

Original article

Influence of gold nanoparticles on the immune response to rift valley fever vaccine and related hepatophysiological toxicity, histological, and immunohistochemical alterations

Background: Vaccination is a very effective method of stimulating the immune response against infections. Adjuvants are employed to enhance the immune response, but they must be safe, inexpensive, and easy to use. **Objective:** This study aimed to evaluate gold nanoparticles as immune enhancers for rift valley fever vaccine. **Methods:** The rats were divided into four groups (10 each): the negative control group, rats immunized with nonadjuvanted rift valley fever vaccine and the last two rat groups immunized with rift valley fever vaccine combined with 40 μ M of spherical gold nanoparticles and combined with 40 μ M of rod-shaped gold nanoparticles, respectively. **Results:** Compared with rats receiving no treatment and rats treated with nonadjuvanted vaccine, rats treated with vaccines combined with gold nanoparticles exhibited toxic biochemical, histological and immunohistochemical changes, as shown by significant elevations in liver enzymatic markers and total bilirubin. The magnitude of the biochemical changes was dependent on the shape of the gold nanoparticles: the elevations in liver enzymatic markers and total bilirubin were greater in the group treated with spherical gold nanoparticles than in the group treated with rod-shaped gold nanoparticles. **Conclusion:** It can be concluded that gold nanoparticles are promising vaccine cellular and humoral immune enhancers/adjuvants via different cytokine pathways.

Keywords: gold nanoparticles, rift valley fever virus, adjuvants, histological and immunohistochemical studies.

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INTRODUCTION

Vaccination is one of the most important ways to control healthcare costs and stimulate a protective immune response against infection in all countries. Adjuvant carriers can deposit antigens at the injection site, improve their appearance to immune-competent cells, stimulate T cells, improve antigen processing, and increase B cell antibody secretion. In the preparation of antibodies and vaccines against pathogens, nanoparticles (NPs) are used as carriers and adjuvants.^{1,2} NPs interact with the immune system and modulate its activity, resulting in immune stimulation, and have promising medical applications. These modulating effects may be beneficial or harmful.^{3,4} Gold nanoparticles (AuNPs) have the potential to be a useful tool in the development of successful vaccines against infectious diseases.⁵ AuNPs penetrate macrophages through receptor-mediated endocytosis and are found primarily in lysosomes and the perinuclear space, depending on their size and shape.⁶

The Rift Valley fever virus (RVFV), a member of the genus Phlebovirus in the Bunyaviridae family, causes Rift Valley fever (RVF). In the Arabian Peninsula and Sub-Saharan Africa, RVFV is a mosquito-borne zoonotic pathogen that causes serious outbreaks in humans and livestock. Fever is a symptom of human diseases, which may contribute to retinitis, encephalitis, hemorrhagic fever, and occasionally death.⁷ RVFV can be detected using a quantitative reverse transcription polymerase chain reaction (qRT-PCR) or an enzyme-linked immunosorbent assay that detects genomic segments (L, M, S)⁸ or surface and non-structural viral proteins⁹, respectively. These tests have a high sensitivity and specificity, but they necessitate the transport of unknown samples from the field to a laboratory, which increases the risk of virus transmission. Zaher *et al.*¹⁰ used unmodified gold nanoparticles (AuNPs) that change color in the presence of RVFV RNA to create a prototype point-of-care diagnostic test specific for RVFV detection, resulting in a simple but sensitive assay. The nanogold assay yields qualitative results that

demonstrate the presence of RVFV RNA in various sample types. With a detection limit of 10 RNA copies/reaction, the assay demonstrated high accuracy and specificity, comparable to quantitative reverse transcription polymerase chain reaction. The assay result could be determined in 30 minutes without the use of any special detection equipment. To protect ruminant populations, three approved veterinary vaccines against RVFV are used: inactivated virus vaccines, live attenuated virus vaccines, and alternative virus vaccines (Clone-13 live and MP-12 live) (OIE, 2015). In the beginning, formalin was used to suppress the virus in mice.¹¹ Beta-propiolactone (PL) is also commonly used in the development of viral vaccines as inactivating reagent.¹² Nearly 100 adjuvants are currently available or in production for approximately 400 vaccines.¹³ AuNPs are a popular antigen carrier used in vaccination and immunization.¹⁴ AuNPs are commonly used in biomedical research due to their unique physicochemical properties, ease of preparation, and low toxicity.¹⁵ We conducted this study to test the activity of spherical and rod-shaped AuNPs as immune enhancers of RVF vaccine.

METHODS

Cell culture

Buffalo green monkey cells (Vero) were kindly provided by the cell culture department, VACSERA, Giza, Egypt. The cell line was maintained according to the methods of Abdel-Gaied *et al.*¹⁶ and dispensed in TC plates and TC flasks (SPL-Korea) at 2×10^5 cells/ml.

RVFV strain

RVFV strain Menya/Sheep/258 was kindly provided by Dr. El Karamany, Former-G.M. of the research and development sector, VACSERA, Egypt. The infectivity titer was $7.5 \log_{10}$ /ml. RVFV seed stock was prepared according to the method of El-Karamany *et al.*¹⁷

Inactivation of RVFV

RVFV was inactivated by β PL (0.0035 M) supplied by Sigma-Aldrich (St. Louis, MO, USA). β PL-treated RVFV was incubated at 37°C for 2 h with continuous stirring. Samples were collected at 15-min intervals to evaluate the inactivation potential of β PL.

AuNPs

Spherical and rod-shaped AuNPs were purchased from Nanotech Company (6th October City, Giza, Egypt) at 1 mM final concentration and 20 nm size.

AuNPs were prepared at a final concentration of 40 μ M.

