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The Effect of Silver Nanoparticles on Cellular and Humoral Immunity of Mice *in Vivo* and *in Vitro*

Rand M. Abd AL-Rhman , Shaim R. Ibraheem , Israa AL-Ogaidi

Department of Biotechnology , College of Science , University of Baghdad

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Abstract: This study investigated the immunological adjuvant effect of silver nanoparticles AgNPs which have been prepared and characterized in previous study. The silver nanoparticales were synthesized by biological method using green (G) and black (B) tea as reducing agent. AgNPs were 50nm and 30 nm respectively with like spherical in shape. The effect of AgNPs as adjuvant were tested both *in vitro* and *in vivo*. The effect of the concentration2mg/kg of AgNPs was evaluated in mice by intraperitoneal immunization. *In vitro* the number of both T and B cells, monocytes and neutrophils populations showed an increase in absolute cell number in 2B and 3G groups in the immunized mice compared to the control group, whereas the relative cell numbers remained constant in eosinophils and basophils between group2 and control. The levels of IL 4 and IL6 showed significant elevation ($P \leq 0.05$) in the serum groups (2B,3G) of animals which were (23.1 ± 0.80, 32.4 ±1.35) pg/ml for group2 (36.7 ±1.76and 50.5 ± 1.76) pg/ml for group3 compared to those that found in control (12.± 10.23and 19.5±0.34) pg/ml respectively. So, the inoculation with AgNPs, showed no signification difference at ($P \leq 0.05$) in group2B while the concentration of IgGgroup3G showed significant elevation which were (4.9±0.11 and 6.8±0.12) mg/ml respectively as compared with control group (4.5± 0.11) mg/ml. Also the results showed an increase in phagocytes of treated mice's blood 36 (32%)and 95 (45%) for group2B and group3G respectively as compared with control 4 (17%). Therefore, we conclude that AgNPs have a significant adjuvant effect and the mechanism of this effect is mainly ascribed to the recruitment and activation of local leukocytes, especially lymphocytes, increase cytokines levels in mice, IgG concentration and phagocytes. The humoral and cellular immunities were significantly enhanced in immunized mice, whichcan be utilized as an effective adjuvant to improve the immunoprotection, the result is beneficial for the future applications, especially in biomedicine.

Key words: Silver nanoparticles, Adjuvant, Antibody, phagocytosis

Corresponding author: should be addressed (Email: quiet_randuna@yahoo.com)

Introduction

Silver nanoparticales (AgNPs) have received important responsiveness owing to their physicochemical, attractive electronic and optical properties (1) that enables to use it in a host of innovative applications. Due to its strong disinfectant properties, silver is one of the most popular substances in nanotechnology (2). The biological techniques, bymicro-organisms, enzymes, and plant or plant extract have been recommended as possible because it is eco-friendly, cost-effective, a single-step method for biosynthesis process and safe for human beneficial use (3). Recently, nanoparticles have been evaluated for their ability to increase the immune responses as adjuvants. In many cases, adjuvants are employed to evoke more powerful immune responses. An optimally-formulated adjuvant must be safe, stable before administration, readily biodegradable, able to promote an antigen-specific immune response, inexpensive to produce and easy to use (4). The effects of silver nanoparticles on cytokine expression by peripheral blood mononuclear cells (PBMC) (5), human mesenchymal stem cells (hMSCs) (6), and macrophages (7). Nanosilver is also recommended by alternative medicine practitioners as a dietary supplement that boosts immunity (8). Phagocytosis process is the engulfment and degradation of microorganisms and foreign particles by cell such as macrophages, monocytes, dendritic cells, neutrophils and even B lymphocytes (prior to their activation). Neutrophils and monocytes circulate in the blood, while macrophages reside in tissue (9). Therefore, the objective of this study was to develop a safe and novel immuneadjuvant (AgNPs) to enhance the cellular and humoral immunity.

Materials and Methods

AgNPs Characterization

The silver nanoparticals were synthesis by biological method using green (G) and black (B) tea as reducing agent. AgNPs were 50nm and 30 nm respectively with like spherical in shape. AgNPs which have been prepared and characterization in previous study (under publication) according to procedure developed by Cataldo (10). (G) represent AgNPs solution which is prepared by green tea as reducing agent and (B) represent AgNPs solution which is prepared by black tea as reducing agent. Both were intraperitonial injected in the mice to study its immunological effects as follow.

Experiment Design

Thirty adult male mice were obtained from the animal house of National center for drug control and research and kept in the animal house of biotechnology Research Center, AL-Nahrain University, where its placed in a separated cages at ($25C^{o}$) room temperature, and fed with suitable diet in addition to water. The experimental animals were randomly divided into three groups as follows:

*First group (C): Control group consisted of 10 animals were injected intraperitonially with PBS for 15 days.

