء الفئران نفس الجرعة وفى نفس المدة مثل المجموعة الثالثة ثم تم ترك الفئران دون اعطائها اى شيء لمدة ٨ اسابيع اخرى. تم عد كريات الدم البيضاء الكلية والنوعية وقياس نسبة الانترلوكين ٢و١٠.كما تم عمل دراسة هستوباثولوجية و دراسة هيستوكيمائية مناعية لقطاعات من الطحال للكشف عن بروتين السى دى ٤ للخلايا اللمفاوية والكشف عن البروتين المسئول عن إفراز اوكسد النتريك علاوة على ذلك تم فحص الحمض النووى لقياس المذنبات.

النتائج : لوحظ فى المجموعة الثالثة (المعالجة بواسطة الجسيمات النانونية لثانى اوكسيد التيتانيوم) انخفاض ذو دلالة احصائية فى كلا من عدد كريات الدم البيضاء الكلية والنوعية ونسبة الانترلوكين ٢و ١٠ ،نقص فى عدد الخلايا اللمفاوية المفرزة للسى دى ٤و زيادة فى الخلايا المفرزة لاوكسيد النيتريك مما يدل على تاثر الحالة المناعية للطحال ووجود اجهاد تاكسدى فى خلاياه.كما لوحظ ايضا تغيرات باثولوجية فى انسجة الطحال ووجود تلف فى الحمض النووى لخلايا الطحال. وعند مقارنة كل هذة التغيرات بالمجموعة الرابعة (عند توقف اعطاء المادة لمدة ٨ اسابيع) وجدت زيادة ذات دلالة احصائية تفيد تحسن فى نسب كريات الدم البيضاء الكلية والمختلفة ونسبة الانترلوكين ٢و ١٠،زيادة فى عدد الخلايا اللمفاوية المفرزة للسى دى ٤و نقص فى الخلايا المفرزة لاوكسيد النيتريك مما يدل على تحسن الحالة المناعية و الاجهاد التاكسدى لخلايا الطحال.كما لوحظ ايضا تحسن فى التغيرات الباثولوجية فى الطحال باستثناء بعض مناطق احتقان الانسجة كذلك وجد تحسن بسيط فى خلايا الحمض النووى التالفة.

الاستنتاج: من النتائج السابقة ، يمكن استنتاج ان استهلاك الجسيمات النانونية لثانى اوكسيد التيتانيوم لة اثار سمية مناعية على خلايا الطحال فى الجرذان البالغة والذى من الممكن ان يكون نتيجة للتلف الجينى والاجهاد التاكسدى, مع وجود تحسن ملحوظ بهذة التغيرات بعد فترة من الانقطاع التام عن اعطاء الجسيمات النانونية لثانى اوكسيد التيتانيوم وننصح بزيادة تلك الفترة لمتابعة امكانية حدوث التحسن التحسن التام.

١ قسم الطب الشرعى والسموم الاكلينيكي,جامعة الزقازيق, مصر.

٢ قسم الطب البا ثولوجي,جامعة الزقازيق, مصر.

٣ قسم الطب التشريح,جامعة الزقازيق, مصر