Laboratory animals

Forty male albino rats (body weight 75-80 g) were supplied from Theodore Bilharz Research Institute, Giza, Egypt. The rats were housed in both STST cages at VACSERA under standard breeding conditions and supplied with dry food and tap water, at a 12 h light/12 h dark cycle and a temperature of 25 °C.

Study design

The rats were randomly divided into four groups of 10 each: the negative control (NC) group, rats immunized with nonadjuvanted RVF vaccine (the Vac group), rats immunized with RVF vaccine combined with 40 μ M of spherical AuNPs (the AuNP-S group), and rats immunized with RVF vaccine combined with 40 μ M of rod-shaped AuNPs (the AuNP-R group).

Immune sera

Blood samples were collected from the retro-orbital plexus of the eye. The samples were kept at 37°C for 30 min for blood coagulation and then kept overnight at 4°C for retraction of the clot. The samples were then centrifuged for 15 min at 3500 rpm in a cold centrifuge (Jouan-ki22-Franc). Sera were removed in empty tubes and recentrifuged for another 10 min to remove any remaining blood clots and red blood cells. The collected sera were aliquoted and stored at -80 °C until use.¹⁸

Biochemical analysis

Serum samples from the immunized groups and NCs were processed for evaluation of liver function according to measurements of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total serum bilirubin (TB) using a standard colorimetric kit following the manufacturer's protocol (Biovision, Mountain View, CA, USA).¹⁹

Immunological studies: cytokine evaluation

The serum concentration of TNF- α was determined by direct enzyme-linked immunosorbent assay according to the method of Brouckaert *et al.*²⁰

Statistical analysis

All results were expressed as means \pm SD. Statistical analysis was performed by one-way analysis of variance according to the method of Dawson and Trapp²¹ using Statistical Package for the Social Sciences software version 25.

Histological and immunohistochemical studies

Fresh specimens of liver were taken from the control and treated groups. The specimens were fixed in 10% formal saline neutral buffer, washed, dehydrated in increasing concentrations of alcohol, cleared in xylene, and embedded in paraffin wax. Sections were prepared at 5- μ m thickness and stained with hematoxylin and eosin (H&E) for histological studies according to the method of Drury and Wallington.²² Another set of slides was processed for detection of caspase 9 and CD68 as immune markers according to the method of Tan *et al.*²³

RESULTS

Inactivation of RVFV

RVFV seed stock prepared in the Vero cell line was inactivated using 0.0035 M of β PL. Inactivation kinetics was determined relative to time (Fig. 1). RVFV was completely inactivated within 2 h after β PL treatment with a mean depletion of virus infectivity titer on the order of 1.2 \log_{10} /15 min.

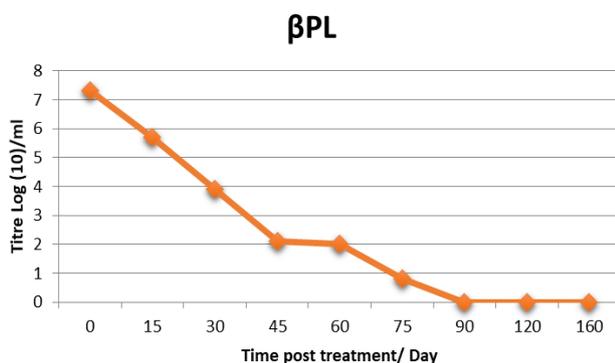


Figure 1. Inactivation kinetics of RVFV post treatment with β PL to time.

Biochemical analysis

Compared with rats receiving no treatment (the NC group) and rats treated with nonadjuvanted vaccine (the Vac group), rats treated with vaccines combined with AuNPs exhibited toxic biochemical changes, as shown by significant elevations in liver enzymatic markers (ALT and AST) and TB ($P < 0.05$) after vaccination in the AuNP-S and AuNP-R groups (Figs. 2–4). The magnitude of the biochemical changes was dependent on the shape of the gold NPs: the elevations in ALT, AST, and TB were greater in the group treated with AuNP-S (spherical NPs) than in the group treated with AuNP-R (rod-shaped NPs). The elevations in ALT,

AST, and TB peaked on day 45 after vaccination and then declined. The biochemical changes were indicative of damage to the plasmalemma of the hepatocytes, resulting in leakage of the enzymes in the liver.

Immunological results

Levels of TNF- α as a proinflammatory marker were detected on day 8 after vaccination and were significantly elevated ($P < 0.05$) in the AuNP-S and AuNP-R groups (Fig. 5). The elevation was significantly greater in the AuNP-R group than in the AuNP-S group and was significantly reduced on days 60 and 95.

Histological and immunohistochemical changes in liver tissue

Histological studies

H&E stain was used to detect hepatic cellular changes. The NC group showed normal hepatic architecture; the hepatic lobule structure of the liver tissue was normal and the central vein was visible. The hepatocytes were arranged radially around the central vein, the structure of the hepatic sinus was clear, and there were no pathological changes (Fig. 6A). In contrast, the normal lobular structure of the liver tissue was changed in all treated rat groups; the greatest effect was detected in the AuNP-R group. There was a large amount of congestion in some hepatic sinuses and the central vein of the liver, and the hepatic sinus was clearly dilated. A large number of inflammatory cells were infiltrated, and the number of Kupffer cells was increased. Some hepatocytes showed dense cytoplasm and dark nuclei, and others showed vacuolated cytoplasm. There were numerous apoptotic figures (pyknosis and karyorrhexis), and some binucleated cells were observed (Fig. 6B–D).

Immunohistochemical studies

Immunohistochemical staining of prepared slides for detection of caspase 9 apoptosis was scored as (–) for normal sections in the NC group, (+3) moderate in the Vac group, (+2) mild in the AuNP-S group, and (+4) highly positive in the AuNP-R group (Fig. 7A–D).