*Second group (B): include 10 animals were injected with 0.1 ml of (2 mg/kg) (B) solution intraperitonially for 15 days.

*Third group (G): include 10 animals were injected with 0.1 ml of (2 mg/kg)

(G) solution intraperitonially for 15days.
The solutions concentration was determined according to the work of Sardari *et al.* (11).

Serum

Blood was collected by heart puncture, **c**lotted blood was centrifuged to produce serum and determine IL4, IL6 and IgG levels. Serum samples from the animals receiving nanoparticles were stored at -20° C.

Determination of Cytokine Levels by Enzyme Linked Immunosorbent Assay (ELISA)

The serum levels of IL4 and IL6 were analyzed by mice IL4, IL6 ELISA kits (Cloud-clone corp./ USA), and by comparing the O.D. of the samples to the standard curve (Figure-1).The cytokines were quantified using manufacturer's protocol.

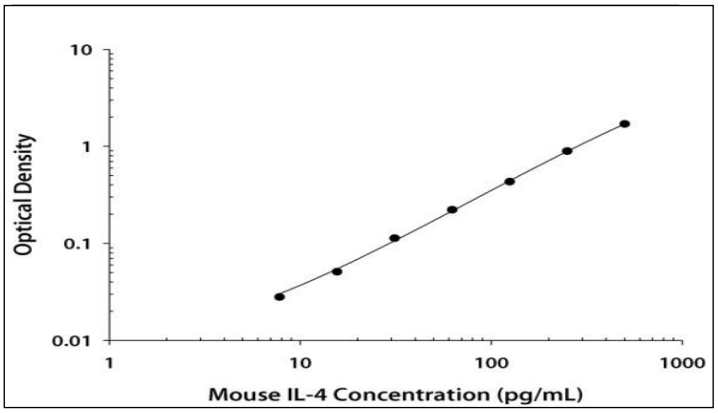

Figure- 1 a :Standard curve of IL4

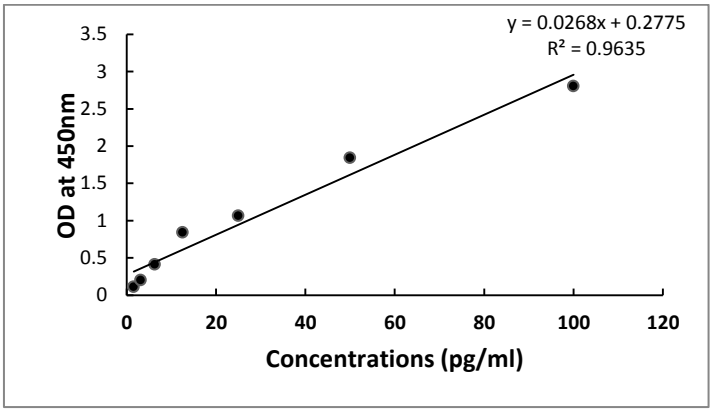

Figure-1 b: Standard curve of IL6

Determination of Immunoglobulin IgG Concentration by ELISA

Quantitative determination of mice IgG was performed using a competitive inhibition enzyme immunoassay technique (Cloud-clone corp./USA)**.** Briefly, the microtiter Polystyrene 96-well plate provided in this kit has been pre-coated with an antibody specific to IgG. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody prepared as a specific for IgG and Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB substrate solution is added to each well. Only those wells that contain IgG, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a change is measured spectrophotometrically at a wave length of 450 nm. The concentration of IgG in the samples is then determined by comparing the O. D. of the samples to the standard curve (Figure-2).

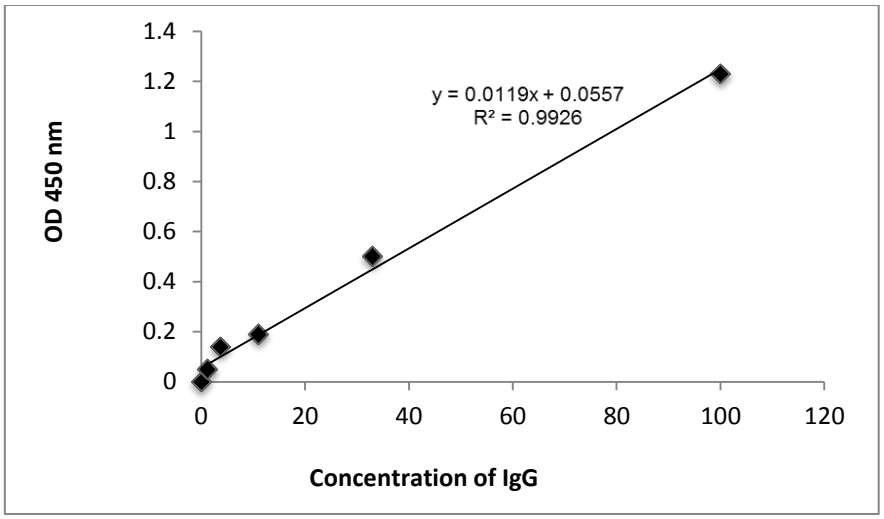

Figure 2- Standard curve of IgG

Phagocytosis Index

It has been done according to Furth*et al.* (12) as follows:

