EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 4
Issue: 6
529-536
Year: 2017

IMMUNE ENHANCING POTENTIAL OF SPHERE AND ROD GOLD NANOPARTICLES TO RIFT VALLEY FEVER VACCINE RELATIVE TO TIME: *IN VITRO* STUDY

Maha G. Soliman[1], Aly F. Mohamed[2], Rasha A. El Sayed[1] and Asmaa A. Abo Elqasem*[1]

[1]Zoology Department, Faculty of Science, Al-Azhar University (Girls branch), Cairo, Egypt.
[2]VACSERA, Cairo, Egypt.

***Corresponding Author: Dr. Asmaa A. Abo Elqasem**
Assistant Lecturer, Zoology Department, Faculty of Science, AL - Azhar University, Cairo, Egypt.

Article Received on 20/04/2017 **Article Revised on 10/05/2017** **Article Accepted on 31/05/2017**

ABSTRACT
Present study aimed to evaluate sphere (S) and rod (R) gold nanoparticles (GNPs) as immune enhancer. Immune response to β-propiolactone (βPL) inactivated Rift Valley Fever (RVF) vaccine enhanced with GNPs was compared with aluminum (Alum) as a current adjuvanted. Regarding inactivation kinetics, βPL showed a fast inactivation kinetic recording depletion rate in order of 1.2 log $_{(10)}$ / 15 min. Also, it was noticed that the immune response post subcutanious (SC) immunization was GNPs, shape and molar concentration dependent. The peak antibody (Ab) level was detected on the 30th day post immunization in case of Alum and 20 nM of RGNPs, while the peak value was reached on the 45th day post vaccination in the rest of test groups. The least value of Ab was in case of non adjuvanted RVF vaccine. In the mean time cellular immune response was influenced where the peak levels of IFN-γ and IL-10 were detected in case of Alum adjuvanted vaccine and 20 nM SGNP enhanced vaccine, while the rest formulae showed lower values. The levels of IL-10 and IFN-γ was time related and declining phase was noticed on the 45th day and in case of 20 nM of SGNPs there was a stationary phase extended throughout the 7th – 30th day post vaccination.

KEYWORDS: Rift Valley fever virus, gold nanoparticles, adjuvants, rats.

INTRODUCTION
Rift Valley fever virus (RVFV) is a mosquito-borne zoonotic pathogen causing severe outbreaks in humans and livestock in sub-Saharan Africa and the Arabian Peninsula. Human infections are characterized by fever, sometimes leading to encephalitis, retinitis, hemorrhagic fever, and occasionally death[1]. Control of RVF in Egypt depends mainly on periodical vector control and vaccination of susceptible animals with binary inactivated RVF vaccine[2]. Several RVF vaccines have been developed and are available in commercial production like Formalin and βPL-Inactivated vaccine, live attenuated Smithburn vaccine and the most recent Clone13[3]. Alum compounds have been approved as adjuvant for human use in several European countries[4]. In the United States, Alum compounds are the most extensively used adjuvants in licensed vaccines. Although they effectively enhance immune responses, there are several disadvantages associated with their use[5]. The disadvantages of Alum-based adjuvants include the severity of local tissue irritation, the longer duration of the inflammatory reaction at the injection site[6]. For these reasons, new adjuvants are being developed to enhance the immunity against antigens. Many other adjuvants are designed to induce minimal side effects, enhance the duration of the immune response, and concurrently stimulate humoral responses. Furthermore, an ideal adjuvant would be biodegradable, economical, and simple to manufacture. In addition, it would have the potential to selectively trigger a defined class of immune response[7]. Nanoparticles can be used to overcome some of the limitations found in traditional vaccines[8]. Nanoparticles can also stimulate immune response by acting as adjuvants[9]. Nanoparticles that have immense applications in industries are of different types, namely, Gold, Silver, Alloy, magnetic etc. GNPs are used in immunochemical studies for identification of protein interactions. Gold nanorods and sphere are being used to detect cancer stem cells, beneficial for cancer diagnosis and for identification of different classes of bacteria[10]. So the present study aimed to evaluate GNPs with different shapes and concentrations as adjuvant against RVFV.

MATERIALS AND METHODS
Rift vally fever virus strain
A seed stock of pan tropic Menya, sheep No. 258 (Menya /Sheep/258) strain was kindly provided by Dr. El Karamany, Ex-G.M. of research and development sector VACSERA, Egypt. The strain had a titre of $10^{9.7}$/ ml

$MICL_{50}$/ml of sucking mice and $10^{7.5}$ / ml ($MIPLD_{50}$/ ml).