CD68, a widely used monocyte/macrophage lineage marker, was positive in all treated groups and showed a weak positive or negative reaction in the NC group (Figure 8A–D).

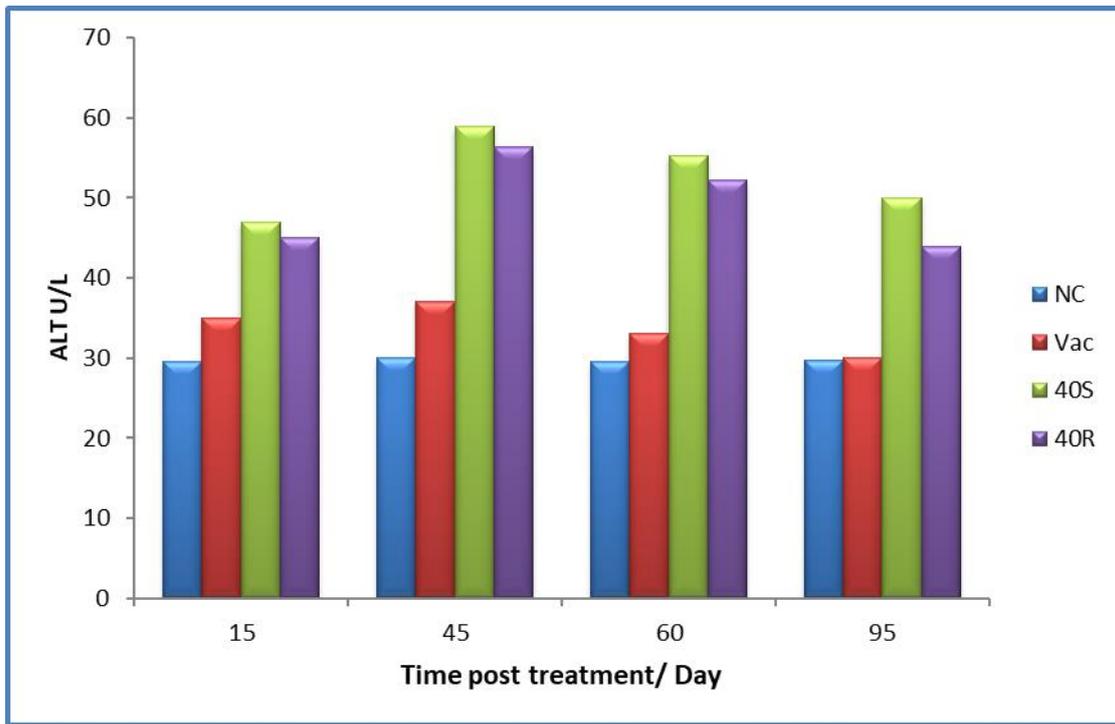


Figure 2. Effect of non-adjuvanted (Vac) and AuNPs adjuvanted vaccine on the activity of ALT.

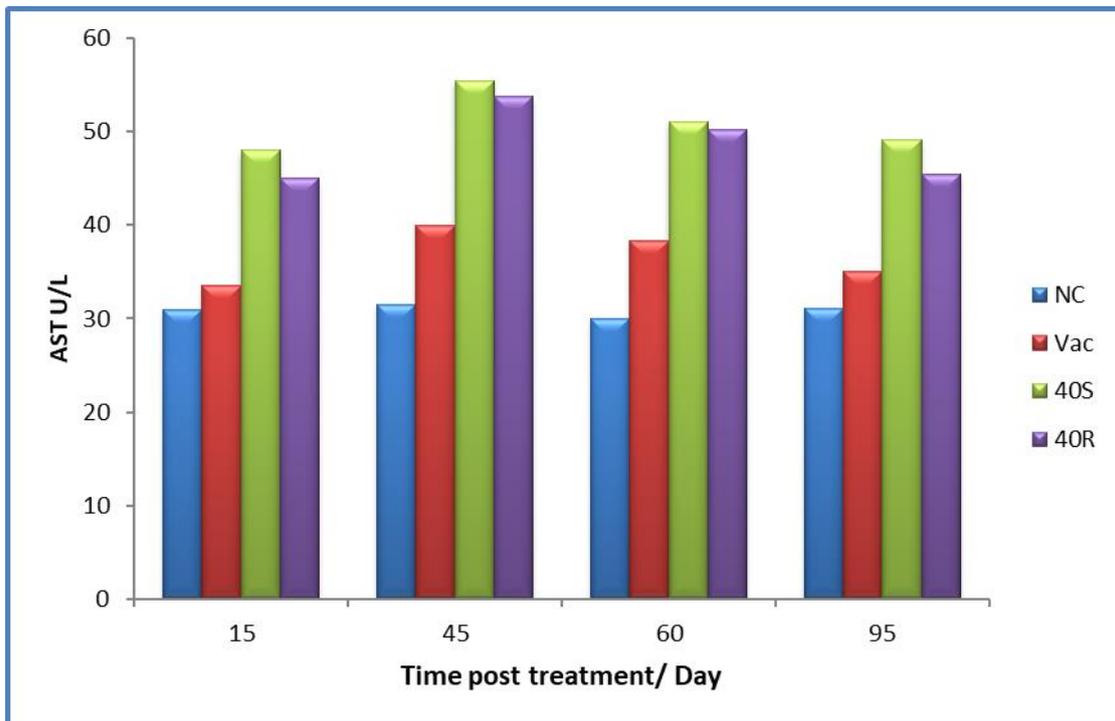


Figure 3. Effect of non-adjuvanted (Vac) and AuNPs adjuvanted vaccine on the activity of AST.

