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Impact of Silver Nanoparticles on Immune Responses as Vaccine against Activated *Staphylococcus aureus* Bacteria

Rand M. Abd Al-Rhman

Department of Biotechnology, College of Science, Baghdad University, Baghdad, Iraq

Abstract: ***Staphylococcus aureus is a gram positive bacterium with a broad host range and ubiquitous distribution. S. aureus is a nosocomial pathogen of hospitalized patients brought about extensive morbidity and mortality. It's responsible for causing pneumonia, osteomyelitis, cystitis, prostatitis, meningitis, bacteremia, skin, urogenital tract, central nervous system, and the best method of control is vaccination with locally prevalent strains. Recently, the ability of nanoparticles were assessed as an adjuvant for increase immune responses.Then again, the main response to immunization by neutralizing antibodies in numerous disease. Therefore, we utilized to assess AgNPs adjuvanticity with antigen inactivated S. aureus bacterin. To prepare 0.2 ml (at 2mg/kg concentration) of vaccine used olive oil (75µl) and inactivated S. aureus (25 µl) were added to AgNPs (100µl) size 88nm. The mice were intraperitoneally immunized by the AgNPs alone as positive control, olive oil alone, inactivated S. aureus only and incomplete Freund's adjuvant only, commercially available adjuvant. The vaccine used two time subcutaneous and intraperitoneal route on first and seventh day. One week later the blood was collected from immunized mice. In the immunized mice, the count of both T and B lymphocytes, neutrophils and monocytes in intraperitoneal and subcutaneous groups were demonstrated an increase in total cell number, but other groups slight increasing, whereas the relative eosinophils and basophils numbers stayed constant contrasted with the control group. Also the results shown that increase in phagocytosis of treated mice's blood {25 (21.36% one dose); 29 (22.48% post dose) } and { 32 (22.2% one dose; 40 (23.2% post dose) } for intraperitoneal and subcutaneous respectively in compared with negative control, and the other groups slight elevation of phagocytosis than negative control 4(18.1%). The quantity of neutralizing antibodies was dictated in serum, the outcomes demonstrated that administered intraperitoneally (i.p.) and subcutaneously were significant increased (P ≤0.05) which were (9.8 ± 0.08 one dose, 12.3 ± 0.23 post dose) and (11.7 ± 0.11 one dose, 16.2 ± 0.11 post dose) mg/ml respectively than those found in the positive control AgNPs (4.1±0.08 mg/ml), negative control (4±0.05 mg/ml), Killed S. aureus alone (5±0.12 mg/ml) and incomplete Freund's adjuvant (6.2 ± 0.17 mg/ml), and significant differences were observed between 1th (one dose) and 7th days (post immunization). It must be noticed that subcutaneously post-immunization had better impact on immune response. Hence conclude the mechanism of AgNPs have significant adjuvant impact is mainly ascribed to produce immune responses against S. aureus microscopic organisms and which is vital in clinical treatment of S. aureus illness by recruitment and activation of leukocytes.***

Keywords: Silver nanoparticles, *S. aureus*, antibody, phagocytosis

1. Introduction

Several reviews were attended with the impact of particles smaller than 100 nm on immune system, were called nanoparticles. Physicochemical properties vary from the mass substances, in light of nano-size(1). Because of nanoparticles high applicability their have been considered as promising tools. Many mechanisms were used to produce nanoparticles biological synthesis, chemical synthesis, physical synthesis, and photochemical (2). Microbial growth was prevented by silver particles have anti-microbial activity. Silver was being protected in reduced dosages for people, therefore truly utilized for burns recuperating and wound (3). For many years, researchers were focused on broad search of more effective adjuvants because of the constant mutation of existing microorganisms, limited adjuavtation impact of aluminum salts, and ever identification of new sickness bringing on organisms. At this time, the selection of adjuvants for people immunization mirrors a trade off distributed an acceptable low level of side effects and a necessity for adjuvanticity (4). When stimulation of innate immunity and maintained release of antigen in injection site, the adjuvants enhance immunogenicity (5). *Staphylococcus aureus* were 0.5–1.5 mm in width, happening in sets, separately, in small chains (3-4 cells), in group of four, gram positive, non motile, non flagellate, non spore-forming cell wall contains peptidoglycan and teichoic acid. Usually un encapsulated or limited capsule formation. Staphylococcacea are aerobic or facultative anaerobes, in medium containing 10% NaCl grows well but ineffectively in 15% NaCl (6). *S. aureus* is in charge of an assortment of human diseases are pneumonia, osteomyelitis, cystitis, prostatitis, bacteremia, toxic shock syndrome, and different intra-stomach organs. Also, nutrition poisoning by staphylococcal enterotoxin (7). Therefore, In this study armed to bring out immune response against *S. aureus* infection by vaccine of silver nanoparticles (AgSNPs) as immune-adjuvant impact with formalin-killed *S. aureus* also the outcomes were contrasted with, industrially ready for use Freund's incomplete adjuvant.