Maintenance of cell line and seed stock preparation

Vero cells were kindly supplied by cell culture department, virology sector, VACSERA, Giza, Egypt. Growth medium was removed from the decanted cell culture flasks, and the monolayer was washed with sterile PBS, pH 7.2. Vero cells were trypsinized with 0.25% (W/V) trypsin - EDTA solution (Invitrogen, San Diego, Calif. USA), by dispensing 10 ml of trypsin solution to the monolayer. Trypsinized cells were left for 15-30 seconds, and trypsin solution was removed. The culture was held at 37 ºC until the cells had pilled apart. The cells were suspended to the proper concentration (200.000 cells/ml) in 199-E medium (GIBCO), supplemented with 10% fetal calf serum (FCS) (Sigma Chemical Co., St Louis, MO, USA in 75 cm^2 tissue culture flasks (Nunc, Roskilde, Denmark). Then incubated at 37 ºC in 5% CO2 saturated atmosphere until confluent monolayer detected. Growth medium was discarded and replaced with 5 ml fresh medium containing 10 % FCS which contain 100 $TCID_{50}$ of RVFV sterile TC harvest and flasks were incubated in CO_2 incubator for an hour. Bottles were shacked at 15 min interval for assurance of well virus distribution. Maintenance media as 100 ml was added to each bottle. Inoculated bottles were daily microscopically observed for 7 days for detection of cellular changes and development of cytopathic effect (CPE). Flasks developed CPE were freeze and thawed three times for virus extraction[11].

Inactivation of RVFV using βPL

Chemical inactivation of concentrated RVFV with βPL (Sigma – Aldrich – USA) was accomplished by adding the reagent to a final concentration of 0.00035 M. After vigorous mixing, the βPL-containing virus suspension was kept at 37 ˚C.Samples of 1 ml of βPL-containing virus were evaluated for interval infectivity titer depletion rate at 15 min for 3 hours according to[12]. Inactivation was achieved 10 fold serially dilution of virus samples from 10^{-1} - 10^{-8} for determination of residual live virus titer at certain time interval. Growth medium was discarded from precultured 96- well plates (Dynatech, Alexandria, VA, USA) and 0.1 ml of each dilution was dispensed to 4 wells- uninoculated cell control. Infected plates were kept at 37 ºC at 5 % CO_2 concentration for 7 days with daily microscopic observation using inverted microscope (Hund-Germany) for detection of cellular changes. Residual live RVF virus titer was determined according to[13]. The mean RVFV infectivity titer depletion / hrwas determined.

Preparation of RVFV cell lysate Ag

RVFV was diluted to final dilution 1:50 in Hank's balanced salt solution (GIBCO- USA). The growth medium was poured off and 2 ml of virus per roller bottle was added and allowed to adsorb for 60-90 minutes. Bottles were allowed to rotate on the roller apparatus (Belco-USA) 5 rpm/ minutes, followed by addition of 100 ml of maintenance medium per roller bottle. Bottles were incubated for 24 hr. The cells were scraped with rubber policemen scraper, followed by decantation into 200-ml centrifuge bottles then spin 15 min. at 3000 RPM at 4 ºC. Pellet was re-suspended in borate saline pH 9 into 50 ml Oak Ridge tube. Pellet was rewashed and rotated for 10 min. at 12,000 RPM at 4 ºC and resuspended in borate saline solution, pH 9, 1%Tritan x 100 (Sigma Chemical Co. USA) , 1% SDS (BIO-RAD). Pellet was sonicated in cup horn apparatus until particulates dispersed, and then centrifuged for 10 min. at 12,000 RPM in cold centrifuge (Jouan France). Supernatant was collected in polypropylene tube, and pellet was discarded. The antigen was a liquated into labeled tubes and store at -70 ºC[14].

Preparation of adjuvants

Sphere and rod gold nano particles

SGNPs and RGNPs were purchased as 2nM from Nano Tech. Company.

Alum adjuvant

Alum phosphate was prepared in 40 ml normal saline according to[5].

Animals

Male albino ratswere supplied from Theodore Bilharz Research Institute, Giza, Cairo. Body weights of rats ranged from 75-80 gm. Animals were housed in metallic cages. All rats were housed in VACCERA animal house and kept under good conditions of light and temperature, allowed free access tap water and dry food diet. Animals were kept under observation for about one week before the onset of the experiment for adaptation. Care and cleaning were important for maintaining the animals in a normal healthy state.

Animals used in the present experiment were divided into seven groups (ten rats in each group):

Group (1): served as the negative control (Neg. con.) group.