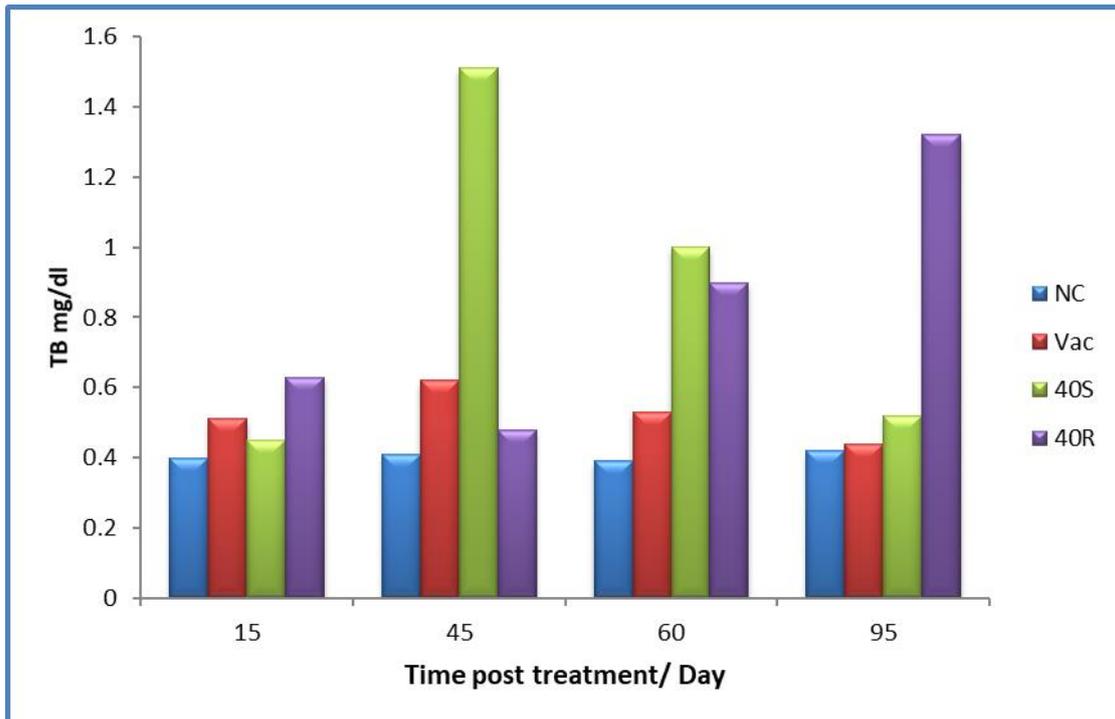


Figure 4. Effect of non-adjuvanted (Vac) and AuNPs adjuvanted vaccine on the activity of TB.

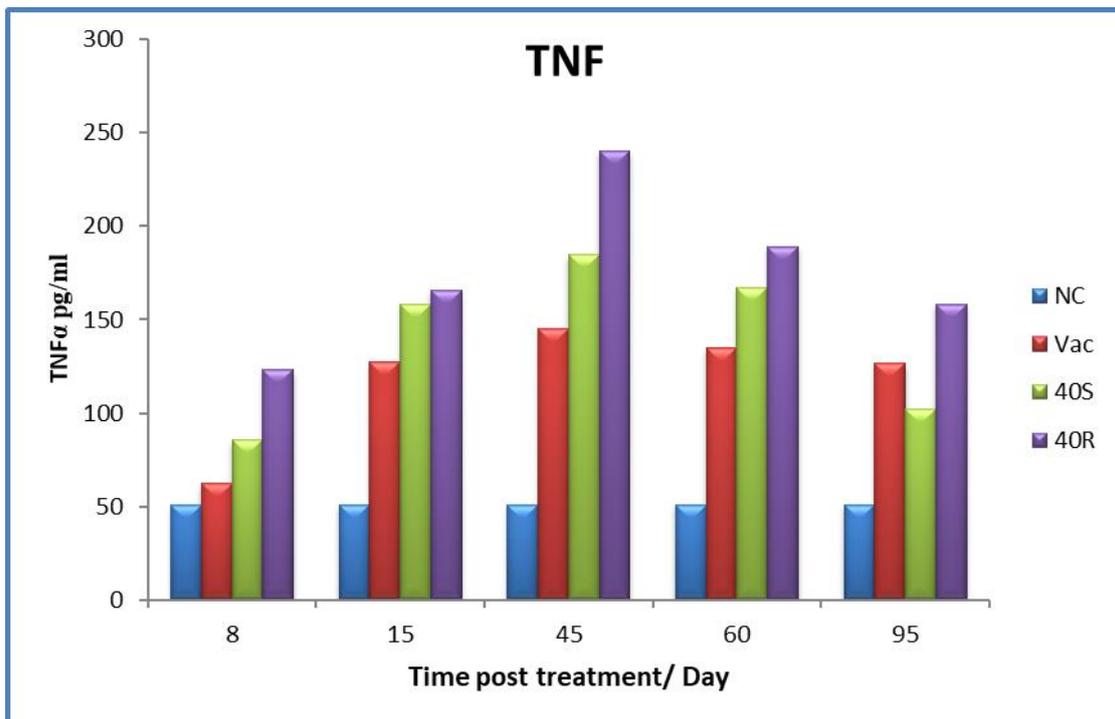


Figure 5. Effect of Vac (nonadjuvanted group), AuNPs (S&R) (adjuvanted groups) on TNF- α level.