2. Material and Methods

AgNPs characterization

The silver nanoparticals were synthesis by biological method using green (G) tea as reducing agent. AgNPs were 88 nm with spherical in shape. AgNPs which have been prepared and characterization in previous study (under publication) according to procedure developed by Cataldo (8). So, (G) represent AgNPs solution which is prepared by Green tea as reducing agent. The solution were injected in the mice to study its immunological effects as follow.

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Bacteria

S. aureus identified from purulent wound was selected, which were represented the most resistant isolates to locally used antimicrobial agent depend on prior study. The isolate was supplied locally by University of Baghdad/ College of Science/ Biotechnology Department. The isolated *S. aureus* was activated on brain heart infusion broth (himedia) and incubated over night at 37 °C, then prepared at a concentration (1.5X 10^8 CFU/ml) according to McFarland tube no. 0.5 to further use.

Preparation of formalin-killed *S. aureus* bacterin (9)

In order to prepare inactivate *S. aureus* bacterin, isolated bacteria was inoculated by loopful of culture brain heart infusion broth media on tryptic soy broth (TSB) (himedia) and incubated for overnight at 37°C. Than formalin was added to TSB culture (40% w/v) at 0.5% (V/V) a final concentration and left two days at room temperature. After that, for their sterility (free from the living cells) the bacterins were tested by streaking them onto trypticase soy agar(himedia) which showed no growth.

Loading of inactivated *S. aureus* on AgNPs (killed *S. aureus*-AgNPs vaccine) (10)

Formalin-killed *S. aureus* bacterin (25 µl) and oil (75µl) were added to AgNPs (100µl) size 88nm at 2mg/kg concentration to prepare 0.2 ml of vaccination and the blend was incubated overnight at 4°C with delicate mixing under high sterility conditions.

Mice animal and immunization (10)

Twenty eight healthy white mice, weighing (20-25 g) were supplied from the animal house of National center for drug control. The mice were kept for 7 days to adapt to the environmental condition in the animal house of the Biotechnology Center/ University of Al-Nahrain/Iraq. They were housed in steel cages in a room with controlled temperature of 20±22ºC, and a12 hr. light/ dark cycle. The mice were fed a commercial mouse food and provided with fresh water and cleaning of the cages were achieved by daily. The animals were divided into seven equal groups:

Group A: negative control

Group B: administered silver nanopartical (AgSNP as adjuvant positive control)

Group C: administered olive oil

Group D: administered incomplete Freund's adjuvant (Sigma /USA)

Group E: administered killed *S. aureus* bacterin only

Group F: administered the vaccine solution intraperitoneally (i.p.).

Group G: administered with vaccine solution subcutaneously.

Mice were injected with 0.2 ml of immunization on the primary day of experiment. After seventh day half intraperitoneal and subcutaneously groups were injected with the similar amount of vaccine (booster dosage) and their blood tests were gathered 1 week coming after (fourteen day).

Serum

One week coming after (fourteen day), the blood tests were gathered by heart puncture. Then centrifugation at 5000 round per minute for ten minute to disengage serum also put away at -20°C for more examination (serological examination using ELISA test).

Microscopic examination

Number immune cells of mice (11)

According the following protocol, the numbers each sort of the leukocytes exhibit in the blood mice were resolved:

To produce a smear of each tested animal blood by place a drop on the clean slide. The spread film ought to be dried quickly, will be thicker at the drop end and thin film at the inverse end. The smear was stained with giemsa stain for 8-10 minutes than with water wash off and dry than under (40X) is analyzed. Choose a region where the cells morphology is plainly visible and moving the slide zone, including the focal and peripheral for numbers of differential leukocyte

Phagocytosis index

It has been done according to (12) as follows:

An aliquot of 500 µl of EDTA immunized mice blood was exposed to the bacterial suspension *S. aureus* a concentration 1.5X 10^8 according to McFarland tube 0.5 in sterile test tube and mixed gently for variable times, and incubated the mixture at 37ºC for 1.5 hours. Taking a drop of the mixture to set up smear on the slide than air dried slide. The smear slide was stained with Giemsa stain for 10 min than used D.W for washed. The slides had been analyzed by oil immersion lens to calculate the number of phagocytes engulfed *S. aureus*-AgNPs vaccine. Also, the percent of phagocytic cells was concluded, concurring the equation:

$$\text{The percentage of phagocytosis \%} = \frac{\text{Number of phagocytic cells}}{\text{Number (phagocytic + non phagocytic cells)}} \text{ X } 100$$

