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Determination of the Immunogenic and Hematologic Effects of Titanium Nanoparticles Manufactured from *Aspergillus flavus in Vivo*

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Abstract

The Titanium dioxide nanoparticles (TiO_2 NPs) have been synthesized via biological methods using *Aspergillus flavus*, and were subjected to numerous tests to confirm the formation of nanoparticles such as UV visible spectroscopy X-ray diffraction (XRD), Fourier transform infrared (FTIR), Atomic force microscope (AFM) and Scanning electron microscope (SEM) analysis. This study evaluates the immunogenic and hematologic effects of TiO_2 NPs *in vivo* in rabbit groups as treatment after infection by two pathogenic bacteria; *Escherichia coli* (G1) and *Staphylococcus aureus* (G2). A dosage of 20 mg/kg of TiO_2 NPs was administrated orally to the rabbit groups (G1, G2) and the results were compared with the control group (G3) which received the same dosage of TiO_2 NPs without any prior bacterial infection. The infected groups showed a significant reduction in the values of RBC, PCV, Hb compared with the control group (G3) and the groups (TG1, TG2, TG3) that were treated with TiO_2 NPs. Neutrophils percentages were higher than Lymphocytes in the infected groups compared with the control group before and after the treatment. The titers of IgG and IgM showed a significant increase in the infected animals compared with the control group and the group that was administrated TiO_2 NPs only. The levels of IL-10, IL-6, IFN-γ and TNF-α showed a significant elevation (P <0.05) in the serum of infected groups (G1, G2) compared to those treated with TiO_2 NPs or that found in the control. The oral dosage of TiO_2 NPs showed no significant differences at (P >0.05) in group (TG3) compared with the control group (G3).

Key words: TiO_2 NPs, Hematological parameters, Immunoglobulin level, Cytokines

1. Introduction

According to recent studies, the effectiveness of nanoparticles has been evaluated in stimulating the immune system. Nanoparticles that target immune cells can manipulate or control the immunological diseases such as infectious diseases or tumor therapy. If the immune system cannot recognize any foreign substance as body-threatening, this substance is ignored or tolerated. Therefore, the immune response must be considered when it deals with nanoparticles within the body of the organism, (Moyano *et al*., 2012). There are critical issues that should be highlighted in this regard; the most important of which is the immune system's rejection of nanoparticles, which the body identifies as foreign substances. The toxicity of nanoparticles should be assessed and considered, as they could introduce pathological changes to the immune system, and that would render the nanoparticles incompatible with the immune system (Boraschi *et al*., 2012). Nanoparticles can be used as adjuvants (Li *et al*., 2014), for example, the HIV-2 virus vaccine in mice consists of Polymethylmethacrylate (PMMA) nanoparticles which are used as adjuvants that can enhance the response of the antibodies one hundred times higher than the conventional adjuvant aluminum hydroxide [Al(OH)3] (Stieneker *et al*., 1991). The mechanism of nanoparticle usage as an adjuvant is not well understood, but nanoparticles are thought to improve the ingestion of antigen, or stimulate the action of antigen-presenting cells (APCs) (Dobrovolskaia and McNeil, 2007). A number of studies are carried out in vitro and in vivo focusing on the direct toxic effects, crystal phase composition, size, charge, the route of administration, and amount of dosage of TiO_2 NPs. These are important factors when considering TiO_2 NPs as a drug (Ivo Iavicoli *et al*., 2012). The American Food and Drug Administration (FDA) has determined that TiO_2 NPs are non-toxic, and have been used in human food, medicine, food contact materials (Wist *et al*., 2004). The continual resistance of microorganisms to conventional antibiotics stimulates scientific researchers to find out alternatives to them especially against the multidrug-resistant bacteria. The unique properties of TiO_2 NPs render them important in the pharmaceutical and medical industries (Gerhardt *et al*., 2007). TiO_2 NPs have a broad spectrum of activity against microorganisms, including Gram-negative, Gram-positive and fungi as well

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(Josset *et al*., 2008). The other prosperity is that when manipulating TiO_2 NPs biologically, no toxic substances will be produced. Consequently, TiO_2 NPs are environmental friendly (Llorens *et al*., 2012).

2. Material and Methods

TiO_2 NPs have been synthesized from *Aspergillus flavus*. The biomass of the fungus was prepared by cultivation according to Baskar *et al*. (2013), and the synthesis of TiO_2 NPs was preformed according to Tarafdar *et al*. (2013). The manufactured nanoparticles were subjected to numerous tests to confirm the formation of nanoparticles such as UV visible spectroscopy ((UV-1800 series-Shimadzu/Japan), X-ray diffraction (XRD) (XRD-6000- Shimadzu/ Japan), Fourier transform infrared (FTIR) (Bruker/ Germany), atomic force microscope (AFM) (Phywe measure nano/ England), and scanning electron microscope (SEM) (Angstrom/ advanced/ USA) analysis.