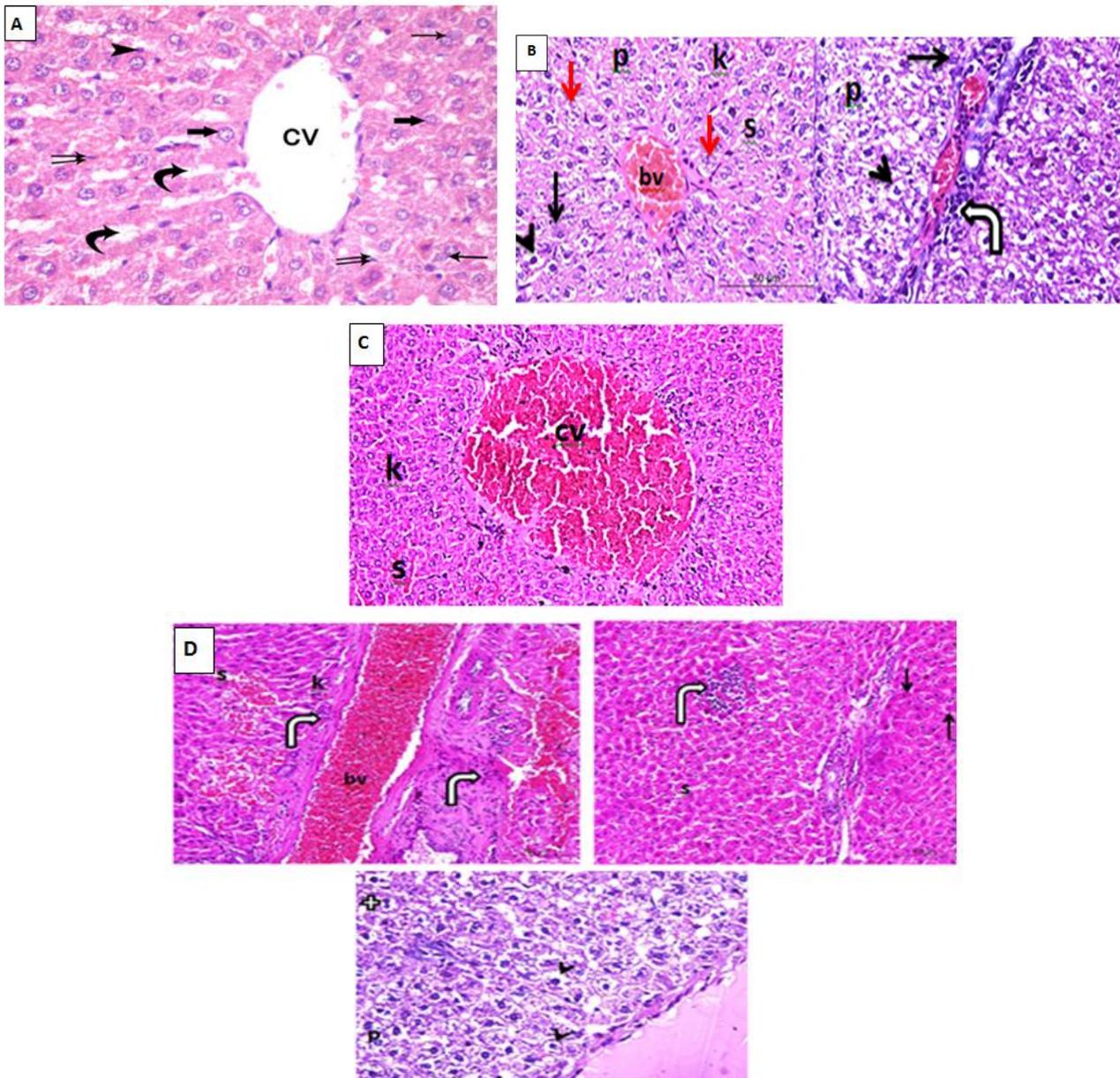


Figure 6. (A) Photomicrograph of rat liver of NC group showing, normal hepatocytes (rounded or polyhedral in shape) contain central rounded vesicular nuclei (thick arrows) and sinusoids (curved arrows). Kupffer cells (arrow head) lining sinusoids, few cells are binucleated (thin arrows) and endothelial (double arrows) cells lining are noticed between the hepatocyte plates (H & E stain X 400). (B): Photomicrograph of rat liver of Vac group showing congested blood vessel with RBCs (bv) and dilated blood sinusoids (s). There are numerous apoptotic figures (pyknosis; p and karyorrhexis; k; red arrows). Hepatocytes show cytoplasmic vacuolation (arrow head) and Kupffer cells (k) are increased in number. There is inflammatory cell infiltration (bent arrow) in the periportal area. Some hepatocytes are with dense cytoplasm and dark nuclei (black arrow) (H&E, X400). (C): Photomicrograph of rat liver of 40S group showing the central vein (cv) and sinusoids (s) are congested with RBCs. The Kupffer cells (k) are increased in number (H&E X400). (D): Photomicrograph of rat liver of 40R group, showing, inflammatory cell infiltration (bent arrow), congested blood vessels (bv), dilated blood sinusoids (s) with increasing number of Kupffer cells (k). Some hepatocytes are with dense cytoplasm and dark nuclei (arrow). Hepatocytes show vacuolated cytoplasm (arrow head), pyknotic nuclei (p) and few hepatocytes are binucleated (plus) (H&E X400).

