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Comparative evaluation of gold nanoparticles and Alum as immune enhancers against rabies vaccine and related immune reactivity, physiological, and histopathological alterations: *in vivo* study

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Purpose: The present study aimed to compare the immune-enhancing potential of gold nanoparticles (AuNPs) to Alum against rabies vaccine and the related immunological, physiological, and histopathological effects.

Materials and Methods: Alum and AuNPs sole and in combination with rabies vaccine were used at 0.35 mg/mL and 40 nM/mL, respectively. Rats used were categorized into six groups (20/each): control rats, rabies vaccine, aluminum phosphate gel, rabies vaccine adsorbed to Alum, AuNPs, and rabies vaccine adjuvant AuNPs.

Results: Liver and kidney functions were in the normal range after AuNPs and Alum adjuvanted vaccine compared to control. Interleukin-6 and interferon-γ levels were significantly increased in groups immunized with Alum and AuNPs adjuvanted vaccine, the peak level was in the case of AuNP adjuvanted vaccine on the 14th day. Ninety days post-vaccination, total immunoglobulin G (IgG) against adjuvanted rabies vaccine showed a significantly elevated anti-rabies IgG with AuNPs and Alum adsorbed vaccine compared with unadjuvanted one. The total antioxidant capacity, malondialdehyde (MDA) levels, superoxide dismutase, and glutathione peroxidase activities were significantly increased post-adjuvanted AuNPs adjuvanted vaccine vaccination than in Alum adsorbed vaccine, while MDA was significantly decreased. The histopathological examination revealed detectable alterations post-AuNPs and Alum adjuvanted vaccine immunization compared with liver and kidney profiles post-administration of unadjuvanted and non-immunized groups, meanwhile, splenic tissue revealed hyperplasia of lymphoid follicles indicating increased immune reactivity.

Conclusion: The AuNPs are promising enhancers of the immune response as Alum, and the undesirable effects of AuNPs could be managed by using suitable sizes, shapes, and concentrations.

Keywords: Rabies, Vaccines, Gold nanoparticles, Alum, Interferons, Interleukin-6, Pathology, Physiology

Introduction

Rabies is a preventable zoonotic disease and causes acute encephalitis. In resource-limited and resource-poor countries, endemic rabid dogs result in an ongoing risk of transmission to humans due to their bites. Prevention of human rabies through the

control of domestic rabid dogs is a realistic goal for large parts of Africa and Asia. Pre-exposure immunization in people is recommended for travelers to high-risk areas in rabies-affected countries, and for people in certain high-risk occupations such as laboratory workers dealing with the live rabies virus and other *lyssa* viruses, and veterinarians and animal handlers in rabies-affected areas [1].

Alum-based adjuvant remains the only adjuvant approved for human use. Aluminum compounds are used as immunologic adjuvants to increase the effectiveness of many vaccines. In some cases, these compounds were associated with redness, itching, and low-grade fever, but their use in vaccines was not associated with serious adverse events [2]. The quality of vaccine production could be improved by incorporating immune modulators or adjuvants with modified delivery vehicles. liposomes, immune-stimulating complexes, and micro/nanosphere apart from Alum, being used as a gold standard [3].

The use of nanoparticles, particularly metal nanoparticles has expanded in biomedical research, diagnosis, therapeutics cosmetics, electronics, innovative food products, and environmental remediation [4]. They are used in various areas due to their unique properties of small size, large surface area to volume ratio, high reactivity to the living cells, stability over high temperatures, and translocation into the cells. They are available in different sizes and shapes due to their ability to react and agglomerate with other nanoparticles in their surroundings. Gold nanoparticles (AuNPs) were reported to be inert and relatively less cytotoxic. Gold was used for various applications including drug and gene delivery indicating the enormous growth in this field [3]. The present study aimed to investigate the use of AuNPs as an Alum alternative adjuvant and to find out the related biological alterations including liver and kidney functions, humoral and cellular immunity; total immunoglobulin G (IgG), interleukin-6 (IL-6), and interferon-gamma (IFN-γ), respectively. Also, histopathological alterations of the liver, kidney, and spleen were considered.

Materials and Methods

Aluminum phosphate gel (Alum)

Aluminum chloride hexahydrate (0.63 M $AlC13.6H_2O$) and sodium phosphate dodecahydrate (0.3 M $Na_3PO_4.12H_2O$) were prepared in 40 mL of normal saline each. Prepared solutions were filtered using a 0.2 μm filter (Millipore, Burlington, MA, USA). Contents were stirred (Saurt, Stone, UK) continuously during the procedure at 40 to 60 rpm; 0.3 M $Na_3PO_4.12H_2O$ solution was added to a mixing bottle. And 300 mL normal saline was added, and the antigen was added followed by the addition of 0.63 M $AlC13.6H_2O$ solution to the mixing bottle. pH was maintained between 6.5–6.8. The final volume was adjusted with sterile normal saline. The suspension was mixed for 2 hours at 37°C [5].

