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ASSESSMENT OF NEUROLOGICAL TOXICITY OF GOLD SPHERE AND ROD NANOPARTICLES USED AS ADJUVANT TO RIFT VALLEY FEVER VIRUS VACCINE (RVFV) AND RELATED PHYSIOLOGICAL CHANGES: *IN VIVO* STUDY

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ABSTRACT
Nanotechnology is a promising, rapidly growing discipline, and many research efforts have so far been focused on using nanoparticles as agents for targeted drug delivery. Although aluminum (Alum) salt is renowned as a vaccine adjuvant against Rift Valley Fever Virus (RVFV), gold nanoparticles (AuNPs) can be tested as alternative adjuvants to avoid associated alum disadvantages. This study was conducted using different molar concentrations of spherical and rod gold nanoparticles (AuNPs); 20 and 40 µM, those were subcutaneously injected into rats as an RVFV vaccine adjuvant to determine the subsequent blood and brain biochemistry changes in concomitant to neurotransmitters alterations. Interestingly, the present study indicated that the utilization of AuNPs adsorbed RVFV vaccine improved the blood and brain physiological changes as well as neurotransmitters disturbances resulted in the alum-adsorbed. Spherical AuNPs were much safer than rod NP with special attention for the NP concentration, therefore further detailed studies are required.

KEYWORDS: Gold nanoparticles, Rift Valley Fever Virus, neurotransmitters, antioxidants.

INTRODUCTION
Nanotechnology is a novel and rapidly growing research area that has caused profound changes in many scientific fields. Recently, there has been a stunning effort for the development of nanotechnology in the field of fundamental biomedicine, as well as some clinical disciplines that have introduced a profusion of nanoparticles.[1] Nanoparticles (NPs) are materials that characterized by their tiny size which is less than 100 nanometers that make it small enough to access almost every part of the human body and some of them have access to the blood-brain barrier.[2] Nanoparticles display unique, superior and indispensable properties that arise specifically from their higher surface to volume ratio and increased the percentage of atoms at the grain boundaries.[3] Over the past few decades, NPs have been attained a considerable interest as effective drug delivery devices.[4] It can target pathophysiological sites, avoiding unnecessary influence on healthy tissues so they are able to deliver drugs to hard-to-target and/or extremely sensitive sites such as the brain.[5] Several types of nanoparticles have been successfully designed and applied as efficient drug carriers.[6] Gold nanoparticles (AuNPs) are the most commonly used nanomaterials in biosensing, biolabeling and are expected to have a wide range of applications in the future.[7] AuNPs have received special attention because of their excellent stability, non-toxicity, non-immunogenicity and high tissue permeability without hampering cell functionality.[8] Therefore, AuNPs have attracted great attention as a novel carriers for delivery of therapeutic substances to the brain across the blood-brain barrier (BBB).[9] The properties of shape, size, and surface chemistry play important roles in mediating the physiological behaviors of AuNPs: blood circulation, targeting, distribution, translocation, metabolism, clearance and inflammation in vivo and cellular pathways in vitro.[10]

Rift Valley Fever Virus (RVFV) is an important arbovirus that lacking attention in recent years. It is a mosquito-borne disease that belongs to the genus *Phlebovirus* of the *Bunyaviridae* family and mainly affects ruminants and humans. Severe outbreaks caused by RVFV results in serious socio-economic and public health impact for endemic communities of the African continent and the Arabian Peninsula.[11] The RVFV infection is characterized by high rate of abortion, the high mortality rate in young animals and significant mortality in adult animals. However, human infections are characterized by fever and sometimes leading to encephalitis, retinitis, hemorrhagic fever and

occasionally death.[12] Human infections have been reported in much of Africa, Saudi Arabia, Egypt and Yemen, with recent outbreaks in Kenya.[13]

Vaccination of livestock would be the most practical way of preventing the disease in animals and its spread to humans in RVF-endemic areas. The basis underlying vaccination is the theory of stimulating the body's immune response that provides protection against the pathogens and prevents the prolonged immunosuppression.[14] Several vaccines and vaccine candidates have been developed against RVF virus, but none are yet approved for public use outside endemic areas.[11] Currently, there are no available licensed vaccines for humans and the widely used live attenuated RVFV vaccines for livestock in Africa have major drawbacks that including abortogenic and teratogenic properties, inadequate immunogenicity and an underlying opportunistic infection.[15] An effective vaccine usually requires administration of an adjuvant to enhance its immunogenicity while keeping the injected foreign material at a minimum dose.[16] Adjuvants have been necessary to promote the uptake of antigens by antigen presenting cells (APC), contribute to the delivery of antigen to lymph nodes and stimulate cytokine release or expression of co-stimulatory signals on APC which are needed to prime T helper cells for B cell proliferation and induction of cytotoxic T lymphocytes.[17] A wide variety of compounds has been evaluated as adjuvants such as saponins, mineral oil and aluminum salts. Currently, the most commonly used adjuvants are the aluminium (alum) based adjuvants that were found to be associated with several disadvantages as the severity of local tissue irritation, the longer duration of the inflammatory reaction at the injection site, minimal induction of cell-mediated immunity, elicit undesirable immunoglobulin E (IgE) responses and increase the undesirable homocytotropic antibodies in animal species.[18] Furthermore, alum-based vaccines are frequently ineffective for the induction of antiviral and anticancer immunity.[19] For these reasons, the potential for introducing of an adjuvant for RVFV vaccine are needed to develop as a better way to target the virus and reduce the vaccine amount. AuNPs can be considered as alternative adjuvant because of its unique properties that can overcome some of the limitations found in traditional vaccines.[20] Unfortunately, there is no scientific data regarding the association of gold nanoparticles with rift valley fever medications, as well as the overall feasibility of this kind of drug delivery platforms as most of the studies on gold nanoparticles (AuNPs) has mainly been focused of brain cancer medications. Therefore, it is quite possible that the current study can provide independent and baseline information on using gold nanoparticles as RVFV vaccine adjuvant by studying the biochemistry of blood and brain as well as neurotransmitters changes.