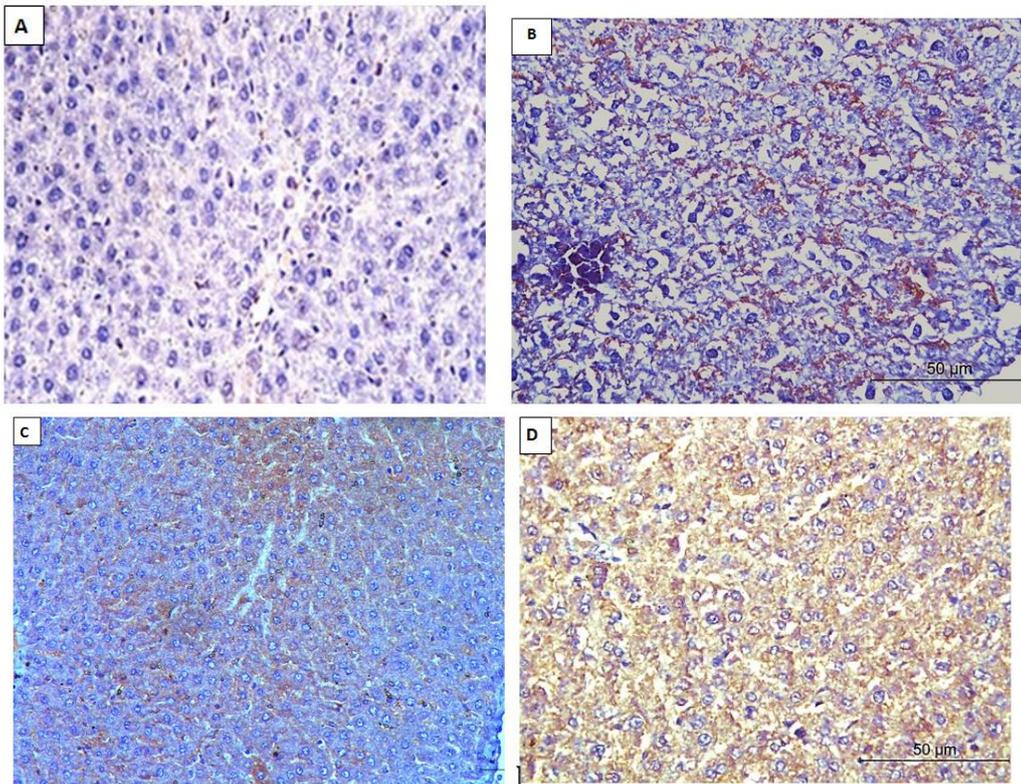


Figure 7. (A): Immunohistochemical staining of caspase 9 in liver tissues of NC group, (B): Immunohistochemical staining of caspase 9 in liver tissues of Vac group showed moderate cytoplasmic caspase 9 positivity (+++), (C): Immunohistochemical staining of caspase 9 in liver tissues of 40S group showed mild caspase 9 positivity (++), (D): Immunohistochemical staining of caspase 9 in liver tissues of 40R group showed highly positive (++++) for caspase 9.

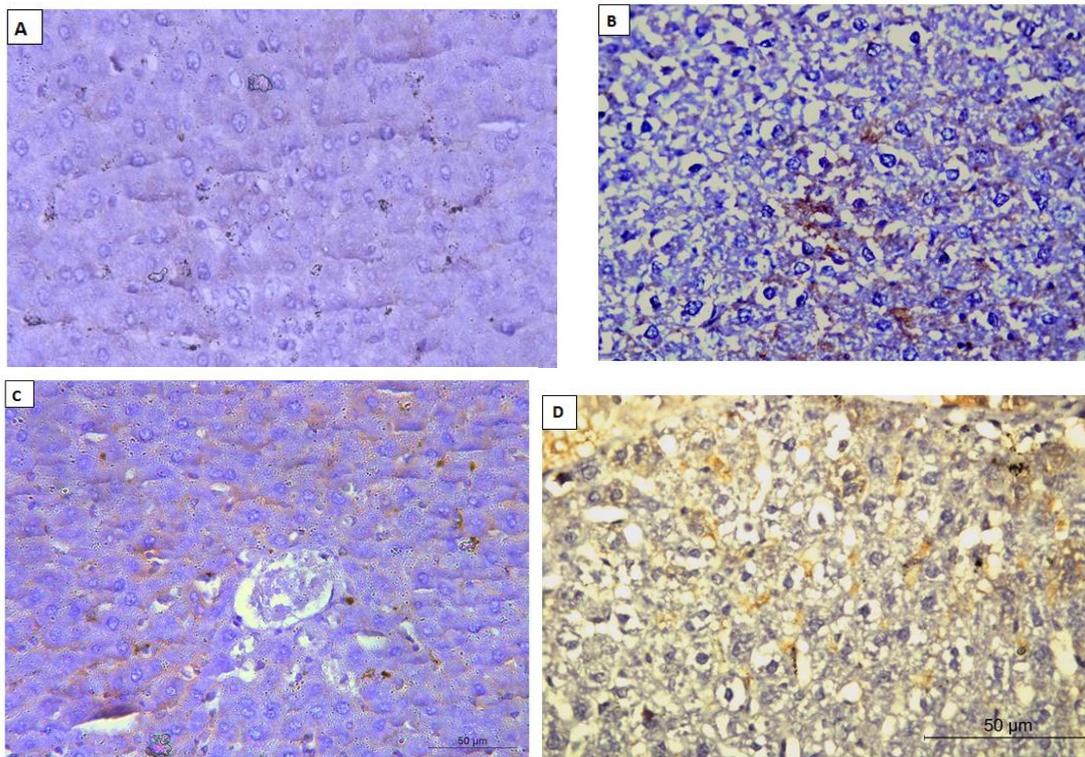


Figure 8. (A): Immunohistochemical staining of CD68 in liver tissues of NC group showed weak positive or negative reaction. (B): Immunohistochemical staining of CD68 in liver tissues of Vac group mild positive reaction. (C): Immunohistochemical staining of CD68 in liver tissues of 40S group moderate positive reaction. (D): Immunohistochemical staining of CD68 in liver tissues of 40R group strong positive reaction.