Sphere gold nanoparticles (AuNPs)

Sphere AuNPs were kindly purchased at 1 mM from Nano-Tek, Giza, Egypt on October 6th. Nanoparticles were diluted to contain 40 nm final concentrations.

Inactivated rabies vaccine

β-propiolactone (βPL) inactivated rabies vaccine (Vero Rab) was kindly supplied from the Holding Company for Production of Vaccines, Sera and Drugs (VACSERA, Giza, Egypt). The rabies vaccine was purchased from Pasteur, France. Vaccine potency was >2.5 IU/dose.

Challenge virus standard virus

The challenge virus standard virus was kindly supplied by the National Control Authority (VACSERA). The vaccine infectivity titer was 106.5/mL, which was used for vaccine potency evaluation.

Anti-rabies immunoglobulin

Home reference rat anti-rabies immunoglobulin was obtained from Rabies Vaccine Research Unit, VACSERA.

Inactivated rabies vaccine potency (ED50)

The potency of βPL inactivated rabies vaccine was evaluated using a mice immunization assay [6]. The effective dose of vaccine that can protect 50% of infected mice was measured, where the experimental vaccine batch was 5-fold serially diluted (1/5–1/625) using sterile phosphate-buffered saline, pH 7.2±2 in addition to the concentrated vaccine. Each dilution was inoculated intraperitoneally as 0.3 mL/mouse using 10 weaning mice of 21 days old (14–16 g) per dilution. Another set of 10 mice was left as a negative control. One week later the 2nd dose of the vaccine dilutions was inoculated as previously. Fourteen days after the 1st shoot, a challenge dose of 12.5–50 minimum inhibitory concentration 50% lethal dose/mL was intracerebrally inoculated. Five weaning mice of the negative control group were challenged in the same way, and the rest of the control mice group was left as the negative con-

trol. Mortality was recorded throughout 14 days and the potency of inactivated rabies viral vaccines was determined [6].

Experimental animals

A total of 120 male Sprague-Dawley albino rats were supplied by the animal house, VACSERA (EGyVac, Giza, Egypt). Rats aged 8–16 weeks with body weights ranging from 80–100 g were housed in metallic cages. All rats were kept in a laboratory animal facility under standard laboratory conditions of 12 hours light/12 hours dark system and a fixed temperature of 27°C. Rats were fed on a standardized pelted chow composed of 11.2% moisture, 25.4% protein, 4.8% crude fibers, 8.5% ash, and 3.4% fat, and tap water was supplied *ad libitum*. Animals were kept under observation for 1 week for adaptation before the onset of the experiment. Care and cleaning were maintained to keep the animals in a normal healthy state. All experiments were done according to the approval of the VACSERA Ethical Committee (approval no., VAC-EC-22-65/R/2020).

Animals grouping and blood sampling

Laboratory animals used were categorized into six rat groups: group 1: negative control group; group 2: sole rabies vaccine (Vac) group; group 3: Alum adjuvanted vaccine (Alum-Vac) group; group 4: sole Alum group; group 5: AuNPs-rabies vaccine mix (AuNP-Vac) group; and group 6: sole AuNPs group. Rats received vaccine formulae, sole adjuvants, and normal saline subcutaneously at 0.5 mL/rat.

Immune sera preparation

Collected blood samples were kept overnight at 4°C for retraction of blood clots. Collected blood samples were cold centrifuged for 15 minutes at 3,500 rpm, using a cold centrifuge (Jouan-Ki-22; Jouan, Jarzé, France). Sera were removed to empty tubes and re-centrifuged for another 10 minutes to remove any remaining blood clots or red blood cells. Collected sera were liquated and stored at -80°C until use [7].

Evaluation of liver and kidney functions

Estimation of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) activity, as liver functions parameters, were undertaken in sera samples using a kinetic rate method, purchased from Spectrum (catalog number 263002 and 261005) for ALT and AST activity. Meanwhile, ALP activity was estimated using commercial kits from Biodiagnostics (Cairo, Egypt). The kidney function was carried out by measuring urea level in rats' sera using kits from Biodiagnostics, while creatinine level was determined by kits purchased from Randox Laboratories (Crumlin, UK).

Evaluation of cellular immune markers

IL-6 and IFN-γ were estimated in sera samples using rat enzyme-linked immunosorbent assay (ELISA) kits purchased from BD Biosciences Pharmingen (San Diego, CA, USA; catalog number 551216).

Immunoglobulin-G level

Specific total anti-rabies immunoglobulin developed against the rabies vaccine was detected using home reference ELISA kits based on the antibody capture technique using polyvinyl chloride (PVC) plates. The antigen was bound to the PVC wells, and the antibodies were allowed to bind to the antigen. The unbound antibodies/antigen were washed out using a wash buffer. The antigen–antibody complex was detected using anti-rat IgG coupled to peroxidase enzyme used as 1/1,000 final dilution. This method was performed, with some modifications [8].