From immunized animals (different groups) 1 ml of blood was pooled from the heart for each tested animal are transmitted to tubes contain EDTA as an anticoagulant, with gently mixed. An aliquot of 500µl of EDTA mice blood were mixed gently with the 200µl of silver nanopartical SNP in sterile test tube. The mixture was incubated at 37ºC for 1.5hours. Smear had been prepared by taking a drop of the mixture on the clean microscope slide; triplicate slides were made for each tube. Slides had been air dried, stained with Giemsa stain for 10 min. and then washed by D.W. The slides had been examined by oil immersion lens of light microscope to calculate the number of phagocytes engulfed nanoparticales. And according the following equation, the percent of phagocytic cells was determined.

$$\text{Phagocytosisindex}(\%) = \frac{No.of\ phagocytic\ cells}{No.(Phagocytic+non\ pagocytic\ cells)}\ X\ 100$$

Calculation Immune Cells of Mice

According to Ceelie *et al.* (13) the number of each type of white blood cell, neutrophils, monocytes, and lymphocytes present in the blood mice were determined, according the following protocol:
A drop of blood sample for each tested animal is placed on the clean slide to produce a smear. The smear film should be dried rapidly. A good blood film preparation will be thicker at the drop end and thin film at the opposite end. Giemsa stain was poured over the smear for 8-10 minutes. Wash off with water and dry. The dry and stained film is examined under (40X). For differential leukocyte counts, choose an area where the morphology of the cells is clearly visible. Differential count by moving the slide in area, including the central and peripheral of the smear.

Statistical Analysis

Statistical analyses were performed by use of IBM SPSS computer program version 21. Differences between the groups were statistically analyzed by ANOVA table. Data are expressed as mean ± standard error (SE). A *p* value of ≤ 0.05 was regarded as statistically significant.

Results and Discussion

Immune Cells Number

The number of immune cells increased in the immunized mice compared to the control group. Silver nanoparticles effect on the lymphocytes production by increasing the mitoses of cells, this effect due to interaction between silver nano-particles with immune component (14). B cells increase in number, this agrees with another study on Ag-NP in rats (15). Both T and B cell, monocytes and neutrophils populations showed an increase in absolute cell number in 2(B) and 3(G) groups, whereas the relative cell numbers remained constant in eosinophils and basophils between group2 (B) and control. So, the results showed there were increase the number of immune cells specially in group3(G) in compared with the group2(B) because nanoparticles had a heterogeneous size group2 (B 30nm) – group3 (G 50nm) (Table 1). Leukocytes are considered as the active cells in carrying out the functions of the immune system, both non-specific and specifically, and their count may give a general picture about the function of the immune system and the results demonstrated that a treatment with AgNPs had an effect on a differential count of leukocytes especially the Lymphocytes, Neutrophils, and Monocytes, the Neutrophils are mainly involved in the innate immune system to carry out phagocytosis, while lymphocytes represent the humeral and cellular arms of specific immunity, Monocytes are involved in carrying out phagocytosis, but they are also professional antigen presenting cells. Eosinophils are involved in allergic and inflammatory reactions, as well as, parasitic infections. Basophils release histamine, heparin and some pharmacological mediators of immunological reactions (16) .

Table 1:The leukocytes number of mice blood samples treated with AgNPs compared with control

Groups	Lymphocytes Cell/field	Monocytes Cell/field	Neutrophils Cell/field	Eosinophils Cell/field	Basophils Cell/field
Control	15	1	3	0	0
Group2(B)	57	2	18	0	0
Group3(G)	69	4	41	3	0