MATERIALS AND METHODS

Rift Valley fever virus strain

RVFV; Pan Tropic-Menya Strain (Menya /Sheep/258) was kindly provided by Dr. Aly Fahmy, Head of R &D sector, VACSERA, Giza, Egypt. RVFV was of an infectivity titer in the order of $10^{7.5}$/ml.

Maintenance of cell line and seed stock preparation

Baby Hamster Kidney cells (BHK), kindly provided by cell culture department, virology sector, VACSERA-Egypt. The cell line was maintained according to[21] and suspended to the proper concentration of $2x10^5$ cells/ml. Virus seed stock was prepared according to.[22]

Inactivation of RVFV using Beta-propiolactone (βPL)

Beta-propiolactone (βPL) were purchased from (Sigma - Aldrich, USA) and prepared as 0.0035 M[21] for use in RVFV inactivation.

Preparation of adjuvants

Sphere and rod gold nanoparticles adjuvants

Sphere and rod gold nanoparticles were purchased (Nano Tech company, 6th October City -Giza -Egypt) as 1 mM final concentration. AuNPs were diluted to a final concentration of 20 and 40 µM.

Aluminum phosphate (Alum) adjuvant

Alum was prepared according to[23] and the pH was adjusted between 6.5-6.8.

Animals

Thirty-five male albino rats of Sprague-Dawley strain, weighing 80-90 gm were obtained from Theodore Bilharz Research Institute, Giza, Egypt. Animals were kept under constant environmental conditions at room temperature, fed a standard commercial diet (balanced diet) and water was available *ad-libitum* for acclimatization one week before onset of the experiment. The guidelines for the ethical care and treatment of the animals followed the regulations of the Animal Care and Use Committee (ACUC).

Study design

Sprague Dawley albino rats were randomly divided into seven groups (5/each); group (1) served as the negative control, the other six groups were immunized subcutaneously by 0.5 ml RVFV/rat as 2 doses at 30 days intervals. However, group (2) received only RVF vaccine, group (3) received RVF vaccine conjugated with aluminum phosphate (Alum). Meanwhile, groups (4) and (5) received RVF vaccine conjugated with the sphere AuNPs as 20µM and 40µM, however, groups (6) and (7) received RVF vaccine conjugated with the Rod AuNPs in the same concentrations.

Animals of all groups were kept fasting for 12 hours before decapitating. Blood sera were separated using cold centrifugation (Jouan, KI-22 - France) post-coagulation and preserved at -80 °C till being used for further biochemical analysis. Brains were quickly

excised and placed in iced normal saline, perfused with the same solution to remove blood cells, blotted on filter paper, weighed and quickly frozen at -80°C until used.

Preparation of tissue homogenate

The frozen brains were cut into small pieces and homogenized in 5 ml cold phosphate buffer saline (pH 7.4) per gram tissue, centrifuged at 11000 rpm for 15 minutes at 4°C and the supernatant was collected for parameters estimation.

Biochemical assays

Blood and brain total protein (TP)[24] and albumin[25] concentrations were determined while globulins concentrations were calculated by subtraction of albumin from TP values. Also, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were assayed according to[26] while alkaline phosphatase (ALP) according to.[27] Also, blood urea and creatinine concentrations were determined.[28,29] In particular, blood and brain malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity were measured according to.[30,31,32] respectively. All kits were purchased from Biodiagnostic, Egypt.

Determination of brain monoamines

Brain norepinephrine (NE), dopamine (DA) and serotonin (5-HT) were measured by HPLC with little modification.[33] Separation was achieved on ODS-reversed phase column (C18, 25 x 0.46 cm i.d. 5µm). The mobile phase consisted of potassium phosphate buffer/ methanol 97/3 (v/v) and was delivered at a flow rate of 1.5 ml/min. UV detection was performed at 270 µM and the injection volume was 20µl. The concentration was determined by an external standard method using peak areas. Serial dilutions of standards were injected, and their peak areas were determined. A linear standard curve was constructed by plotting peak areas versus the corresponding concentrations. The concentration in samples was obtained from the curve.

Statistical Analysis

All data were presented as a mean ± standard error. Data analysis was performed with one-way ANOVA using SPSS (Version 23). Post hoc test was used to assess differences between means. A significant difference for all statistical analysis in this study was considered at the level of $P \leq 0.05$.