Quantitative determination of IgG antibodies by ELISA

The immunoassay procedure was performed to identification of IgG titer utilizing mice IgG ELISA kit (Cloud-clone corp./USA) **.** Briefly, in this kit was provided with plate microtiter polystyrene 96-well has been covered with the capture antibody particular to IgG. Then samples or standards with a biotin-conjugated polyclonal antibody preparation specific arrangement particular for IgG were added to microtiter plate in the fitting wells. Aliquot of a Avidin joined to Horseradish Peroxidase (HRP) solution to catalyze a colorimetric reaction with amount of chromogenic substrate reagent (TMB) (3,3,5,5- tetramethylbenzidine) were added to each well. The colorimetric enzyme-substrate reaction produces a blue color develops changing to yellow by add dilute sulphuric acid solution to terminate the reaction. The absorbance (shading change) at a wavelength of 450 nm was measured by ELISA reader. The quantity of IgG present in the samples are then dictated by the optical density of the samples comparing to the standard curve figure (1).

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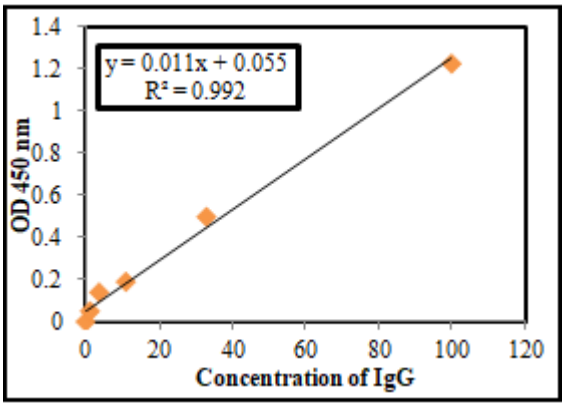


Figure 1- Standard curve of IgG

Experimental Challenge (13)

From each immunization groups and control groups were subjected to challenge by scratched dorsal skin with 0.1 ml (1.5×10^8 CFU/ml) of virulent *S. aureus* strain. The challenged mice were observed for a period of 2 weeks post-challenge for mortality/clinical signs. all mice survived were examined to check the pathological lesions of the skin brought on by the disease with bacteria *S. aureus.*

Statistical analysis

Data are displayed as mean ± standard error (SE). The significance of differences between groups were statistically analyzed by ANOVA table, when a P ≤0.05 was regarded the differences were significant. IBM SPSS programming (version 21) was utilized for computer analyses.

3. Results and Discussion

Changes of the number of immune cells

The interaction between silver nanoparticles with immune component impact on the lymphocytes creation by increasing the mitoses of cells (14). Both T and B lymphocytes**,** monocytes and neutrophil were demonstrated an increase in absolute cell number, whereas the relative eosinophils and basophils numbers stayed constant in all groups than the control group (table 1). Active cells that completing the work of immune system, both non-specific and specifically, are leukocytes, and the results demonstrated that a treatment with subcutaneously (post dose) had an effect on a differential count of leukocytes especially the lymphocytes, neutrophils, and monocytes. In the innate immune system is mainly required neutrophil cells to carry out phagocytosis, while the humeral and cellular arms of specific immunity represent by lymphocytes. Monocytes are professional antigen presenting cells, also are included in completing phagocytosis. Parasitic infections and inflammatory reactions, and additionally, hypersensitive were included by eosinophils. Basophils release some pharmacological mediators of immunological reactions, heparin and histamine (15).

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

Groups		Lymphocytes Cell/field	Monocytes Cell/field	Neutrophils Cell/field	Eosinophils Cell/field	Basophils Cell/field
Negative control		14	1	3	0	0
Positive control AgNPs		15	5	10	1	0
Freund's Incomplete adjuvant		20	4	5	0	0
Olive oil		16	2	4	0	0
Killed S. aureus		18	4	6	0	0
Intraperitoneal	One dose	53	18	20	1	0
	Post dose	60	20	21	1	0
Subcutaneously	One dose	69	22	23	1	0
	Post dose	75	26	30	1	0