Other sex groups were immunized subcutaneously by 0.5 ml RVF vaccine/rat as:

Group (2): RVF vaccine only (vac)

Group (3): RVF vaccine conjugated with Alum phosphate

Group (4): RVF vaccine conjugated with 20 Nm SGNPs

Group (5): RVF vaccine conjugated with 20 Nm RGNPs

Immunological Studies

1-Detection of IgG antibody against RVF vaccine

Wells of polystyrene micro titer plates (96-flat bottomed wells, M 129A Dynatech) were coated with 100 µl /well of RVF antigen prepared as 1 µgm/ ml total protein in carbonate buffer, pH 9.6 and incubated overnight at room temperature. Plates were washed 3 times with washing buffer (PBS - 0.5%Tween 20), blocked with 100 µl /well of 4% BSA (Sigma-Aldrich -USA) in 0.1 M PBS, pH 7.4 and incubate for 1 hr at 37°C. Plates were

washed as previous. Sera samples were 2 fold serially diluted (starting dilution was 1/25) in the reciprocal wells in dilution buffer (1% BSA- 100 ml PBS). Hundred µl of immune sera were dispensed to each well and incubated for 1 hr at 37°C. Plates were washed as previous. Rat anti rat horseradish Peroxidase conjugate (Sigma-Aldrich -USA) diluted in 1% BSA. Hundredµl/well of conjugate were dispensed to the whole plates. Plates incubated for 60 min at 37°C, and were washed as previous. Hundredµl/well of substrate solution (one tablet of O-phenylenediaminedihydrochloride; OPD) was added as 100 µl/ well. Plates were incubated in the dark at room temperature for 30 min. Hundredµl/well of 2N H_2SO_4 were added to stop the enzyme substrate reaction. The absorbance was measured at 450 nm using ELISA reader (Bio-Rad microplate reader, Richomond, Co.).

2-Cytokine determination

Serum IFN-γ and IL-10 levels in rat sera were determined according to the manufacturer instruction. Plates of ELISA were coated with 100 µl /well of serial dilute sera samples in PBS buffer and the plates incubated overnight at room temperature. Plates were aspirate to remove liquid and washed 4 times with washing buffer (PBS - 0.5%Tween 20). Plates were blocked by adding 300 µl /well blocking buffer and incubated for 2 hr at room temperature. Plates were aspirate and washed as previous. Antibody was diluted in diluent to a concentration of 1ug/ml then added 100 ul/well. Plates were incubated for 2 hr at room temperature. Plates were aspirate and washed as previous. Avidine-HRP conjugate was diluted 1:2000 in diluent and 100 ul/well was added. Plates were incubated for 30 min at room temperature, aspirate and washed as previous. Hundred ul/well of substrate solution were added and plates incubated in dark at room temperature for color development. Color development with an ELISA reader was monitored at 405 nm.

Statistical Analysis

The statistical analysis was carried out using analysis of variances (one way ANOVA). For the comparison of significance between groups a post hoc test was used according to the statistical package program (SPSS version 22).

RESULTS

Inactivation of RVFV using chemical inactivants

RVFV stock prepared in Vero cell line was inactivated using 0.0035 M of βPL. Inactivation kinetics was determined relative to time (Fig. 1). RVFV was completely inactivated within 2hrs post βPL treatment recording a mean depletion of virus infectivity titer in the order of 1.2 $\log_{(10)}$/ 15. Min.

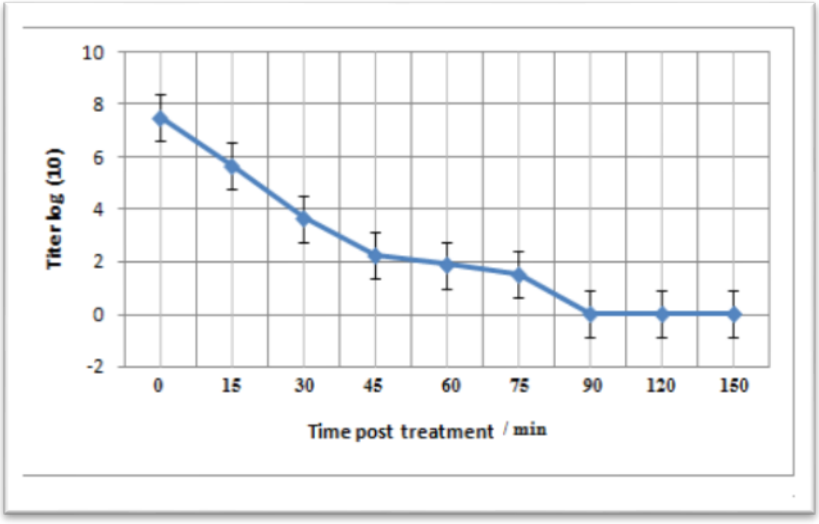

Fig 1: Evaluation of inactivation kinetics of RVFV using 0.0035 M of βPL

RVF vaccine potency (ED_{50})

Regarding the immune potential of the prepared experimental Rift valley fever viral vaccines, the effective dose that protected 50% of challenged mice [ED_{50}] was evaluated using the mouse immunization-challenge assay. It was found that the ED_{50} of the βPL was recording 0.0163 ml [fig. 2].