RESULTS

Blood and brain biochemistry Analysis were performed for the control and all treated groups to investigate the effect of using alum and gold nanoparticles as RVFV vaccine adjuvants. No significant changes were detected in the levels of serum TP in all vaccinated groups except for the alum-treated one that revealed a significant ($P< 0.05$) reduced TP compared to the serum of control group [Fig.1]. However, brain homogenate TP levels showed a significant decrease ($P< 0.05$) in all treated groups except for 40µM (sphere or rod) AuNPs treated groups that were within the normal control values [Fig.2].

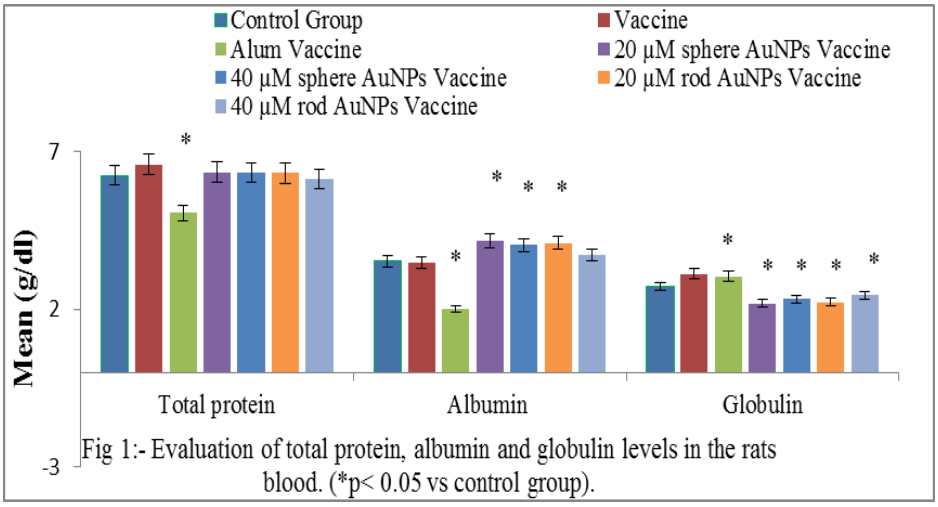

Fig 1:- Evaluation of total protein, albumin and globulin levels in the rats blood. (*p< 0.05 vs control group).

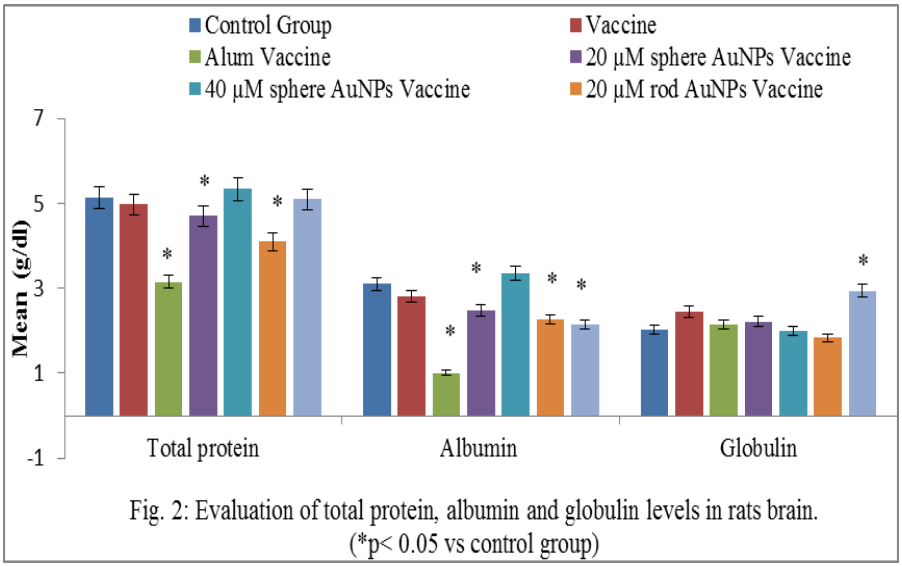

Fig. 2: Evaluation of total protein, albumin and globulin levels in rats brain. (*p< 0.05 vs control group)

On the other hand, serum albumin exhibited a significant (P< 0.05) increase post gold nanoparticles administration excluding for the 40µM rod AuNPs group that values very close to those of the control group. Meanwhile, a significant (P< 0.05) reduction was detected in the brain albumin in all treated groups except for 40µM sphere AuNPs groups that were not significantly different from those of the control values [Fig.2]. Moreover, all AuNPs treated groups showed a significant (P< 0.05) decrease in serum globulins levels whereas the brain globulins level increased in 40µM rod AuNPs treated group but remained unchanged in the other AuNPs treated groups.

Serum ALT, AST, and ALP levels recorded a drastic elevation (P< 0.05) in the alum-adsorbed group [Fig.3]. Both of 40µM sphere and 20µM rod AuNPs groups ALT and AST values were within the normal ranges relative to control group, however,20µM sphere and 40µM rod AuNPs groups were significantly increased. Meanwhile, ALP values were significantly (P<0.05) reduced post AuNPs administration excluding 20µM rod AuNPs group that showed an increase in ALP level as compared to control [Fig.3]. Similarly, the brain ALT values were statistically (P< 0.05) elevated in both the alum and 40µM rod AuNPs groups but reduced for all other treated groups as compared to control [Fig.4]. A significant (P< 0.05) elevation was recorded in levels of brain AST in all treated groups whereas the brain ALP levels were also elevated except for both of the 40µM sphere and 20µM rod AuNPs groups as their values non-significantly reduced compared to control group value [Fig.4].