Evaluation of oxidant/antioxidant biomarkers

The malondialdehyde (MDA) content and the total antioxidant capacity (TAC) were estimated in rats' sera using kits from Global Institute Research Services Solution (Cairo, Egypt). Both superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity were determined using kits from Global Institute Research Services Solution. Biochemical assays were performed in triplicates.

Histopathological examinations

Fresh specimens of the spleen, liver, and kidney were collected from the control, and the treated groups were prepared [9]. The specimens were examined using an oil lens and cellular changes were pictured using a digital camera (Canon, Tokyo, Japan) with a magnification of ×200.

Statistical analysis

Analysis of data were performed using the IBM SPSS ver. 26.0 (IBM Corp., Armonk, NY, USA) to compare all the treated groups, using one way analysis of variance [10]. Data were expressed as mean±standard errors of the mean. The quantitative results with a parametric distribution of Leven's test were determined for analysis data. Statistical differences with values of $p<0.05$ were considered statistically significant. Per-

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centage differences representing the percent of variation with respect to the control group were also calculated.

Ethics approval

All experimental protocols and steps of the tests were conducted in compliance with the regulations of the Research Ethics Committee of Egyptian Ethical Guidelines for the use of animals in research. Additionally, all animal experiments were obtained from the animal house of VACSERA, in accordance with protocols approved by the US National Institutes of Health (1978).

Results

Vaccine potency

The effective dose 50 (ED_{50}) of the Vero cell rabies vaccine was 4.5 IU/mL, which complied with the World Health Organization recommendations that the potent vaccine ED_{50} is not less than 2.5 IU/dose.

Evaluation of liver functions

As illustrated in Fig. 1, Alum and AuNPs, as adjuvants used either in sole form or in combination with Vero cell rabies vaccine, showed variable levels of ALT and AST values of liver functions. Although the variation was detected, these values were in the normal range compared with the unvaccinated control group. The % of ALT change on the 3rd day post-administration of Vac, Alum, and AuNPs treated groups was significant ($p<0.05$) compared with unvaccinated control group values recording a percentage difference of 20.83%, 17.26%, and 20.83%, respectively; while on the 7th day, only AuNPs group showed an increase of 15.68% with a significant change at $p<0.05$. ALT levels post-vaccination with Alum-Vac at the 3rd, 7th, and 14th days, showed an increase in the order of 20.24%, 15.68%, and 16.04%, respectively, and in the case of immunization with AuNPs-Vac, the change recorded was 22.62%, 17.30%, and 18.72%, respectively, with significant change at $p<0.05$ as compared to control. Both Alum-Vac and AuNP-Vac adjuvanted groups indicated a significant ($p<0.05$) increase in ALT level on the 14th day of immunization compared to the ALT level of the sole vaccine immunized group.

Estimation of aspartate aminotransferase activity

As depicted in Fig. 1, sera from rats collected on the 3rd, 7th, and 14th days indicated a significant change ($p<0.05$) in AST activity in the order of 27.60%, 28.30%, and 51.90% post-administration of rabies vaccine, and of 43.52%, 43.62%, and 51.90% post-administration of Alum and 55.41%, 54.89%, and 64.43% in case of AuNPs administration compared to control group AST values. Moreover, recorded data revealed that AST level at the 3rd, 7th, and 14th days was significantly changed ($p<0.05$) recording 45.44%, 52.55%, and 61.97%, respectively post-administration of Alum-Vac, and AST increased level was 57.32%, 53.40%, and 61.07%, respectively post-administration of AuNP-Vac compared to control AST values.

Estimation of alkaline phosphatase activity

As indicated in Fig. 1, analysis of data collected from different rat groups concerning ALP activity showed a significant ($p<0.05$) elevated ALP activity post-sole vaccine administration on the 3rd and 7th days, recording 19.63% and 15.61%, respectively, compared to control values. Meanwhile, sole administration of Alum and AuNPs showed a significant change ($p<0.05$) recording (18.16%, 21.62%, and 12.97%) and (20.74%, 23.00%, and 13.75%) post-administration of Alum and AuNP, respectively, compared to control group values. On the contrary, no changes were detected on the 14th day. In the meantime, Alum-Vac and AuNPs adjuvanted rabies vaccine indicated a significant change ($p<0.05$) in ALP levels recording (33.25%, 33.02%, and 11.23%) and (40.86%, 24.26%, and 23.04%) post-immunization with Alum-Vac in AuNPs on the 3rd, 7th, and 14th day, respectively, compared to control group values. Meanwhile, Alum-Vac exhibited a significant increase in ALP level on the 7th day of vaccination compared to the level of the sole vaccine immunized group, while immunization with AuNP-Vac indicated the same effect on the 3rd and 14th days versus the same group.