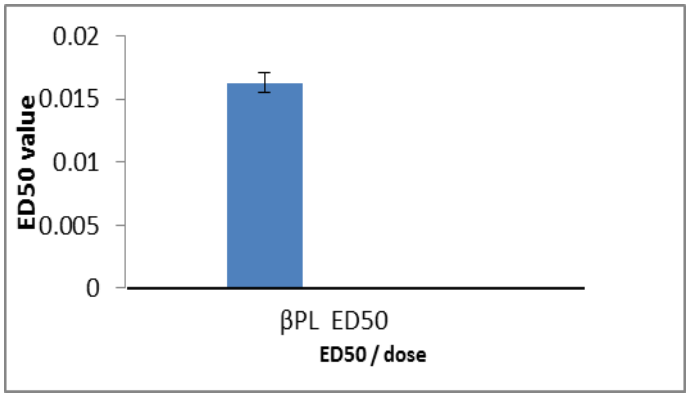

Fig 2: Evaluation of effective dose of RVF vaccine candidates [ED_{50}] using mice inoculation assay.

Evaluation of antibody (IgG) level

Regarding the immune potential of prepared RVF vaccine candidate, recorded data indicated that antibody titre of IgG level showed a time dependent. It was noticed that, IgG level of Vac group was significantly increased compared to Neg. con. group and 20 RGNP adjuvanted group (P>0.05). The level of IgG was detected post vaccination with Alum adjuvanted group and it was significantly increased than Neg. con., Vac groups and 20 RGNP adjuvanted group (P>0.05). Alum recorded the higher value during the whole experiment from all groups except at 45th and 60th days it was lower than 20 SGNP group.Also, concerning the enhancing potential of the GNPs; both S&RGNPs used as immune enhancer to RVF vaccine. IgG level in case of 20 SGNP adjuvanted group recorded a higher results than control and Vac groups while 20 RGNP adjuvanted group was still lower than other vaccinated groups (P<0.05) [fig. 3].

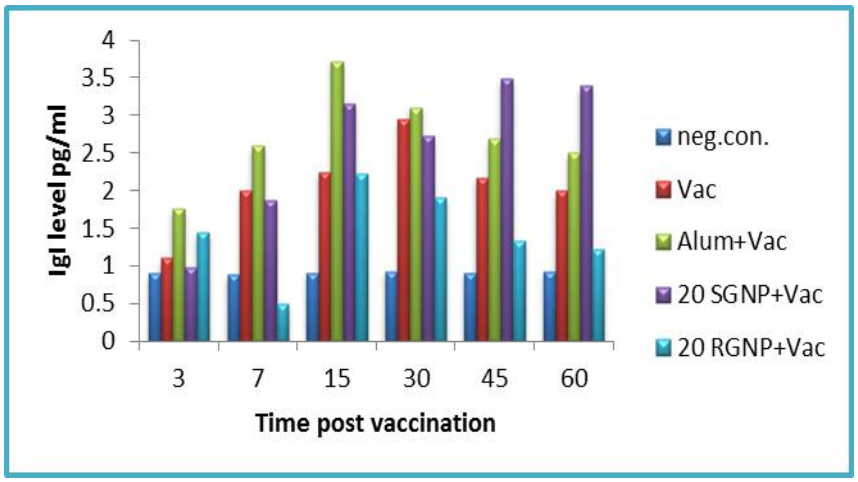

Fig 3: Comparative evaluation of Ab level post vaccination of rats with vac. (non–adjuvanted),Alum-adjuvanted RVF vaccine and 20 of S&RGNPs-adjuvanted vaccine

Serum cytokine levels

Sera samples collected from RVF vaccine immunized rat groups were tested to evaluate some sera cytokines levels like Th^1 (INF-γ) and Th^2 (IL-10) cytokines using ELISA.Both INF-γ and IL-10 cytokines levels were detected 3 days post vaccination using different formulations of RVF vaccine candidates. It was noticed that there was a significantly increased level of IL-10 in all vaccinated and enhance vaccine groups relative to time (P<0.05). IL-10 level was elevated in Vac group compared with Neg. con. group, 20 SGNP and 20 RGNP adjuvanted groups. There was a significant elevation of IL-10 level in Alum adjuvanted group compared to Neg. con. group, Vac group, 20 SGNP and 20 RGNP adjuvanted groups with highest value at the 45th and 60th day post vaccination. IL-10 level of 20 SGNP adjuvanted group was significantly higher than Neg. con. group and 20 RGNP adjuvanted group but still lower than the level in case of vaccination with Vac. group and Alum adjuvanted group (P>0.05)[fig. 4].