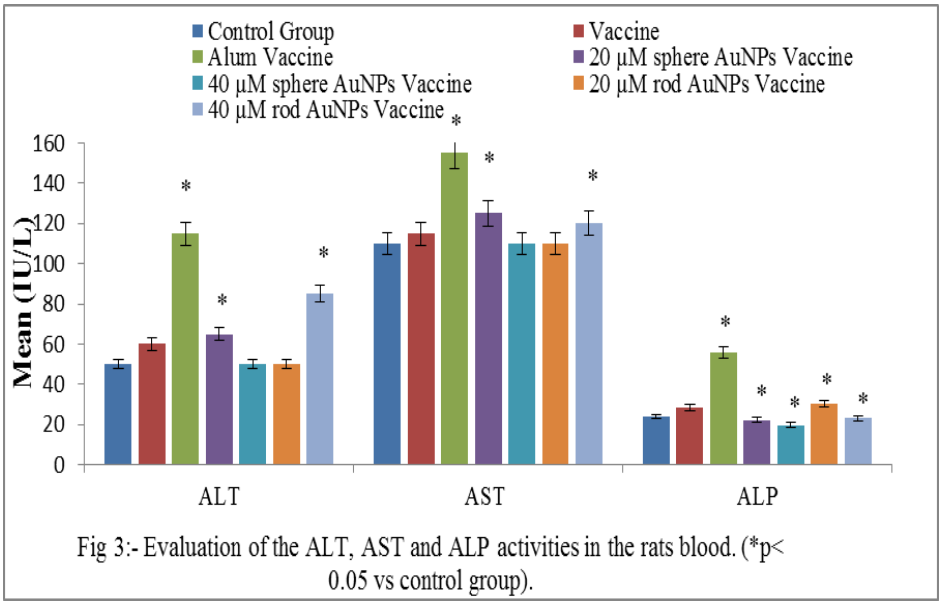

Fig 3:- Evaluation of the ALT, AST and ALP activities in the rats blood. (*p< 0.05 vs control group).

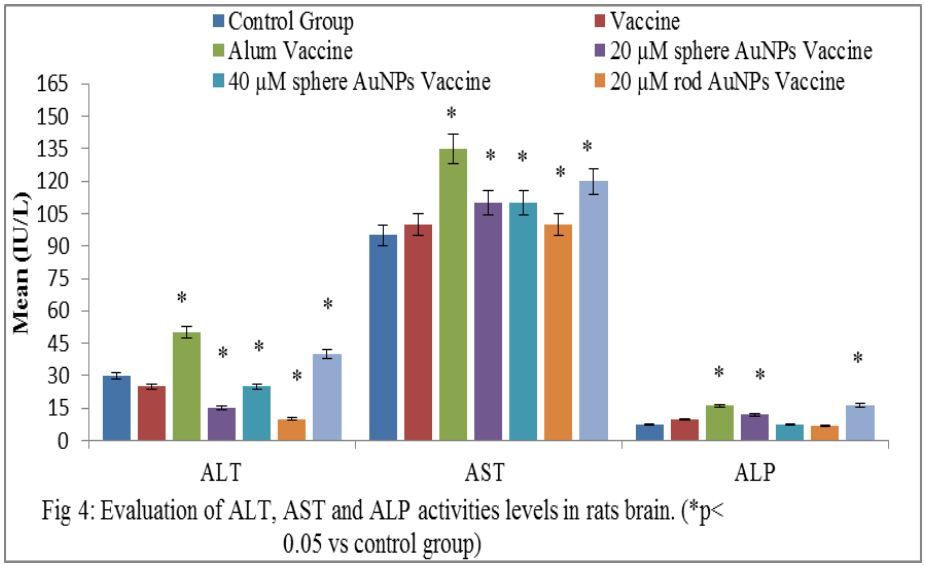

Fig 4: Evaluation of ALT, AST and ALP activities levels in rats brain. (*p< 0.05 vs control group)

Furthermore, blood urea and serum creatinine levels increased in a significant way (P<0.05) in alum-treated group while their levels in AuNPs treated groups showed no significant difference than the control group (P<0.05) [Fig. 5,6].