2.1. Preparation of Bacterial Suspension

The bacterial suspension was prepared according to (Baron *et al*., 1998), which used to induce experimental infection in rabbits.

2.2. Preparation of Laboratory Animals

Twelve males of local rabbits (*laguscaniculus orycto*/ SDI/ Samarra) were used in this experiment. Their ages are 6 months, and their weights range from 700 to 950 g. To ensure their safety, they were examined by a specialist veterinarian, and were set under observation for seven days before the experiment, providing adequate ventilation and lighting conditions and a temperature between 23 and 25°C. The rabbits were fed the optimal formula according to (NAS-NRC, 2002). Food was provided, and the cleanliness of the cages has been observed, and sawdust was periodically changed throughout the experiment.

2.3. Distribution of Experimental Animals

As illustrated in Figure 1, three laboratory experiments were carried out as follows:

1. **First experiment**: Incubation Period
   In the first stage of the experiment, all the animals were incubated under the same conditions.
2. **Second experiment**: Feeding
   The animals were divided into three groups (each group has four animals): G1 was given the bacterial suspension of *Escherichia coli (E. coli)*, and G2 was given a bacterial suspension of *Staphylococcus aureus* (S. *aureus*) with an amount of 1 mL per day (1×10^8 cell/ mL). G3 was left without being given any kind of dosage. The health status of the animals was monitored and the changes were recorded daily. Symptoms began to appear clearly on the fifth day, and were clearer on the seventh day; these include inactivity, solitude, lack of movement, lack of appetite, as well as diarrhea in the animals infected with *E. coli*, and conjunctivitis in the animals infected with *S. aureus.* All the animals have been examined by a specialist veterinarian to ascertain their infection. Blood samples were withdrawn from the animals, and these samples were dispensed in two tubes, one without EDTA for serological tests, and the other with EDTA for hematological tests (Theml, *et. al*., 2004).
3. Third Experiment: Treatment
   The animals that were subjected to the infection (G1, G2) and the control group (G3) were administrated TiO_2 NPs with a concentration of 20 mg/ mL (Sankar *et al*., 2013) via mouth. The health status of all animals was monitored, and the procedure continued until the complete disappearance of the disease symptoms. Again, their safety was checked by a specialist veterinarian. Then, the blood samples were withdrawn in two sets of tubes.

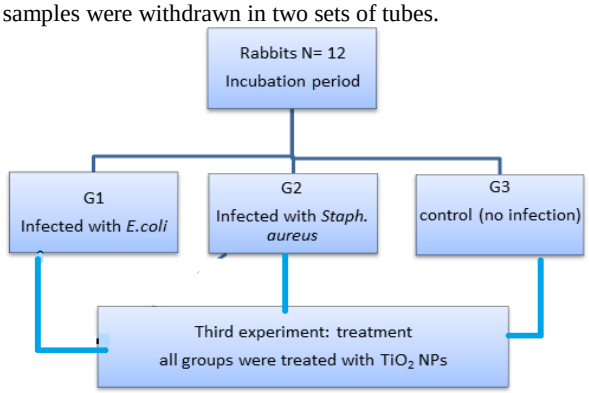

Figure 1. Illustrate the experiment design

G1: animals infected with *E. coli;* G2: animals infected with *S. aureus;* G3: control group; TG1: Infected G1 treated with TiO_2 NPs; TG2: Infected G2 treated with TiO_2 NPs; TG3: G3 treated with TiO_2 NPs.

2.4. Parameters Studies

2.4.1. Hematological Parameters

Blood was withdrawn from all groups to perform the hematological tests. One mL of blood was withdrawn by cardiac puncturing using sterile medical syringes. Blood was collected in plastic tubes containing anticoagulant EDTA. The total number of white blood cells and the differential number of white blood cells (the lymphocyte and granulocyte), RBC, Hb, PCV and PLT were calculated using automation automatic analysis (Company/ Minaray BC-3000 plus/ Germany).

2.4.2. Serological Parameters

Immunological tests were carried out on the serum of the rabbits to detect the presence of IL-6, IL-10, IFN-γ, TNF-α, IgG and IgM in all groups. Blood was collected by cardiac puncture. 5 ml of blood was collected in plastic tubes free of anticoagulant to obtain the serum using centrifuge 3000 rpm for five minutes. The serum samples were kept at -20°C until the time of testing using (Elisa kit/ eBioscience and Abnovo / UK).