Evaluation of kidney functions

Sole rabies vaccine and Alum administration, exerted a normal variation in urea level at the 3rd, 7th, and 14th compared with its level in the control group and around the border levels of kidney function levels, on the contrary, there was a significant elevation post-administration of AuNPs and AuNPs-Vac as illustrated in Fig. 2; recording (42.38%, 55.76%, and 54.14%), (38.49%, 59.93%, and 56.07%), (53.15%, 66.79%, and 60.39%), (57.96%, 63.97%, and 52.78%), and (60.94%, 69.36%, and 64.47%) for the sole vaccine, Alum, Alum-Vac, AuNPs, and AuNPs-Vac immunized groups, respectively, compared to control values. Urea levels of the AuNPs-Vac-immunized group increased significantly ($p<0.05$) versus the sole vac-

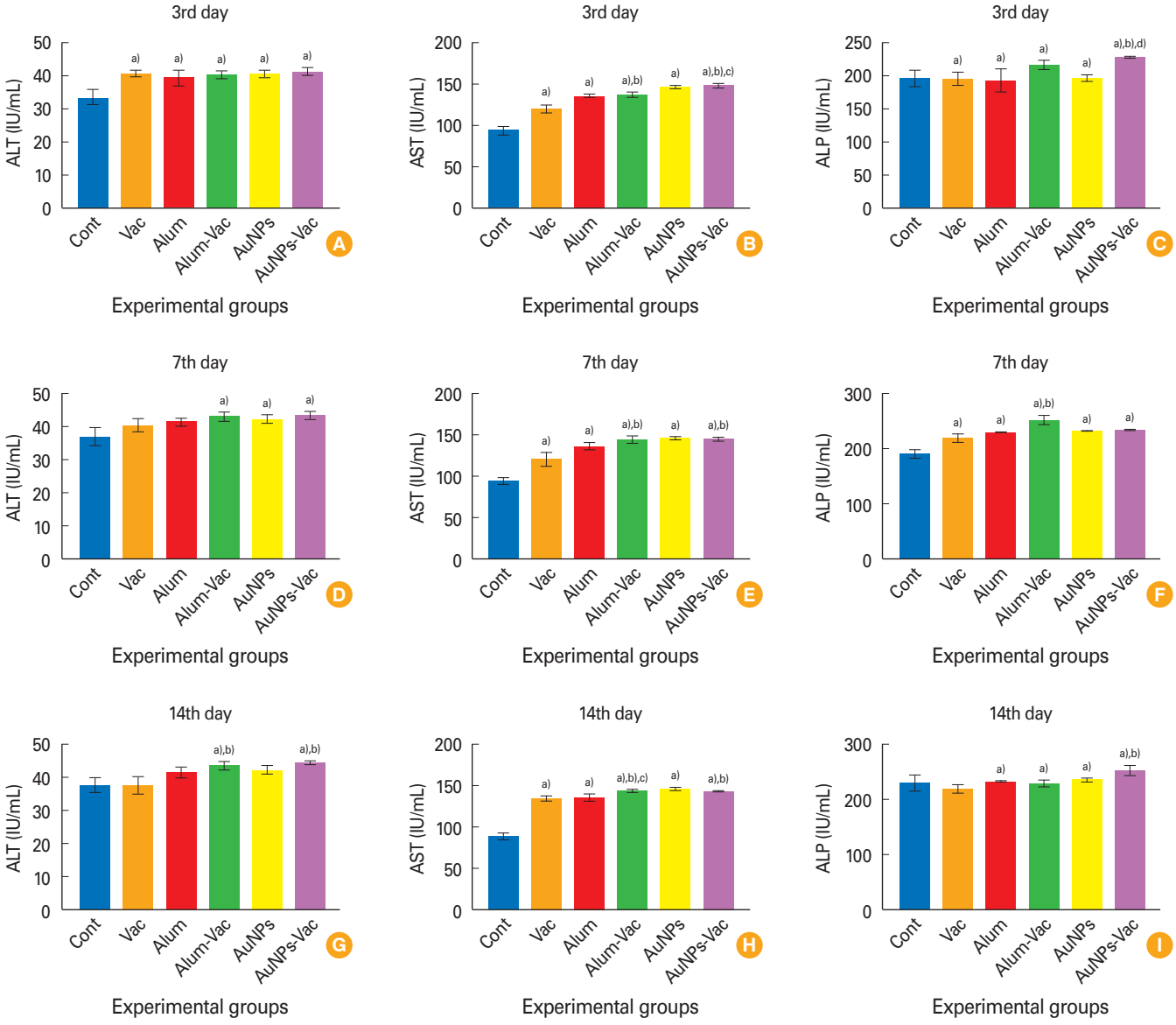

Fig. 1. (A–I) Comparative evaluation of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) activity (U/L) in sera of adult male rats immunized with either Alum, gold nanoparticles (AuNPs) sole, or adjuvant with rabies vaccine at the 3rd, 7th, and 14th days after vaccination. [a]Significant changes at ($p<0.05$) compared to control (Cont) group. [b]Significant changes at ($p<0.05$) compared to vaccine (Vac) group. [c]Significant changes at ($p<0.05$) compared to Alum group. [d]Significant changes at ($p<0.05$) compared to AuNPs group.

cine-immunized group on 3rd day of immunization.