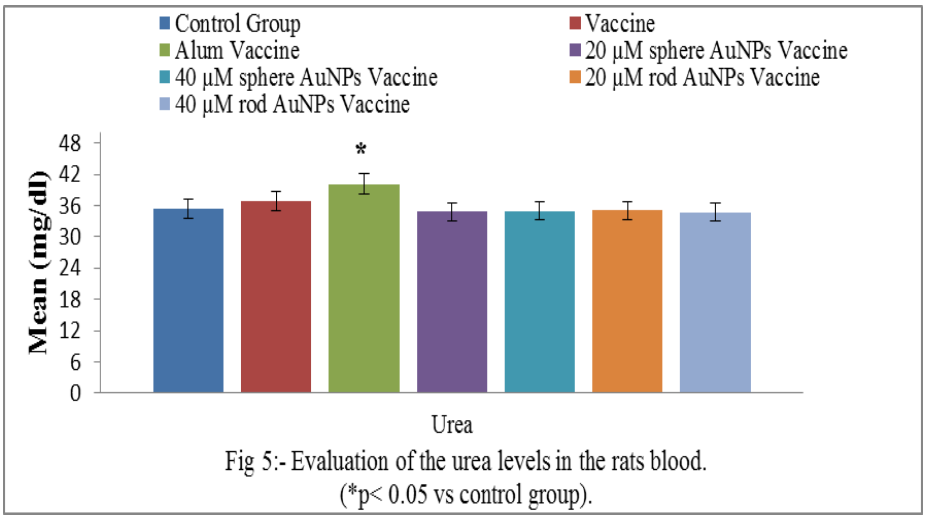

Fig 5:- Evaluation of the urea levels in the rats blood. (*p< 0.05 vs control group).

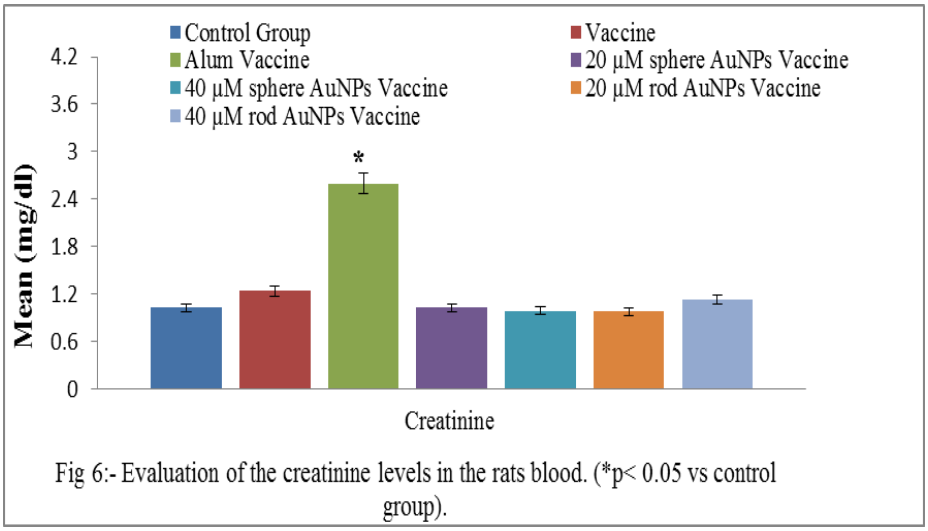

Fig 6:- Evaluation of the creatinine levels in the rats blood. (*p< 0.05 vs control group).

Administration of alum-adsorbed vaccine induced a significant (P<0.05) elevation in serum MDA levels along with a marked reduction in GPx levels [Fig.7]. Co-administration of different concentrations and shapes of gold nanoparticles with RVFV vaccine insignificantly changed serum MDA levels compared with the control values except for 20µM rod AuNPs group its value was significantly increased. In parallel, the serum GPx values for all gold nanoparticles treated groups were insignificantly less than the control value (P<0.05) [Fig.7]. Distinctively, the alum-adsorbed group revealed a significant (p<0.05) increased brain MDA levels along with significant (p<0.05) reduction in the GPx and SOD activities [Fig.8]. No significant (p<0.05) changes were detected in the levels of brain MDA, GPx and SOD after sphere AuNPs administration with RVFV vaccine as compared to the control values as shown in Fig [8].

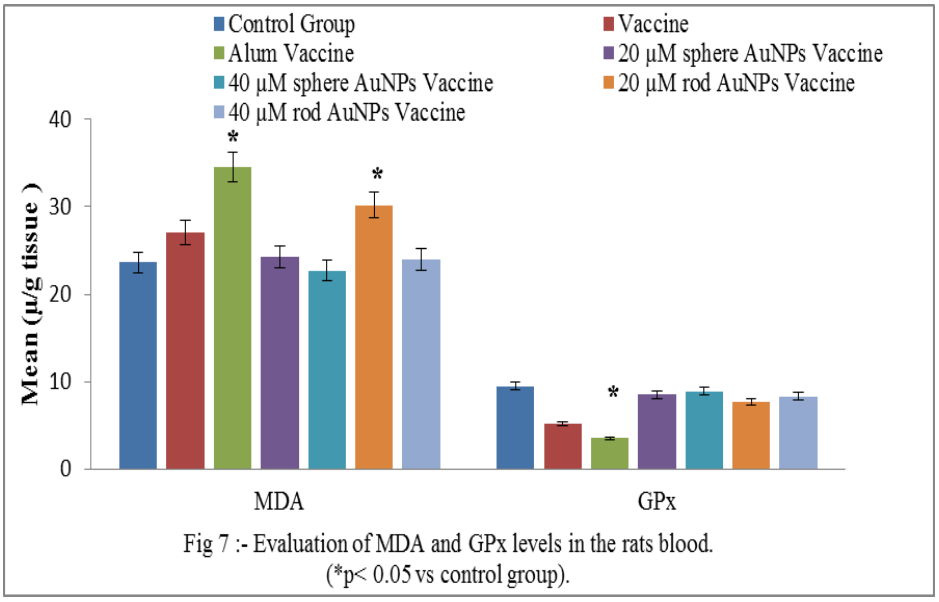

Fig 7 :- Evaluation of MDA and GPx levels in the rats blood. (*p< 0.05 vs control group).