3. Results and Discussion

3.1. Hematological Parameters

Only few studies have investigated the influence of TiO_2 NP exposure on the hematological parameters (Ivo Iavicoli *et al*., 2012). Table 1 illustrates some of these parameters in the treated rabbit groups. The results revealed that there are many effects of *E. coli* and *S. aureus* infection in male rabbit's blood parameters. The hematological parameters in the infected groups (G1, G2)

which are administered 1 mL of *E. coli* and *S. aureus* suspension, showed significant changes ($p<0.05$) when compared with the normal control group (G3). The total white blood cells (WBC) count was found to be increased with the reduction in the total red blood cell (RBC) count, hemoglobin (Hb), PCV and PLT. The total white blood cells count in the group infected with *E. coli* and *S. aureus* reached 17.5, 16.1($\times 10^3$ cell / mm^3) respectively compared with 3.9 ($\times 10^3$ cell / mm^3) in the control group.

The increase in the number of white blood cells after being infected with bacteria is attributed to inflammation, which usually occurs after bacterial infections. The lipopolysaccharide (endotoxin) of gram negative and exotoxin of gram positive was found to be immunogenic and mitogenic for many immune cells such as B cells and T cells (Pulendran *et al.*, 2001; Su *et al*., 2002). However, there were no significant changes of these parameters in the treated groups (G1 and G2) which received oral doses of 20 mg/mL of TiO_2 NPs compared with the control group. The results of this study agree with (Aysa, 2017).

Table 1 shows the significant reduction in the hemoglobin level ($p< 0.05$) for the study groups compared with the control group; for the groups infected with *E. coli* and *S. aureus* it decreased to 9.1, 8.9 (dl/mL) respectively while for the control group it was 12.5 (dl/mL). The groups infected with *E. coli* and *S. aureus* showed a significant reduction in the percentage of PCV reaching 29.1%, 27 % respectively, compared to 41% for the control group. Also, there was a significant decrease in platelets count in the infected groups compared with the control group. This table shows that the treatment of the groups, (G1, G2), with TiO_2 NPs restored the number of white blood cells close to the control groups. This can be ascribed to the positive effects of these inorganic oxides in removing the harmful effects of bacteria and the recovery of the natural parameters' values. Accordingly, there was a significant decrease in the white blood cells numbers in the infected groups after being treated with TiO_2 NPs reaching 4.9, 4.6 ($\times 10^3$ cell / mm^3) respectively. The reason for this significant decrease in the level of Hb and PCV can be attributed to the ability of the pathogenic bacteria to produce hemolysin enzyme (Nester *et al*., 2001). Since PCV is a measure of the volume of packed cells, the hemolysin in red blood cells leads to a decrease in their level. It can also be attributed to the bacterial infection impact on the animals' appetite and the lack of nutritional elements that are necessary for the formation of red blood cells such as minerals and vitamins. The results were consistent with those found in Al-Zamely and Shaymaa (2011) regarding the experimental infection in male rats with *E. coli* which resulted in a significant decrease in PCV and Hb compared to the control group. Also, the results showed that the treatment of the infected groups (G1 and G2) with TiO_2 NPs leads to a significant increase in Hb and PCV levels. Table 1 indicates an improvement in the condition of animals and a recovery of red blood cells to their natural composition and formation of hemoglobin. This can be ascribed to the ability of TiO_2 NPs to control the infection in a short time, and the high oxidizing power of the free radicals that can directly destroy bacteria. TiO_2 NPs can inhibit the activity of ATPase which leads to the reduction of the essential ATP to sustain the bacterial cell life (Cui *et al*., 2011).

Table 2 shows that the types of white blood cells (eosinophils, monocytes, and basophils) of G1 and G2 after infection have no significant changes in their levels when compared to the control group ($P >0.05$). The results showed that the percentages of neutrophils in the G1 and G2 were 19.0 %, 22.3 % respectively, while in the control group (G3) it was 53.3 % demonstrating a significant decrease. The Lymphocytes showed a significant increase in the percentages in G1 and G2 after infection reaching 47.6 %, 50.7 % respectively compared to the control group which reached to 22.6 %. The increased number of lymphocytes may be attributed to the infection by the two types of bacteria which stimulated the immune system to form defensive cells that caused an increase in the total number of white blood cells generally and lymphocytes particularly. These cells play an important role in immunity by secreting the chemical immunoglobulins consequently, controlling the pathogenic microorganisms (Stites, 1987). The reason behind the decrease of neutrophils is the production of leucocidin that destroys neutrophils through the lysis of the granule cytoplasm (Todar, 2004). When the bacterial infection is severe, these cells cannot resist the infection, and the body is forced to use lymphocytes as a second line of defense. After treatment with TiO_2 NPs, the results showed a significant increase in the neutrophils proportions at 43.0 % and 47.3% respectively compared to the proportion in the infected groups (G1 and G2) before treatment. The outcomes of the treatment resulted in the lymphocytes reduction reaching 30.3, 26.6 in G1 and G2, respectively.