Effect of vaccine on phagocytosis

The results shown that increase in phagocytosis of treated mice's blood which were{25 (21.36%one dose); 29 (22.48%post dose) } and { 32 (22.2% one dose; 40 (23.2% post dose) } for intraperitoneal and subcutaneous respectively in compared with negative control 4 (18.1%),and the other groups slight elevation of phagocytosis than negative control (table 2).Nano particles with antigen inactivated *S. aureus* increased phagocytosis compared with negative control**.** With increasing of the number of immune cells, the phagocytosis was also increased was observed between Intraperitoneal and Subcutaneously groups. In addition, adjuvanticity impact is comparable with Incomplete adjuvant 10 (21.2%) and Killed *S. aureus* alone 6 (18.18 %). This observation might be because of used antigen sort as our antigen is a complex antigen. (16).Sort of mice utilized might be another reasons, it was reported mice had more resistant immune responses. In addition to protocol of immunization (17). It is critical that our antigen is more immunogenic as opposed to Incomplete adjuvant and increase of immune cellular response.

Table 2: Phagocytosis index

Groups immunization		Total No. (Nonphagocyte+ phagocyte cell)	No. phagocytes and Efficiency (%)
Negative Control		22	4 (18.1%)
Positive Control		38	8 (21.05%)
Incomplete adjuvant		47	10 (21.2%)
Olive oil		27	5 (18.5%)
Killed S. aureus		33	6 (18.18 %)
Intraperitoneal	One dose	117	25 (21.36%)
	Post dose	129	29 (22.48%)
Subcutaneously	One dose	144	32 (22.2%)
	Post dose	172	40 (23.2%)

Effect of vaccine on antibody level

IgG titers in the serum of animals active immunization with vaccines (Formalin-killed *S. aureus*-AgNPs) administered intraperitoneally (i.p.) and subcutaneously were significant increased (P ≤0.05) which were (9.8 ± 0.08 one dose, 12.3 ±

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0.23 post dose) and (11.7 ± 0.11 one dose, 16.2 ± 0.11 post dose) mg/ml respectively than those found in the positive control AgNPs (4.1±0.08 mg/ml), negative control (4±0.05 mg/ml), Killed *S. aureus* alone (5 ± 0.12 mg/ml) and incomplete Freund's adjuvant (6.2 ± 0.17 mg/ml), and significant differences were observed between 1th (one dose) and 7th days (post immunization), (table 3).The route of administration plays critical role in stimulation of humoral immune response, that was illustrated by many reviews. It must be noticed that subcutaneously post-immunization had better impact. By Xu et al were previously appeared the adjuvant watched impact was affirmed because of AgSNPs because that AgNPs which formed just of silver and oxygen molecules (16).The mechanism of activity for NPs were dependent upon surface changes and composition (18).On the other hand, high IgG levels are usually associated with increased production of lymphocytes, specially type B. The formation of IgG, a proper marker of increasing the number of immune cells, was modestly instigated by Killed *S. aureus* or nano particles alone, and was especially improved by antigen plus nano particles as contrasted with nano particles (AgNPs) or Killed *S. aureus* (Ag) alone. The immunogenicity of vaccine was evaluated in mice. Therefore, to assess AgNPs adjuvanticity, we utilized inactivated *S. aureus* bacteria. Immunoglobulin plays a critical role in host by averting colonization of pathogens and guard at mucosal sites.

Table 3: IgG concentrations in mice immunized

IgG mg/ml								
Negative control	Positive Control AgNP	Olive oil	Killed S. aureus	Incomplete adjuvant	Intraperitoneal		Subcutaneously	
4±0.05	4.1 ± 0.08	5.5±0.11	5 ± 0.12	6.2 ± 0.17	One dose	Post dose	One dose	Post dose
					9.8 ± 0.08	12.3 ± 0.23	11.7 ± 0.11	16.2 ±0.11

Challenge test

Freshly grown *S .aureus* strain (1.5×10^8 CFU/ml) was used. The results of challenge test showed a survival rate of 95-100% in all mice vaccinated especially subcutaneously post-immunization, and all is healed no lesion of skin during one week in comparison with the incomplete Freund's adjuvant, Killed *S. aureus* alone and negative control groups were found to cause 80 to 100% mortality in mice within 2 to 5 days following the bacterial challenge. These results indicated the hyperimmune responses are occurring due to the immunization by AgNPs-killed *S .aureus* vaccine, and these results also agree with the previous results of increase the number of immune cells and IgG elevation that led to protect animals from infection, thus AgNPs, has been found to exert as adjuvant effect with inactivated *S. aureus* bacteria to evaluate AgNPs adjuvanticity. This results was agreed with (13) who reported that when mice immunized with vaccine, they were protected against disease caused by *S. aureus*.

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