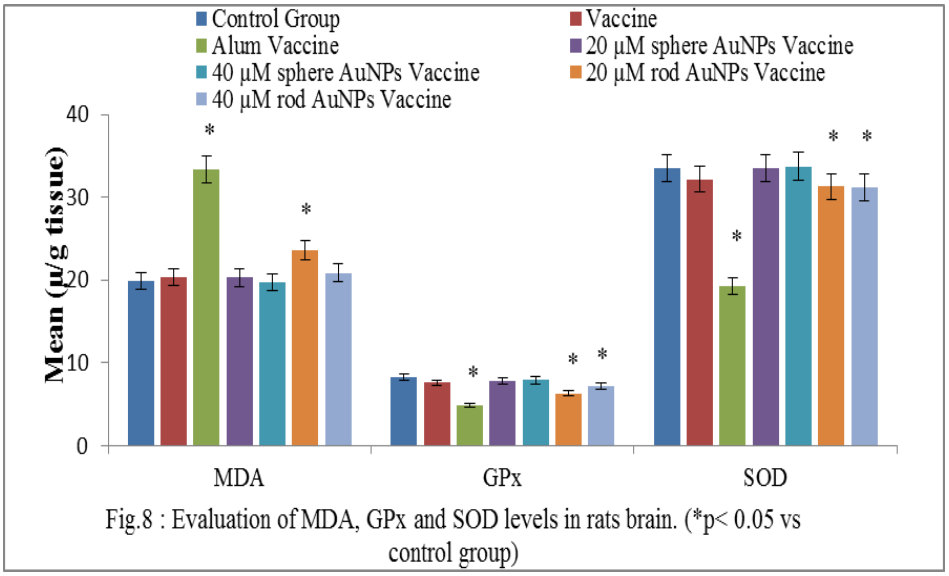

Fig.8 : Evaluation of MDA, GPx and SOD levels in rats brain. (*p< 0.05 vs control group)

A significant (P<0.05) increase was recorded in the noradrenaline (NE) content of alum-treated group compared to control, however, NE was not changed in the other treated groups compared to control [Fig.9]. In contrast, serotonin and dopamine levels were found to be significantly (P<0.05) decreased in the alum-adsorbed vaccine group [Fig.9,10]. Interestingly, the administration of AuNPs was found to be significantly elevated the levels of serotonin and dopamine as compared to the control values [Fig.9,10].

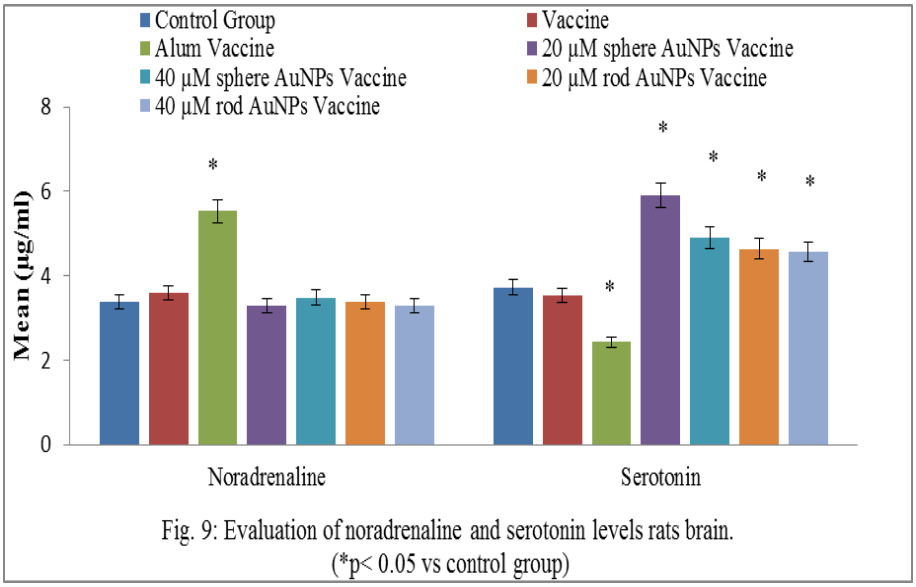

Fig. 9: Evaluation of noradrenaline and serotonin levels rats brain.
(*p< 0.05 vs control group)