The reason for the ability of the cells to recover their normal level is the action of nano-oxides in inhibiting pathogenic bacteria. The small size enables the nanoparticles to penetrate the cell membrane and to destruct it leading to loss of minerals, proteins, genetic material and finally to the cell death (Foster *et al*., 2011). Adams *et al*., (2006) noted that ZnO NPs can inhibit about 90 % of the Gram-positive bacteria and less than that in the case of Gram-negative bacteria. The mechanisms of cellular toxicity of nanoparticles, including ROS, increase the antioxidant system; consequently the cells enter oxidative stress that leads to the breakdown of cellular components such as lipids, proteins, and genetic material (Lovric *et al.*, 2005).

Table 1. Effect of TiO_2NPs in some hematological parameters in infected animals.

Parameters / Groups	WBC ($\times10^3$ cell / mm^3)	PCV %	RBC ($\times10^3$cell / mm^3)	Hb (dl/ml)	PLT ($\times10^6$ cell / mm^3)
infection stage					
G1	17.5± 1.17 a	29.1± 1.2 b	2.6± 0.35 b	9.1± 1.1 c	34.6± 14.1c
G2	16.1± 1.08 a	27.0± 2.7 b	2.5± 0.50 b	8.9± 0.4 c	28.0± 4.3 c
G3	3.9± 0.64 b	41.2± 4.1 a	4.8 ± 0.34 a	12.5± 0.8 a	253.6±55.5a
treatment stage with TiO_2NPs					
TG1	4.9± 0.50 b	37.4±3.0 a	3.7± 0.20 a	11.7± 0.6ab	136.7± 41.6 b
TG2	4.6± 0.80 b	36.4± 1.2 a	3.4± 0.5 ab	10.5± 1. cb	123.3± 25.1 b
TG3	3.7± 1.00 b	39.1± 2.7 a	4.3± 0.9 a	11.6± 0.7ab	190± 45.8 ab

a,c: Values within columns followed by different letters differ significantly at ($p<0.05$).

Table 2. The effect of oral administration of TiO_2 NPs in the differential number of white blood cells in rabbits infected with bacteria.

Parameters / Groups	Neutrophils %	Lymphocytes %	Monocytes %	Eosinophils %	Basophils %
infection stage					
G1	19.0± 3.6 c	47.6±2.6 a	0.0± 0.0 a	0.0± 0.0 a	0.0± 0.0 a
G2	22.3± 3.0 c	50.7±6 a	0.0± 0.0 a	0.0± 0.0 a	0.0± 0.0 a
G3	53.3± 4.7 a	22.6±2.5 c	1.0± 0.0 a	0.0± 0.0 a	0.0± 0.0 a
treatment stage with TiO_2NPs					
TG1	43.0± 3 b	30.3±3.2 b	0.6 ±0.5 a	0.0± 0.0 a	0.0± 0.0 a
TG2	47.3±6.8 ab	26.6± 2.8 b	1.0± 1.0 a	0.0± 0.0 a	0.0± 0.0 a
TG3	50.6± 4 a b	23.6± 4 bc	1.0± 0.0 a	0.0± 0.0 a	0.0± 0.0 a

a, b: Values within columns followed by different letters differ significantly at ($p<0.05$).

3.2. Immunological Parameters

Table 3 demonstrates the levels of immune-globulins; IgG, IgM that are produced as a result of the infection with pathogenic bacteria and treatment with TiO_2 NPs using Elisa kit. The effects of the pathogenic bacteria *E. coli* and *S. aureus* on the level of IgG and IgM are clear through the significant increase ($P < 0.05$) in the titers values 10.3, 12.3 for IgG and 15.3, 13.3 for IgM in comparison to the results obtained from the samples before infection which were 6.7 and 10.3 respectively. It was found that the oral administration of TiO_2 NPs significantly increased the titer of IgM, and IgG in G1 and G2 respectively. Table 3 reveals that the value of IgG in G3 treated with TiO_2 NPs was higher than that in the control group, while IgM is the same in both groups. However, the statistical results show that there are no significant differences between G3 and TG3. The slight elevation can be attributed to different factors such as the genetic effects on the IgG levels including the immune state and environmental factors. The results concerning the elevation of the immunoglobulin might be interpreted as a beneficial host response for recovery from the injury.