DISCUSSION

Because of their promising chemical and physical properties, AuNPs have piqued researchers' interest in recent years. AuNPs are noncorrosive and, depending on their size and shape, have optical, electronic, and biological properties that have made them useful in a variety of biomedical applications. They have been found to boost immunity.²⁴ Gold nanospheres, nanorods, nanobelts, nanocages, nanoprisms, and nanostars are some of the shapes that AuNPs can be made in.²⁵ The interaction of AuNPs with immune cells is extremely important. AuNPs can also be used for immunomodulation and targeted drug delivery.²⁶

Nanoparticles (NPs) are perfect for delivering antigens, acting as adjuvant platforms, and mimicking viral structures, and they play a big role in vaccine development. In this study we used AuNPs as enhancers to maximize the immune response to RVF vaccine and preparation of inactivated vaccine was performed using β PL. Within 2 hours of treatment, RVFV was completely inactivated, and the vaccine potency (ED50) was 0.0163 ml, which was in line with WHO recommendations that the ED50 be less than 0.02 ml and Soliman et al.²⁷ findings. In previous studies Al-Olayan et al.²⁸ reported that β PL had higher vaccine potency than formalin and ascorbic acid inactivants. This was due to β PL minimal effect on the viral epitope configurations that are responsible for antigenicity, as well as the fact that β PL had no harmful influences on the immune response. Emarat et al.²⁹ also showed that β PL rapidly inactivated rabies virus within 2 hours, with a depletion rate of 1.8 log₁₀/15 min and an ED50 of >2.5 IU/dose, as recommended by WHO.

The use of AuNPs has been linked to liver toxicity and injury, as evidenced by elevated serum ALT and AST levels. Hepatocytes produce these enzymes, which are released into the bloodstream following hepatocellular injury and cholestasis. In our study, the levels of these enzymes were higher in the groups receiving vaccines combined with AuNPs than in the NC group. ALT, AST, and TB activities were more elevated after administration of AuNP-R than after administration of nonadjuvanted vaccine or AuNP-S. Other researchers found elevated liver enzymes after being exposed to AuNP.³⁰ In another research El Sayed and Mohamed³¹ found that the degenerative effect of AuNPs on liver cells significantly increased serum levels of ALT and AST in rats treated with 20 μ M of spherical and 40 μ M of rod-shaped AuNPs. In addition, when 10 and 50 nM AuNPs were administered, AST levels increased when compared

to controls.³² Similarly, AST and ALT levels were significantly higher in animals receiving AuNPs compared to the control group two days after intervention, according to Doudi and Setorki.³³ In the current study TNF- α levels were significantly higher in all treated groups on day 8 compared to the NC group, and significantly lower on days 60 and 95 ($P < 0.05$). The highest TNF- α level was detected after administration of AuNPs-R compared with AuNP-S and nonadjuvanted vaccine. Large amounts of TNF- α were secreted in response to a wide variety of inflammatory stimuli, suggesting a potential role for this cytokine in the increased activation of the peripheral immune system. In accordance with the current findings, Kenichi et al.³⁴ concluded that rod-shaped AuNPs uptake into cells was as effective as spherical or cubical AuNPs uptake and released significant amounts of inflammatory cytokines such as TNF- α , IL-6, IL-12, IL-10, and granulocyte macrophage colony-stimulating factor (GM-CSF). When rabbits were immunized with 50 μ g of the HIV-1 Gag p17 peptide conjugated to 2-nm gold glycol-NPs, the proliferation of HIV-specific CD4+ and CD8+ T cells increased and secretion of the highly functional TNF- α and IL-1 β cytokines was induced as compared with administration of the unconjugated peptide.³⁵ AuNPs combined with CpG ODNs considerably enhanced the intracellular penetration of NPs into macrophages and the secretion of the proinflammatory cytokines TNF- α and IL-6, according to Wei et al.³⁶ Stone et al.³⁷ investigated the relationship between NP size and shape and immune response. Liu et al.³⁸ reported that NPs had a low cytotoxic effect on immune cells but significantly stimulated the release of TNF- α , which is essential in the removal of abnormal cells. In accordance with our findings, Bastús et al.³⁹ found that AuNPs enhanced the induction of proinflammatory cytokines such as TNF- α , IL-1, and IL-6 in macrophages. Malaczewska⁴⁰ found that mice given AuNPs orally had increased phagocyte activity and some changes in lymphocyte phenotypes, including a higher percentage of B and CD4+/CD8+ double-positive T cells. The lowest dose increased the synthesis of the proinflammatory cytokines IL-1, IL-2, IL-6, and TNF- α , and had a proinflammatory or immune-stimulating effect. PVP-AuNPs stimulated the synthesis of proinflammatory TNF- α , according to a recent study.⁴¹ TNF- α and IL-6 are cytokines produced by macrophages that affect the systemic inflammatory state.⁴² The current study's histological findings largely corroborate the results of biochemical and immunological tests. When compared to the NC