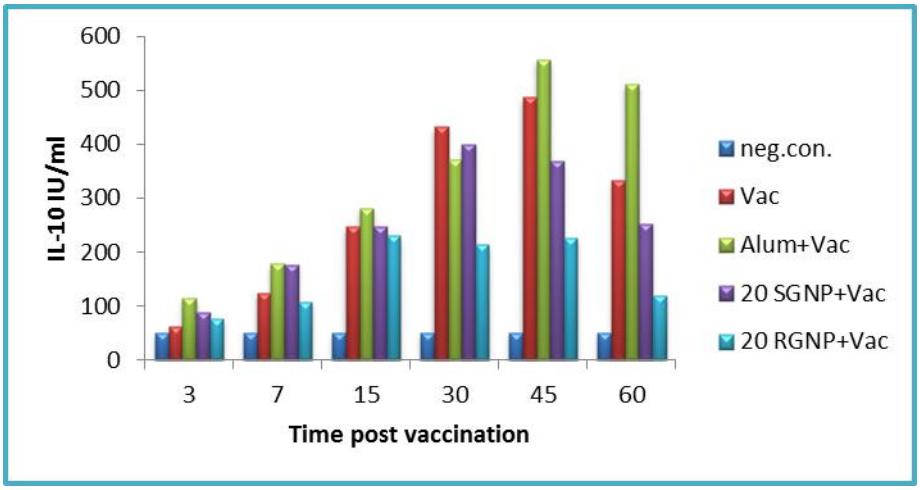

Fig 4: Comparative evaluation of IL-10 level secreted post vaccination with vac. (non–adjuvanted),Alum-adjuvanted RVF vaccine and 20 of S&RGNPs-adjuvanted vaccine

Also, INF-γ level was elevated in Vac group compared with Neg. con. group. There was a significant elevation of INF-γ level in Alum adjuvanted group compared to Neg. con. group, Vac group and 20 RGNP adjuvanted group. Using of 20 SGNP showed a significantly elevated stimulation of INF-γ release at all time intervals except at 60th day compared with its values in Neg. con. group, Vac group, Alum and 20 RGNP adjuvanted group ($P<0.05$). INF-γ level was significantly elevated in 20 RGNP adjuvanted group compared with Neg. con. group [fig. 5].

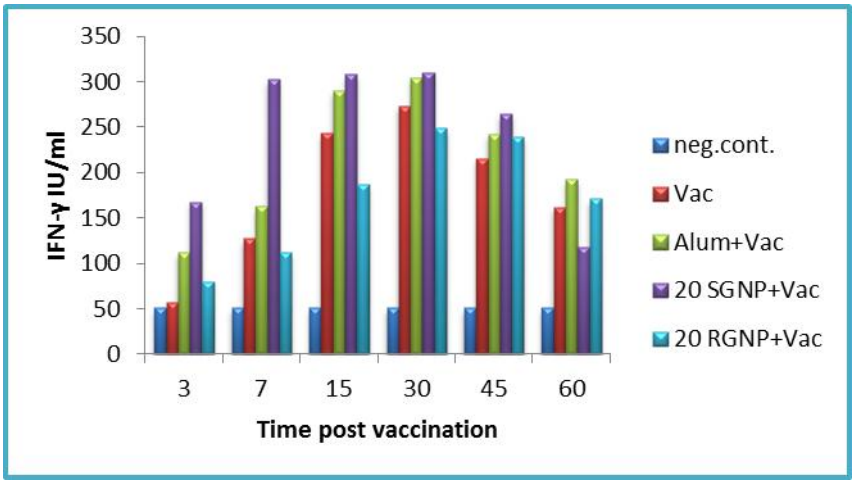

Fig 5: Comparative evaluation of INF-γ level secreted post vaccination with vac. (non–adjuvanted),Alum-adjuvanted RVF vaccine and 20 of S&RGNPs-adjuvanted vaccine

DISCUSSION

RVFV is an arbovirus circulating between ruminants and mosquitoes to maintain its enzootic cycle. Humans are infected with RVFV through mosquito bites or direct contact with materials of infected animals. As vaccination is an effective tool to control RVFV epidemics, βPL inactivated and live-attenuated RVFV vaccines have been used in endemic areas[15]. βPL inactivated elicits dentretic cells (DC) maturation without viral replication /transcription. This suggests that βPL may be a useful tool for vaccine therapy and has potential adjuvant activity[16]. A highly pure antigen preparations are used to improve vaccine safety has led to reduced vaccine immunogenicity and efficacy. This has led to the need to use alternative adjuvants to improve vaccine immunogenicity[17]. After almost a century, Alum salts maintain their dominance as adjuvants in human vaccines this reflects the fact that Alum adjuvants are extremely effective at enhancing both cellular and humoral immune responses and have the strongest safety record of any other human adjuvant[18]. Hence, Alum salt adjuvant remain the gold standard against which all new adjuvants, it provides better protection, tolerability or safety[19]. Recently, nanotechnology may increasingly play a significant role in vaccine development. The use of NPs in vaccine formulations may improve antigen stability and immunogenicity, targeted delivery and slow release. From these NPs, the GNPs used as immune adjuvant to improve immune responses[20]. The present study noticed that RVFV was completely inactivated within 2hrs post βPL treatment with a concentrations of 0.0035 M while