As shown in Fig. 2, serum creatinine levels post-administration of the sole vaccine, sole adjuvants, and adjuvanted vaccine showed a non-pathological variation and recorded values were within the normal range of kidney function throughout the defined durations.

Interleukin-6 levels

As depicted in Fig. 3, on the 3rd day post-sole rabies vaccine administration induced a decreased IL-6 level (-8.74%), followed by a significantly ($p>0.05$) increased level (78.79% and 270.87%), on the 7th and 14th days post-vaccination, respectively, compared to their control group values. Sole Alum and AuNPs administration induced a significant ($p<0.05$) elevated IL-6 level on the 3rd, 7th, and 14th days recording (12.94%, 19.00%, and 5.55%) and (27.74%, -25.39%, and -14.39%), respectively, compared to control group values. Likely, Alum-Vac and AuNPs-Vac immunized groups induced a significant ($p<0.05$) elevation of IL-6 level, compared to control, sole Vac, Alum, and AuNPs groups on the 3rd, 7th, and 14th days. Moreover, the Alum-Vac group indicated a significant elevation in IL-6 levels ($p<0.05$), compared to IL-6 level post-im-

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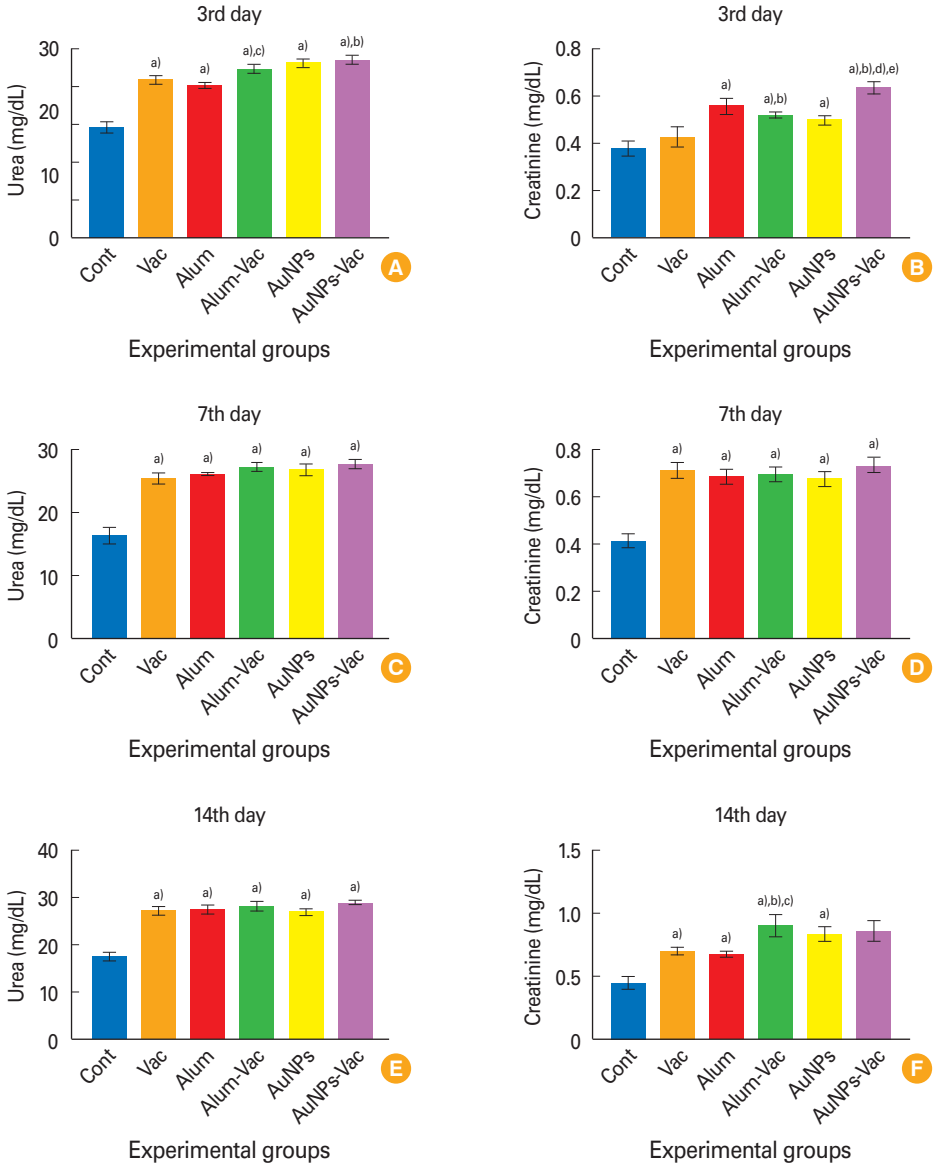