group, the groups receiving nonadjuvanted vaccines and vaccines combined with AuNPs had a lot of histological changes in their liver tissues. After receiving 40 μ M of AuNP-RVF vaccine, damaged tissues seemed to return to their usual pattern. Histological changes included large numbers of infiltrated inflammatory cells. Guo et al⁴³ found that giving mice NPs intravenously caused hepatic, renal, and pulmonary inflammation, implying that NPs have their own mechanisms for causing peripheral inflammation. The activation of Kupffer cells resulted in an increase in the number of Kupffer cells in our study. The activation of Kupffer cells has been linked to the toxic potential of AuNPs.⁴⁴ Hepatic sinuses and central vein congestion were also caused by the administration of AuNPs. Binucleated cells which found in our study, indicating the regeneration process, as stated by Abdelhalim and Jarrar.⁴⁵ Also dense cytoplasm, dark nuclei, and vacuolated cytoplasm were found in some hepatocytes, which may suggest AuNP-induced acute and subacute liver injury. There were a lot of apoptotic figures as well (pyknosis and karyorrhexis). These observed changes were confirmed by Patlolla et al,⁴⁶ who stated that as compared to the NC, uncoated and PEG-coated AuNPs caused major morphological changes, such as damage to the central vein; vacuolation in hepatocytes, pyknosis, or karyomegaly; and condensed nuclei of hepatocytes and necrosis. Hepatocyte swelling can be caused by disruptions in membrane function caused by AuNPs, resulting in a large influx of water and Na⁺, as well as leakage of lysosomal hydrolytic enzymes, cytoplasmic degeneration, and macromolecular crowding.⁴⁷ Ibrahim et al⁴⁸ concluded that the histopathological changes in the liver of AuNPs-treated mice consisted of steatosis, micro- and macrovesicles, cytoplasmic degeneration, necrotic foci, activation of Kupffer cells, hemorrhage, and infiltration of inflammatory cells were similar to our findings. Macrophages and dendritic cells absorbed more nanorods, resulting in increased IL-1 β and TNF- α output.⁴⁹ In line with our findings, Doudi and Setorki³³ showed that spherical AuNPs caused significant changes in the histopathology of liver hepatic damage and the aggregation of basophilic cells around central vein tissues in all treated groups. Another study found that after receiving AuNPs, which have direct effects on liver function, the liver could be slightly impaired.³² Hwang et al⁵⁰ also published research on healthy and impaired rodent livers during AuNPs-induced hepatotoxicity. Cloudy swelling, vacuolar

degeneration, hyaline droplets and casts, karyorrhexis, and karyolysis were all observed after exposure to AuNP doses. Hepatocyte swelling may also be caused by a malfunction of the cell membrane caused by the large influx of water and Na⁺ caused by AuNPs.⁵¹ The pathological changes are related to the time between doses: AuNPs administered every 48 hours demonstrated possible therapeutic benefits without toxicity, while AuNPs administered every 24 hours caused significant parenchymal changes, leukocyte infiltration and cell necrosis.⁵² Citrate- and pentapeptide-coated AuNPs (20 nm, 700 g/kg) were rapidly extracted from the bloodstream and accumulated primarily in the liver after injection in rats.⁵³ A previous study proposed that silymarin-coated AuNPs could be used to treat CCl₄-induced liver injury and cirrhosis.⁵⁴ In our study, immunohistochemical staining of liver sections for detection of caspase 9 apoptosis in NC group was rated as (-), (+3) moderate in the Vac group, (+2) mild in the AuNP-S group, and (+4) strongly positive in the AuNP-R group. These results matched those of Baharara et al⁵⁵, who looked at apoptosis in HaCaT cells induced by PEG-decorated rod-shaped AuNPs and mercaptopropylsulfonate (MPS)-decorated spherical AuNPs. As a result, hexagonal and rod-shaped AuNPs seem to be more likely to cause apoptosis than spherical AuNPs. In Calu-3 epithelial cells, triangular, circular, and hexagonal AuNPs caused higher levels of reactive oxygen species and proapoptotic markers, such as caspase 3 and caspase 9, according to Tian et al.⁵⁶ AuNPs induced apoptosis in MCF-7 breast cancer cells through the p53, bax/bcl-2, and caspase pathways (caspase 3 and 9).⁵⁷ According to another study, quercetin AuNP enhanced caspase activation (caspase 3 and 9).⁵⁸ PEG-coated AuNPs induced acute inflammation and neutrophil influx in the mouse liver, and the percentage of cells undergoing apoptosis in the liver increased on day 7 after treatment.⁵⁹ Caspase 9 acts as an initiator caspase that is activated by death-inducing tumor necrosis family receptors and cytochrome C, both of which are involved in immune and apoptosis signaling.⁶⁰ In a variety of pathophysiological settings, apoptosis, or programmed cell death, is a critical activity in mammalian cells. It is in charge of getting rid of unwanted cells.⁶¹ CD68 was found to be positive in all of the treated groups in the current study: it was mildly positive in the Vac group, moderately positive in the 40 AuNP-S group, and strongly positive in the AuNP-R group. The increase in CD68 in the treated groups' serum

explained the increase in TNF- α level. This may be because the nanorods were targeted to the tissues at the same rate as the nanospheres, and Alfranca et al.⁶² came to the same conclusion. The induction of TNF- α may be related to the curvature of the sphere; the elongated nanorods have a larger surface area in contact with the surface of the endothelial cells, so more of the antibodies that coat the nanorod will attach to receptors on the surface of endothelial cells, resulting in more efficient cell adhesion and a greater impact on the target tissues.⁶³ Rod AuNPs were more easily taken up by tumor cells, resulting in particle distribution in the tumour.⁶⁴ Bartucci et al⁶⁵ corroborated these observations. Previous studies have shown that AuNP administration causes a mild acute hepatic inflammatory response and apoptosis.⁵⁹ Different kinds of NPs are used as delivery mechanisms, and they induce alterations in macrophage phenotypes that affect the disease's progression. By inhibiting regulatory pathways activated by macrophages, lipids and polymeric NPs as RNAi vectors can have therapeutic effects.⁶⁶ Adult albino rats exposed to AuNPs for a long time developed histological changes, with an immunohistochemical analysis revealing a substantial increase in the number of CD68-positive cells.⁶⁷

CONCLUSION

AuNPs are promising vaccine cellular and humoral immune enhancers/adjuvants via different cytokine pathways. The immune response depends on the shape of the AuNPs, which influences the activation of dendritic and other antigen-presenting cells. For a better safety measure, further long-term studies involving several apoptotic signaling pathways and other biomarkers based on transcriptomics and proteomics should be conducted.

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CONFLICT OF INTEREST

The authors declare that there is no Conflict of interest. The authors alone are responsible for the content and writing of this article.

ETHICAL USE OF ANIMALS IN RESEARCH

All applicable international, national, and institutional guidelines⁶⁵ for the care and use of animals were followed. We respected the welfare of animals, and excluded situations when animals were in pain.

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