vaccine potency [ED_{50}] was 0.0163 ml which is in accordance with the WHO regulations. These recommended that the related vaccine potency (ED_{50}) must be less than 0.02 ml. These results are in agreement with[21] who obtained that, potency (ED_{50}) of βPL was (0.006) and the inactivation time of RVFV was also 2 hrs post treatment.[22] reported that 0.0035 M of βPL could inactivate Sabin polio virus within 40 minutes.[23] recorded that, βPL showed higher vaccine potency than Formalin- and Ascorbic acid inactivants. This was attributed to the limited effect on the configurations of the viral epitopes to which the antigenicity is attributed, and it also had no deleterious effect on the immune response. Data recorded was due to the stimulation of Th2 immune cells in accordance with the study of[24] who reported that humoral immune response induced by Alum- βPL RVF vaccine was monitored revealing a time and route of administration dependence.[25] noticed that, the Alum showed activating DCs and macrophages which stimulate Th2 immune responses.[26] suggested that, the Alum adjuvants selectively stimulate a Th2 immune response and other immunomodulatory molecules. Another study of[27] demonstrated that, Ab production depends on T-cell help and those Th-cell subsets with different functions exist led to studies to determine the effects of Alum salts on different Th-cell subsets. Alum salts were found to preferentially induce Th2 cells, which mediate the differentiation of B cells that secrete Th2-cell-associated antibody isotypes IgG1 and IgE.[28] obtained that, eosinophils appear at the Alum injection site within 24 hours, and IL-4 produced by these cells was shown to increase B-cell proliferation and promoted an increase in the production of IgM. In addition, administration of an Alum salt-containing vaccine resulted in the generation of IgG-producing B cells. In addition a study of[29] showed that, Alum salt adjuvants enhance vaccines to induce high titer IgG with a relatively long lasting immunity. Also[30] indicated that, higher neutralizing potency of the antibodies had been induced in the presence of the Alum as adjuvant. In the present study, 20 nM S&RGNPs stimulate humoral immune response and showed the highest peak values of Ab level at 30th day post vaccination ($P>0.05$).[31] noticed how the shape and size of GNPs affect immunological responses *in vivo* and *in vitro* and their finding proved that, SGNPs induced the highest level of Abs, while RGNPs induced only 50% of that of SGNPs. As well[32] concluded that, *in vitro*and *in vivo* GNPs showed activation of B cells and enhancement0f IgG secretion. Th2 cells drive the humoral immunity and unregulated Ab production to fight extracellular organisms. Th2 cells produce large amounts of IL-3, IL-4, IL-5, IL-6, and IL-10 and a small amount of TNF. In the present study, regarding the cellular immune response it was clear that IL-10 level showed variable pattern relative to formulation. These data was in accordance with[33] who concluded that, Alum adjuvants induce strong innate immune responses that consist of an influx of neutrophils, eosinophils, natural killer (NK) cells, dendritic cells (DCs) and monocytes that produce IL-10.[24] proved that, there was a gradual significantly elevated level of Th2 cytokine (IL-10) post immunization with both NPs and Alum adjuvanted rabies vaccines compared with that detected post immunization with non adjuvanted One [$P<0.05$]. The maximum level was detected 72 hrs post immunization followed by a declining phase till end of the experiment. The results of[20] agreed with present results that, using of GNP as an adjuvant can stimulate potent cellular immune responses against antigens, which may promote better understanding of cellular immune response and facilitate potential applications for cancer and viral vaccines. Recently[34] concluded that, the GNPs can penetrate into various immune cells and activate the production of proinflammatory cytokines as IL-10. Also[35] demonstrated that small (<50nm) nanoparticles administered subcutaneously distribute through lymphatic drainage into draining lymph nodes where they stimulate antigen-presenting cells, lymphocytes and adaptive immune response.[31] noticed that, GNPs treated cells produced significant levels of interleukins, also they indicated that, RGNPs showed activation ofinflammasome-dependent cytokine secretion. Meanwhile, SGNPs and cube both significantly induced inflammatory cytokine production, including tumor necrosis factor-α (TNF-α), IL-6, IL-12 and IL-10. On the other hand present data was in agreement with[36] and[37] who found that, the Th1-cell responses *in vivo*may enhance IFN as a cellular immune response by Alum salts.[24] demonstrated that, there was a gradual significantly elevated level of Th1 cytokine (INF-γ) post immunization with both NPs and Alum adjuvanted rabies vaccines compared with that detected post vaccination with non adjuvanted group and the maximum level was detected 96 hrs post immunization. In the present study, the rod and sphere GNP groups showed a significant elevation of INF-γ level ($P<0.05$) but 20 nM SGNP group was the highest compared to Neg, con., Vac, Alum and 20 RGNP group. In agreement with present results,[38] reported that, release of IFN-γ from lymphocytes was increased with GNPs. Moreover, the results of[39] indicated that, SGNPs enhance elevated secretion of T helper1 cytokines such as IFN-γ. These results suggested that SGNPs are effective vaccine adjuvants and enhance the immune response. Recently[34] concluded that, RGNPs induced the macrophages activation and hence induced the release of cytokines and the activation of immune response.

CONCLUSION

It can be concluded that sphere and rod shape GNPs as well as Alum have stimulating potential of cellular and humoral immune response despite the variation of reactivity. Immune response post vaccination with GNP is shape and molar concentration dependent.