Fig. 2. (A–F) Comparative evaluation of urea and creatinine levels (mg/dL) in sera of adult male rats immunized with either Alum, gold nanoparticles (AuNPs) sole, or adjuvant with rabies vaccine at the 3rd, 7th, and 14th days after vaccination. [a]Significant changes at ($p<0.05$) compared to control (Cont) group. [b]Significant changes at ($p<0.05$) compared to vaccine (Vac) group. [c]Significant changes at ($p<0.05$) compared to Alum group. [d]Significant changes at ($p<0.05$) compared to AuNPs group. [e]Significant changes at ($p<0.05$) compared to Alum-Vac group.

munization with AuNPs-Vac.

Interferon-γ levels

INF-γ levels in rats' sera showed a significant ($p<0.05$) increment at different time intervals post-vaccination in all groups, and the highest increase was noticed post-administration of AuNPs-Vac group as clearly demonstrated in Fig. 3. The recorded increase of INF-γ level was (15.13%, 147.58%, and 375.81%), (36.31%, 15.84%, and 31.59%), (65.40%, 160.26%, and 292.34%), and of (81.04%, 198.61%, and 307.26%) and (119.71%, 219.02%, and 471.14%) sole vaccine, sole Alum, sole AuNPs, Alum-Vac, and AuNPs-Vac, respectively, compared with IFN-γ of the unvaccinated control group.

Immunoglobulin G levels

Regarding the immune potential of the prepared rabies vaccine, data represented in Fig. 4 revealed the immunogenicity of the inactivated rabies vaccine which was monitored 90 days post-vaccination. The antibody IgG level showed an elevation of 111.96% with a significant change at $p<0.05$, compared with the control value. A positive enhancement of the immune reactivity was recorded in either the ALUM group or AuNPs group,