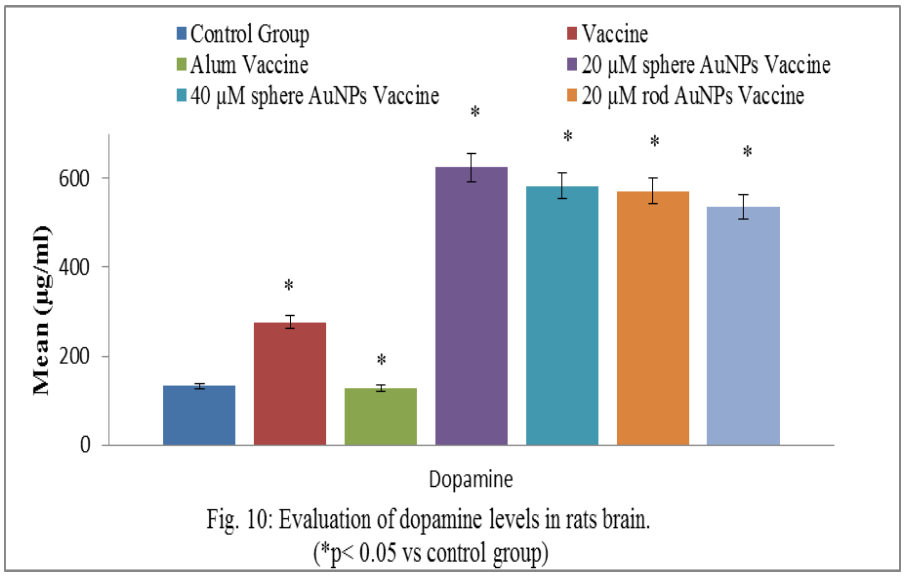

Fig. 10: Evaluation of dopamine levels in rats brain.
(*p< 0.05 vs control group)

DISCUSSION

There is an increasing application of nanoparticles for biomedical purposes.[34] Nanoparticles can serve as efficient drug vehicles to various cells, tissues and organs when adequately functionalized.[35] This way, only small doses are requested for therapeutic efficiency that would not require the administration of a large amount of potentially toxic materials avoiding unwanted systemic adverse effects, such as nephrotoxicity and hepatotoxicity.[1] Gold nanoparticles (AuNPs) have recently emerged as an attractive candidate for delivery of various payloads into their targets. As data on nanoparticles (NP) effects on the brain, especially AuNPs are scarce and greatly controversial[36,37], therefore, it has become imperative to investigate the effect of different shapes and sizes of gold nanoparticles treatments on the brain in concomitant to blood biochemistry changes. Concerning the toxic effect of different shapes and concentrations of gold nanoparticles used in our study, rats did not show any significant behavioral changes during the study period indicating that there was no toxic effect due to the administration of AuNPs that come in accordance with.[38]

In the present study, regarding serum total protein levels of AuNPs administrated groups, our data was in agreement with many studies.[39,40] Total protein percent reflects the balance between protein biosynthesis and catabolism, the significant decrease in both serum and brain total protein of alum-adsorbed vaccine as brain TP of most of AuNPs vaccine treated groups might be attributed to decreased synthesis, increased loss, increased catabolism or liver disease consequent upon the administration of the nanoparticles.[41] In addition, the alteration in serum albumin levels may denote stress on the liver that imposed by the nanoparticles.[34] On the contrary to the previously reported finding[42] which demonstrated that nanoparticles exposure caused a decrease in albumin level as a result of the inflammatory reaction of liver tissues, using of AuNPs in our study elevated the serum albumin levels.

In addition, serum levels of ALT and AST in rats treated with the 20µM sphere and 40µM rod AuNPs particles were significantly increased that may be attributed to the metabolism of gold nanoparticles in the liver.[43] It is well-known that NPs are taken up by Kupfer cells in the liver and by macrophages in other places, regardless of the particle size.[44] Interestingly, there was no corresponding increment on serum levels of ALT and AST of 40µM sphere and 20µM rod AuNPs administrated groups relative to those levels of the control group that comes in agreement with the findings of.[45] This may point in the direction of an adaptive mechanism by animals trying to cope with stress imposed by the nanoparticles exposure.[30] On the other hand, the ALT and AST are important enzymes of the brain; their activities were related to the maintenance of amino acid homeostasis and might be an indicator of mitochondrial injury.[46] A significant reduction in the brain ALT activities was estimated in all treated groups except for alum-absorbed vaccine and 40µM rod AuNPs groups that increased as compared to the control group that may infer to mitochondrial injury in both groups.

ALP is a membrane-associated enzyme, which predominantly concentrated in the brain vascular endothelium forming a more or less continuous sheath of ALP covering all internal and external surfaces of the central nervous system and thus it may functionally be part of the blood-brain barrier mechanism.[47] No significant changes were observed in the brain ALP activities in response to the 40µM sphere and 20µM rod AuNPs injection that come in concurrence with.[48] However, all other treated groups especially alum-absorbed vaccine group showed a significant increase in brain AST and ALP activities which is in agreement with[49] while earlier observation[50] did not notice any alteration in these parameters.

Furthermore, to evaluate the effect of the nanoparticles exposure on kidney function, the blood urea and serum creatinine levels were estimated as a gold standard for the assessment of nephrotoxicity.[51] No significant difference was detected in the creatinine levels between AuNPs treated groups and control group indicating that there was no acute and/or subchronic kidney damage.[51] These findings are in agreement with.[52,53] Meanwhile, a significant rise was revealed in the creatinine and urea levels of the Alum-treated groups that may imply impaired renal excretion.[54]