ACKNOWLEDGEMENT

Authors wish to thank Prof.Dr.AlyFahmy Mohamed, Professor of Virology, VACSERA, The Holding Company for Biological Products & Vaccines.We are

also grateful to Department of Zoology, Faculty of Science (Girls branch), Al- Azhar University for providing laboratory facilities.

REFERENCES

1. Bonto F,Juergen AR.(Short Interfering RNA Inhibits Rift Valley Fever Virus Replication and Degradation of Protein Kinase R in Human Cells). FrontMicrobiol, 2016; 7: 1889.
2. Mohamed GK. (Effect of some minerals on sheep vaccinated with binary inactivated RVF vaccine). Hurghada, Vet Med Zag Congress, 1998; 39: 26-28.
3. Alhaj M. (Safety and Efficacy Profile of Commercial Veterinary Vaccines against Rift Valley Fever: A Review Study). J Immunol Res, 2016; doi, 7346294.
4. He Q, Mitchell AR, Johnson SL, Wagner-Bartak C, Morcol T. (Calcium phosphate nanoparticle adjuvant).ClinDiagn Lab Immunol, 2000; 7: 899-903.
5. Gupta, R. K. (Aluminum compounds as vaccine adjuvants). Adv Drug delivery rev, 1998; 32: 155-172.
6. Confavreux C, Suissa A, Saddier P, Bourdes V,Vukusic S. (Vaccinations and the risk of relapse of multiple sclerosis. Vaccines in Multiple Sclerosis Group). N. Engl J Med, 2001; 344(5): 319-326.
7. Qing H, Alaina R, Mitchell SL, Johnson CWB, Tulin M,Steve JDB.(Calcium Phosphate Nanoparticle Adjuvant).ClinDiagn Lab Immunol, 2000; 7: 899-903.
8. Zhang L, FX GuJM, Chan AZ, Wang, RS. (Nanoparticles in Medicine: Therapeutic Applications and Developments).Clinical pharmacology and therapeutics, 2008; 83: 761-769.
9. Kononenko V, Narat M, Drobne D. (Nanoparticle interaction with the immune system).ArhHigRadaToksikol, 2015; 66: 97-108.
10. AlkilanyAM, Thompson LB, Boulos SP, Sisco PN,Murphy CJ. (Gold nanorods: Their potential for photothermal therapeutics and drug delivery, tempered by the complexity of their biological interactions). Adv Drug Deliv Rev, 2012; 64: 190-199.
11. Bussereau F, Flamand A,Pese-Part D. (Reproducible plaquing system for rabies virus in CER cells). J Virol Meth, 1982; 4: 277-282.
12. Shu de J, Pye D, Cox JC. (Inactivation of Poliovirus with ß-Propiolactone). J Virol Standardization, 1986; 14(2): 103–109.
13. Reed LT, Muench HA. (Simple method of calculating, percent end point). Am J Hyg, 1938; 27: 493-498.
14. Mohamed FA, El-Kaschef AH, Reda IM, Tantawey HM, Hassen RA. (Biological studies for the importance of Rift Valley Fever Vaccine). ph.D thesis, Suez Canal Univ.Zool. Depart.1996.
15. Terasaki K, Tercero BR,Makino S.(Single-cycle replicable Rift Valley fever virus mutants as safe vaccine candidates).Virus Res, 2016; 2: 55-65.
16. Kenichiro H, Masayuki F, Junpei O, Mitsuo K,Tetsuya N. (Human Parainfluenza Virus Type 2 Vector Induces Dendritic Cell Maturation Without Viral RNA Replication/Transcription).Hum GeneTher, 2013; 24: 683–691.
17. Nikolai P. (Comparative safety of vaccine adjuvants: a summary of current evidence and future needs).Drug Saf, 2015; 38: 1059–1074.
18. Petrovsky N, Aguilar JC. (Vaccine adjuvants: current state and future trends).Immunol Cell Biol, 2004; 8: 488–496.
19. Lima HJR, Jacobson LS, Goldberg MF, Chandran K, Diaz-Griffero F, Lisanti MP.(Role of lysosome rupture in controlling Nlrp3 signaling and necrotic cell death). Cell Cycle, 2013; 12: 1868–1878.
20. Yuhua C, Yufei M, Mengxin Z, Haiming W, Xiaolong T, He Shen, Jianwu Dai, Huichen G, Zhijun Z. (UltrasmallGraphene Oxide Supported Gold Nanoparticles as Adjuvants Improve Humoral and Cellular Immunity in Mice).AdvFunct Mater, 2014; 24: 6963–6971.
21. Abd el-Razek EN, Shoman AS, Mohamed FA.(Nanocapsulated Rift Valley Fever Vaccine Candidates and Relative Immunological and Histopathological Reactivity in Out Bred Swiss Mice). J Vaccines Vaccin, 2011; 2: 115.
22. Ali H,El Gawad HA, El Tayeb O, Mohamed FA. (Evaluation of Inactivation Efficacy of Sabin Polio Virus Using Different Inactivating Agents and it's Immunogenicity Post Nano and Micro Encapsulation). International Journal of Microbiological Research, 2010; 1: 114-122.
23. Al-Olayan, ME, Mohamed FA, El-Khadraqy FM, Sheb IR, Yehia MH. (An Alternative Inactivant for Rift Valley Fever Virus using Cobra Venom-derived L-Amino Oxidase, which is Related to its Immune Potential):. Braz arch bioltechnol, 2016; 59: e16160044.
24. Emera, ME, Abdel-Rahman M, Mohamed FA, Nady S. (Physiological and Immunological Evaluation of Rabies Vaccine Adsorbed to Two Different Vaccine Vehicles). Egyptian Journal of Medical Microbiology, 2012; 21: 99-110.
25. Mirjam K, Kaat F, Lambrecht NB. (Alum adjuvant: some of the tricks of the oldest adjuvant). Journal of Medical Microbiology, 2012; 61: 927–934.
26. Harm HE. (Mechanism of Immunopotentiation and Safety of Aluminum Adjuvants).Front Immunol, 2012; 3: 406.
27. Kopf M, Le Gros G, Bachmann M, Lamers MC, Bluethmann H, Köhler G. (Disruption of the murine IL-4 gene blocks Th2 cytokine responses).Nature, 1993; 18: 245-248.
28. Wang HB, Weller PF. (Pivotal advance: eosinophils mediate early alum adjuvant-elicited B cell priming and IgM production). J LeukocBiol, 2008; 83: 817–821.
29. Barbaud A, Deschildre A, Waton J, Raison-Peyron N, Trechot P. (Hypersensitivity and vaccines: an update). Eur J Dermatol, 2013; 23: 135–141.