Assay of Interleukin in Immunized Mice

The levels of IL4 and IL6 showed significant elevation ($P \leq 0.05$) in the serum groups (2B, 3G) of animals administered intraperitoneally with silver nanoparticles which were (23.1± 0.80, 32.4±1.35) pg/ml for group2, (36.7 ±1.76, 50.5±1.76) pg/ml for group3 than those found in control (12.±10.23, 19.5±0.34) pg/ml respectively (table2). These results indicated the increasing of different leukocytes because the cellular source of this interleukin from lymphocytes, mast cell, eosinophil, natural killer and endothelial cells in addition to its function is the proliferation and differentiation of hematopoietic precursors1(17). IL4 is a key regulator in humoral and cellular immunity. IL4 induces B-cell class switching to IgE, and up-regulates MHC class II production. Overproduction of IL4 is associated with allergies (18). Thus this interleukin is related with hypersensitivitytype1 and phagocytosis. IL6 is also produced by T and B lymphocytes, fibro blasts, endothelial cells, keratinocytes, hepatocytes, and bone marrow cells. Under the influence of IL6, B lymphocytes differentiate into mature plasma cells and secrete immunoglobulins. Mononuclear phagocytic cells are the most important source of IL6 (19).

Table 2:IL4 and IL6 concentration in mice immunized by silver nanoparticles

Intraperitoneal Immunization	IL4 pg/ml	IL6 pg/ml
Control	12.± 10.23	19.5 ± 0.34
Group 2	23.1 ± 0.80	32.4 ±1.35
Group 3	36.7 ±1.76	50.5 ±1.76
Total	23.97 ± 3.60	34.15 ± 4.54

However, nanoparticles had a heterogeneous size group2(B 30 nm) – group3 (G50 nm) with like spherical in shape: The results (Table2) showed there were significant high elevation in the level of this interleukin specially in group3 (G) in compared with the group2 (B), so it is establish the effect is related to the chemical nature and the size. The effects and mechanism of action for NPs were dependent upon composition and surface modifications (20).The mechanism of adjuvant effect of AgNPs is not clearly known. But investigations suggested that AgNPs act via cytokines release, recruitment of leukocytes and up-regulation of Major Histocompatibility (MHC) class II expression of peritoneal macrophages. In addition it reported that AgNPs like Alum stimulate the T helper II depended response leads to neutralizing antibodies. Although it was not argued but it was reported that AgNPs in tend to be aggregated (21). Also Becker *et al.* (22) referred that, the presence of NPs signals the influx of more phagocytic cells to the alveolar region for removal of NPs, partly involving release of cytokines and chemokines, which are produced by cells such as Mph, immune cells (Imm), comprising neutrophils, and lymphocytes in varying amounts.

Determination of Antibody Titers by ELIZA

The results (table 3) showed that the concentration of IgG in serum of mice that intraperitoneal immunized with silver nanoparticles, shown no signification difference at ($P \leq 0.05$) in group2Bwhile the concentration of IgG group 3G shown signification elevation which were (4.9±0.11 and 6.8±0.12) mg/ml respectively as compared with control group (4.5±0.11) mg/ml.In addition to, the differences in size, the effect was also differed as observed between (group2B 30 nm) and (group3G 50 nm), the antibody response to group3G (bigger size) was found to be higher compared with group2B. Different factors effect on the IgG serum levels, such as genetic effects, immune state and environmental factors, and may be due to used antigen type as our antigen is a complex antigen. Another reason may be related to protocol of immunization and type of employed mice. Furthermore, it was reported balb/c mice had more potent immune humoral responses (23). It is note worthy that antigen more immunogenic and increase of immune responses by adjuvant AgNP.

Effect of Ag SNP on Phagocytosis

Phagocytes index (PI) for mice blood samples treated with silver nanoparticle after 15 days of injection were measured and it compared with control. The results (table 4) shown that increase in PI of treated mice's blood 36 (32%) and 95 (45%) for group2B functions *in vitro* during the induction of immune responses or inflammation as compared with control. (24) showed that NPs are significantly taken up by macrophages. And cellular uptake of particles is influenced by particle type, size, and surface charge (25). (30 nm) and group3G (50 nm) respectively in compared with control 4 (17%) group. In the current study, was provided an understanding of the influence of different size silver nanoparticles on leukocytes

Table 3: IgG mean ±standards error in mice that immunized with silver nanoparticles

		Control mg/ml	Group2 mg/ml	Group3 mg/ml	Total
IgG	Intraperitoneal immunization	4.5 ± 0.11	4.9 ± 0.11	6.8 ± 0.12	5.42 ± 0.37

Table 4: Results ofphagocytosis index

Groups Intraperitoneal immunization	Total No. (Nonphagocyte+phagocytecell)	No. phagocytes and Efficiency (%)
Control	23	4 (17%)
Group2	113	36 (32%)
Group3	307	95 (45%)

Cellular uptake has been considered to be composed of two processes: delivery and adhesion of NPs onto the cell and uptake of NPs by the cell via phagocytosis. Adhesion of NPs onto the cell surface is a function of particle size, surface zeta potential, and cell type (26).

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