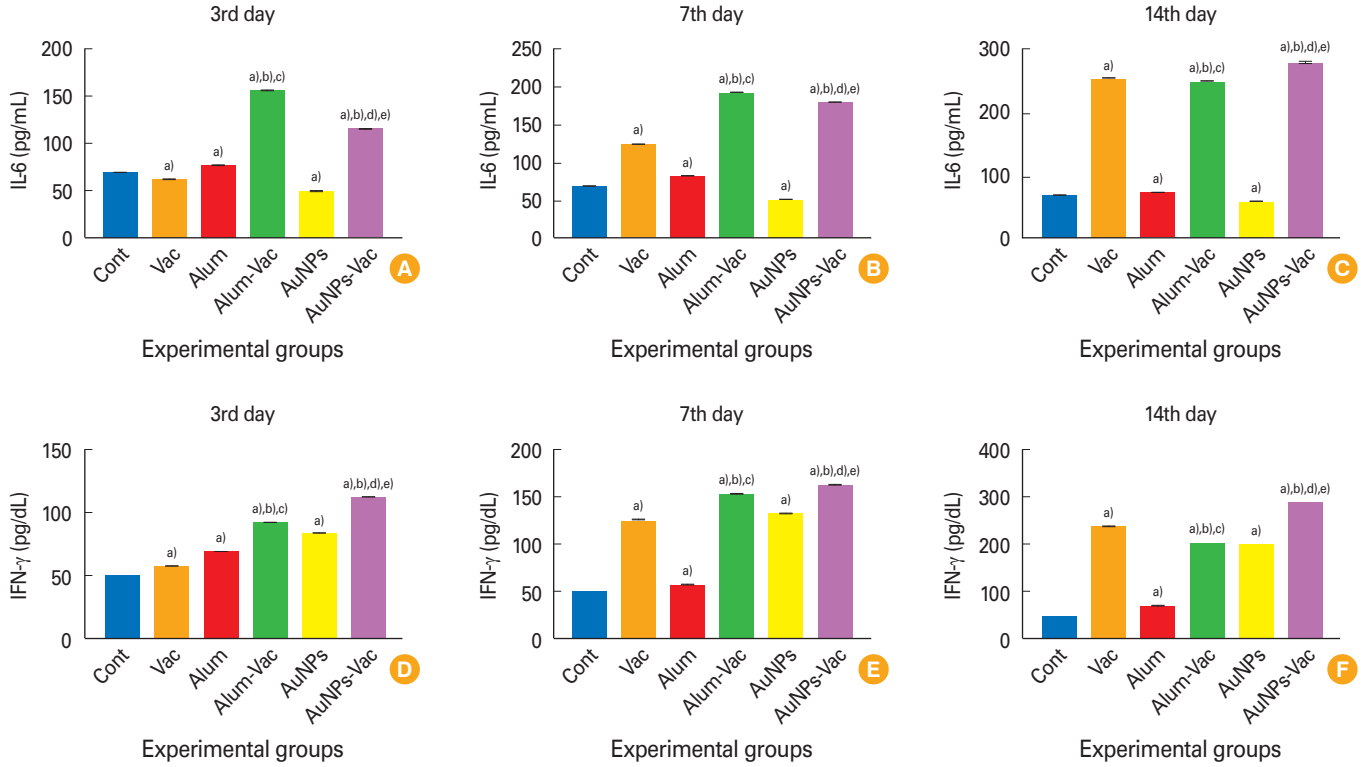

Fig. 3. (A–F) Comparative evaluation of interleukin-6 (IL-6) and interferon-γ (IFN-γ) levels (pg/mL) in sera of adult male rats immunized with either Alum, gold nanoparticles (AuNPs) sole, or adjuvant with rabies vaccine at the 3rd, 7th, and 14th days after vaccination. [a]Significant changes at ($p<0.05$) compared to control (Cont) group. [b]Significant changes at ($p<0.05$) compared to vaccine (Vac) group. [c]Significant changes at ($p<0.05$) compared to Alum group. [d]Significant changes at ($p<0.05$) compared to AuNPs group. [e]Significant changes at ($p<0.05$) compared to Alum-Vac group.

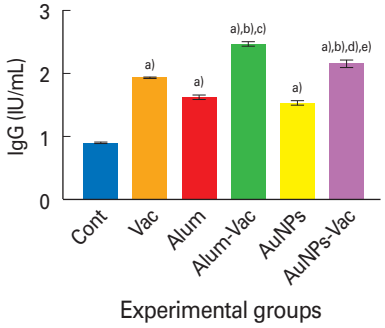

Fig. 4. Comparative evaluation of anti-rabies immunoglobulin (IgG) concentrations (ng/mL) in sera of adult male rats immunized with either Alum, gold nanoparticles (AuNPs) sole, or adjuvant with rabies vaccine at 90 days post-vaccination. [a]Significant changes at ($p<0.05$) compared to control (Cont) group. [b]Significant changes at ($p<0.05$) compared to vaccine (Vac) group. [c]Significant changes at ($p<0.05$) compared to Alum group. [d]Significant changes at ($p<0.05$) compared to AuNPs group. [e]Significant changes at ($p<0.05$) compared to Alum-Vac group.

with values of 79.35% and 68.48%, respectively versus the control group, with significant change at ($p<0.05$). Meanwhile, the immune reactivity was not elevated in the VAC-immunized group. Whereas, injection of either Alum or AuNPs adsorbed vaccine recorded strong enhancing effects in the total antibody level IgG post-immunization, with values 171.74% and 138.04%, respectively, with a significant increase at $p<0.05$, compared to the control group and significant versus VAC group. Therefore, both Alum & Vac and AuNPs-Vac groups revealed a significant increase in antibody IgG level estimated post-immunization compared with either sole Alum or AuNPs groups as well. In addition, the long-lasting immune response indicated significant high levels of IgG in the Alum-Vac group than in the AuNPs & Vac group.

Evaluation of oxidant/antioxidant biomarkers

Total antioxidant capacity level

Exploration of data on the 90th-day post-vaccination with sole Alum and unadjuvanted rabies vaccine indicated no change in the antioxidant capacity level in their sera. Meanwhile, sera of the AuNPs injected rats group showed an increase in TAC level of 33.33% with significant change at $p<0.05$, compared with the control group value. Both Alum-Vac and AuNPs-Vac groups recorded increased TAC levels in their sera, of values 66.67% and 50.0%, respectively, with a significant change at

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Table 1. Comparative evaluation of antioxidant/oxidant status in sera of adult male rats immunized with either Alum, AuNPs sole, or adjuvant with rabies vaccine at 90 days post-vaccination

Variable	Control	Vac	Alum	Alum+Vac	AuNPs	AuNPs+Vac
TAC (mmol/L)	0.06±0.00	0.06±0.00 (0)	0.06±0.00 (0)	0.10±0.00 (66.67)[a),b),c)]	0.08±0.00 (33.33)[a)]	0.09±0.00 (50.0)[a),b),d)]
MDA (nmol/mL)	4.76±0.03	4.02±0.18 (-15.55)[a)]	4.30±0.05 (-9.66)[a)]	3.17±0.10 (-33.40)[a),b),c)]	2.62±0.26 (-44.96)[a)]	3.30±0.10 (-30.67)[a),b),d)]
SOD (U/mL)	95.15±0.23	97.33±0.37 (2.29)[a)]	102.2±0.40 (7.39)[a)]	110.3±0.20 (15.94)[a),b),c)]	105.9±0.5 (11.32)[a)]	113.4±0.41 (19.12)[a),b),e)]
GPx (mU/mL)	5.13±0.08	5.46±0.07 (6.43)[a)]	6.48±0.13 (26.32)[a)]	7.31±0.04 (42.50)[a),b),c)]	6.53±0.09 (27.29)[a)]	7.52±0.04 (46.59)[a),b),d)]