During normal aerobic metabolism, the balance of intracellular reactive oxygen species (ROS) depends not only on its production but also its removal by the antioxidant defense system in cells.[55] Because of the large surface area/volume ratio and reactivity, nanoparticles can bind to and interact extensively with bio-molecules within the cell, which leads to several consequences as producing ROS and oxidative stress.[56] Accordingly, there is an urgent need for studying the nanoparticles influence on living cells particularly the blood and nervous system. The brain and nervous system are especially vulnerable for the initiation of free radical reactions because of the brain's high oxygen consumption, its abundant unsaturated lipid content and the relative paucity of antioxidant protection as compared with other tissues.[57] Mammalian brain cells are equipped with both non-enzymatic (GSH) and enzymatic antioxidant defense systems as glutathione reductase (GR), catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) that reduce the formation of ROS and repair their damaging effects.[58] It has been affirmed that these substances play an important role in maintaining brain physiological levels of oxygen and hydrogen peroxide by hastening the dismutation of oxygen radicals and eliminating peroxides and hydroperoxides as well as disruption of brain energy metabolism.[57]

In the present study, there were significant increases in both of serum and brain MDA in alum-adsorbed vaccine group concomitant with a reduction in GPx and SOD that strongly suggest a hydroxyl radical activity and reflect oxidative stress.[59] Such results are in harmony with those obtained by[60] who indicated that the Alum treatments (even in low concentration) resulted in serum hyperaluminemia that could lead to the excess accumulation of alum in the brain tissues. This enhanced the neuronal lipid peroxidative damage with concomitant alterations in the enzymatic antioxidant defense status and contributed to neurotoxicity and alteration in the information processing.[49] In contrast, blood GPx showed little difference between control and all AuNPs-RVFV vaccinated groups, however, none of these changes were statistically significant for blood MDA values except for 20µM rod AuNPs group. The weakly negative correlation between GPx activities and MDA may show that different shapes of AuNPs might parallel decreased oxidative stress.[61] Meanwhile, brain MDA levels showed a meager increase in concomitant with a slight decrease in GPx and SOD levels in all AuNPs adsorbed vaccine groups except for 40µM sphere AuNPs group that remain in the control range. This was contradictory to[56] that estimated a tremendous increase in MDA accompanied by significant decrease in GPx as an evidence of brain antioxidant defenses impairment by the free radicals generated by AuNPs that was size dependent with the most widespread organ distribution for the smallest size.

Neurotransmitters are chemical substances that work as messengers conduct information throughout body and brain, in addition to carrying and tuning the signals between nerve cells and other cells affecting brain function. Neurochemical changes are greatly involved in chemical neurotoxicity.[62] Consequently, the present study used a neurochemical approach to investigate the effect of used gold nanoparticles as RVFV vaccine adjuvants on brain function. Dopamine (DA) and noradrenaline (NE) belong to catecholamines group that is greatly famed for their roles in the control and

regulation of brain functions and implicated in different neurodegenerative disorders.[60, 63] Meanwhile, Serotonin has been reported to have an inhibitory action in brain and disturbances in its level can affect the serotonergic system, leading to incorrectly connecting neural circuits.[64] The observed significant increase in the noradrenaline (NE) content of alum-adsorbed vaccine group may infer to induced learning impairment as it is known that NE negatively regulates learning and memory process.[65] However, no significant change was recorded in NE content in all AuNPs treated groups compared to control that come in parallel to the previously reported data by.[65,66]

Moreover, both brain serotonin and DA contents recorded significant reduction in alum-adsorbed vaccine group as compared to control rats group that come in accordance with.[67] It is interesting to note, that administration of different concentrations and shapes AuNPs were found to be significantly elevated the levels of serotonin and dopamine in all gold injected groups and the highest level was concentration dependent. This was in agreement with[68] that demonstrated hyperexcitability and increased neurotransmission in some brain regions by AuNPs administration. In contrast,[67] reported a significant decrease in the levels of dopamine and serotonin by administration of 20µM AuNPs. The discrepancy between our study and results of others is most likely attributed to differences in the size, shape and concentration, synthesis methods, surface composition of the nanoparticle and also exposure period of gold nanoparticles used. Size and shape are critical factors that significantly influence AuNPs cellular interactions[69] and in turn their applications. AuNPs size is a key factor in the biological responses to AuNPs; the smaller particles than 5µM tend to be more toxic than the larger ones as it chemically inert like the bulk and lesser in vivo tissue distribution.[70] Overall, AuNPs can be manufactured into a variety of shapes where the shape dependent abilities of AuNPs provides an extra dimension for study. Several studies were examined shape specific effects on cellular uptake and toxicity of AuNPs, some found that uptake of spherical AuNPs by cells was considerably easier than rod-shaped AuNPs.[71] On the contrary, greater *in vivo* biodistribution exhibited by rod-shaped particles that offer several advantages in the field of drug delivery, however, it exhibits increased accumulation.[71] The uptake and toxicity of many sized gold nanospheres and gold nanorods were tested. No toxic effect from all tested nanospheres, however, marked increase in toxicity from all gold nanorods was observed that may be related to increased tissue invading ability of nanorods.[72]

CONCLUSION

In conclusion, using AuNPs adsorbed RVFV vaccines showed great improvement in the measured blood and brain parameters compared to alum-adsorbed RVFV vaccine results, by increased activities of natural antioxidants by quenching the free radicals thus reduces oxidative stress especially the neuro-oxidative stress and ameliorated the disturbances in brain neurotransmitters. According to the findings, it could be concluded that the utilization of low concentration of spherical AuNPs is preferable to rod AuNPs as a promising adjuvant for RVFV vaccine, but further biological changes must be traced relatively to dose and duration to know the best mode of application and time of relief of the residual nanoparticles.

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