30. Juergen Z, Georgios T, Johanna J, Christian K, Karin S, Franz XH. (Aluminum Hydroxide Influences Not Only the Extent but Also the Fine Specificity and Functional Activity of Antibody Responses to Tick-Borne Encephalitis Virus in Mice). JVirol, 2013; 87: 12187–12195.
31. Kenichi N, Tatsuya M, Tadaki S, Shintaro K, Hiroki Y, Yasuko O, Akira K, Hideki H, Kiichi K, Takafumi N, Kuniharu I, Hirofumi S. (Gold Nanoparticles as a Vaccine Platform: Influence of Size and Shape on Immunological Responses *in Vitro* and *in Vivo*). ACS Nano, 2013; 7: 3926–3938.
32. Chia-Hui L, Shih-Han S, Yu-ShiunCh, Saber M H, Andrei AO, Wen LCh, Huang SG. (Gold nanoparticles regulate theblimp1/pax5 pathway and enhanceantibody secretion in B-cells). Nanotechnology, 2014; 25: 125103.
33. Calabro S, Tortoli M, Baudner BC, Pacitto A, Cortese M, O'Hagan DT, De Gregorio E, Seubert A, Wack A. (Vaccine adjuvants alum and MF59 induce rapid recruitment of neutrophils and monocytes that participate in antigen transport to draining lymph nodes). Vaccine, 2011; 29: 1812–1823.
34. Lev AD, Nikolai GK.(Immunological properties of gold nanoparticles).ChemSci,2017; Doi: 10.1039/C6SC03631G.
35. Manolova V, Flace A, Bauer M, Schwarz K, Saudan P, Bachmann MF. (Nanoparticles target distinct dendritic cell populations according to their size). Eur J Immunol, 2008; 38: 1404–1413.
36. Brewer JM, Conacher M, Satoskar A, Bluethmann H, Alexander J. (In interleukin-4-deficient mice, alum not only generates T helper 1 responses equivalent to freund's complete adjuvant, but continues to induce T helper 2 cytokine production). Eur J Immunol, 1996; 26: 2062–2066.
37. Amy SM, Michael WM, Megan KLM, Courtney JF. (Alum Induces Innate Immune Responses through Macrophage and Mast Cell Sensors, But These Sensors Are Not Required for Alum to Act As an Adjuvant for Specific Immunity). J Immunol, 2009; 183: 4403-4414.
38. Neill JL, Emily K, Daniel JN, John F, Steve R, David GF, Andrew O. (Partial mitigation of gold nanoparticle interactions with human lymphocytes by surface functionalization with a 'mixed matrix'). Nanomedicine, 2014; 9: 2467-2479.
39. Qianqian Z, Yulong Z, Juan D, Yuan L, Yong Z, Qiuxia F, Jingang Z, Xiaohui W, Linsheng Z. (Different-Sized Gold Nanoparticle Activator/Antigen Increases Dendritic Cells Accumulation in Liver-Draining Lymph Nodes and CD8+ T Cell Responses). ACS Nano, 2016; 10: 2678–2692.