Table 3. The effect of oral administration of TiO_2NPs on immunoglobulin values in the infected animals.

Parameters / Groups	IgG. mg/dl	IgM mg/dl
infection stage		
G1	10.3± 1.5 ab	15.3± 1.5 a
G2	12.3± 2.3 a	13.3± 1.4 ab
G3	6.7± 0.5 c	10.3± 1.5 c
treatment stage with TiO_2NPs		
G1T	11.0± 1.0 ab	11.0± 1.0 bc
G2 T	10.6± 1.5 ab	10.0± 2.0 c
G3 T	9.0± 1.0 bc	10.0± 1.0 c

a, c: Values within columns followed by different letters differ significantly at ($p<0.05$).

The treatment of animals with TiO_2 NPs showed lower levels of TNF-α and IL-6 in the control group (G3) and in the pre samples that were collected before the beginning of the experiment compared with G1 and G2 before treatment with TiO_2 NPs (Table 4). For cytokines TNF-α and IL-6, high expression levels were observed in rabbits seven days following the bacterial infection. Thus seven days post-infection, the TNF-α was 11.0, 11.2 pg/mL in the infected groups (G1 and G2) respectively, while IL-6 level was 31.6, 27.6 pg/mL in the same groups. Under such conditions, feeding the infected animals with TiO_2 NPs can reduce the expression level of both cytokines (TNF-α, IL-6) significantly, and enhance the anti-inflammatory cytokines that could reduce the inflammatory response of animals (Chih-Yuan *et al*., 2013). A recent study (Rossi *et al*., 2010) demonstrated that, in ovalbumin-sensitized mice, silica, coated rutile TiO_2 NP inhalation decreased TNF-α and IL-13 expression in spleen cells. The results of the present study show a significant increase in the IL-10

concentration (P <0.05) 32.4, 31.1 pg/ mL for the G1 and G2 after infection compared with 18.3 pg/ mL values obtained from control group (G3) before infection. These results are similar to those obtained by Moore *et al*. (2002) and Yoshida *et al.* (2001) who indicated that the level of IL-10 increased in rats injected with *K. pnemaniae*. The IL-10 is characterized by an immunological regulation that includes positive and negative effects. As shown in Table 4, the level of IFN-γ significantly increased in the infected groups (G1, G2) compared to the control group (G3). This finding agrees with the results of (AL-Refaei, 2016) who found that the concentration rate of the IFN-γ in serum after the injection with (O), and (K) antigen of *K. pneumoniae* was elevated in the experimental animals. When all groups are treated with TiO_2 NP, the level of IFN-γ significantly decreased to be closer to the value of the control group. IFN-γ is an immune regulator of macrophages and T and B cells (Talaro, 2012).

Table 4. The effect of oral administration of TiO_2NPs in some levels of cytokines in the infected animals.

Parameters / Groups	IL-6 pg/mL	IL-10 pg/mL	IFN-γ pg/mL	TNF-α pg/mL
Infection stage				
G1	31.6± 3.2a	32.4± 3a	53.0± 8.1a	11.0± 1.0 a
G2	27.6± 3.7a	31.1± 4.1a	46.3± 3.2 a	11.2± 0.6 a
G3	2.9± 0.6 b	18.3± 1.5c	28.3± 1.5c	1.26± 0.25d
treatment stage with TiO_2NPs				
TG1	5.8± 1.5 b	27.6± 4.9 ab	37.0± 3b	6.4± 0.5b
TG2	4.8± 0.3 b	24.0±4.5 bc	36.3± 2.8b	4.8± 0.3c
TG3	1.8± 0.3 b	22.3± 2.5 bc	32.0±3.6bc	1.8± 0.3d

a,d: Values within columns followed by different letters differ significantly at (P<0.05).

4. Conclusion

The laboratory animals that have been infected with pathogenic bacteria and treated with TiO_2 NPs showed improvement in the values of hematological and immunological parameters in addition to the significant restore of the rates of white blood cells, lymphocytes, and granules after treatment with TiO_2 NPs. Also, the results indicate stimulating the immune response of immunoglobulins by increasing the value of IgG and IgM in the rabbits treated with TiO_2 NPs. Cytokines showed different levels of cellular interleukins, IFN-γ and TNF-α.

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