Values are presented as mean±standard errors of the mean (% difference with respect to control value).
AuNPs, gold nanoparticles; Vac, vaccine; TAC, total antioxidant capacity levels; MDA, malondialdehyde contents; SOD, superoxide dismutase activity; GPx, glutathione peroxidase activity.
[a)]Significant changes at ($p<0.05$) compared to control group. [b)]Significant changes at ($p<0.05$) compared to Vac group. [c)]Significant changes at ($p<0.05$) compared to Alum group. [d)]Significant changes at ($p<0.05$) compared to AuNPs group. [e)]Significant changes at ($p<0.05$) compared to Alum-Vac group.

$p<0.05$ versus the control value, but both groups showed a significant slight increase versus Vac group as shown in Table 1.

Malondialdehyde content

Results illustrated in Table 1 showed that sera of Vac, Alum, and AuNPs groups exhibited a decrease in MDA level, with values -15.55%, -9.66%, and -44.96%, respectively with a significant change at $p<0.05$, compared to the control group value. Meanwhile, both Alum & Vac and AuNPs-Vac groups exhibited a sharp decrease in sera MDA contents of values of -33.40% and -30.67%, respectively, compared against the control group value with significant change at $p<0.05$ versus the control value in the Vac, Alum, and AuNPs treated groups.

Superoxide dismutase activity

Rabies vaccination, Alum, and AuNPs administration showed significantly ($p<0.05$) elevated SOD levels by 2.29%, 7.39%, and 11.32%, respectively, compared to control group value (Table 1). Likely, Alum-Vac and AuNP-Vac groups induced a significant elevation of SOD level as well ($p<0.05$) recording (15.94%) and (19.12%), respectively versus control. In addition, the AuNP-Vac group indicated a significant elevation in sera SOD activity compared with the Alum-Vac group.

Glutathione peroxidase activity

Data illustrated in Table 1 revealed that AuNP level post-administration of the sole vaccine, Alum, AuNPs, Alum-Vac, and AuNP-Vac groups recorded significant ($p<0.05$) increased values in the order of 6.43%, 26.32%, 27.29%, 42.50%, and 46.59%, respectively, compared with the negative control value. Also, glutathione level detected post-Alum-Vac and AuNPs-Vac administration showed a significant increase ($p<0.05$), compared to Vac, Alum, and AuNPs group values.

Histopathological examinations

Photomicrographs of rats' liver tissue (Fig. 5) specimens taken from the control group showed a normal arrangement of hepatic lobules which comprised radiating plates or strands of polyhedral hepatocytes vertical to the central vein with bile canaliculi between adjacent hepatocytes (Fig. 5A). Sections from animals exposed to rabies non-adjuvant vaccine showed mild swelling of hepatocytes. Dilatation of hepatic sinusoids and central veins was also noticed (Fig. 5B). Animals exposed to Alum showed mild swelling of hepatocytes and hyperplasia of Kupffer cells (Fig. 5C). Alum adjuvant rabies vaccine group revealed swelling and granularity of cytoplasm (Fig. 5D). Sections from the AuNPs group showed swelling and granularity of hepatocytes. The hepatic sinusoids appeared narrow with hyperplasia of Kupffer cells. (Fig. 5E). Livers of animals exposed to AuNPs adjuvant rabies vaccine showed degenerative changes of hepatocytes and hyperplasia of Kupffer cells (Fig. 5F). Sections of kidney tissue (Fig. 6) got from the control group showed the normal histological structure of renal parenchyma which characterized by intact glomeruli and renal tubules (Fig. 6A). Kidneys of animals exposed to rabies non-adjuvant vaccine in the VAC group showed a mild shrinkage of glomerular tufts and swelling of the epithelial lining of tubules (Fig. 6B). On the other side, kidney sections from the Alum group & Alum adjuvant rabies vaccine group showed resemblance to the control group (Fig. 6C, D). Meanwhile, kidney sections obtained from the AuNPs group showed intact glomeruli and capillary tufts. The renal tubules, especially the proximal convoluted, showed degenerative changes of the epithelial lining (Fig. 6E). Animals of the AuNPs-Vac group revealed shrinkage of glomerular tufts and necrosis of tubular epithelial lining (Fig. 6F). Investigation of spleen tissue sections (Fig. 7) of the control group showed the normal histological structure of the spleen parenchyma tissue character-

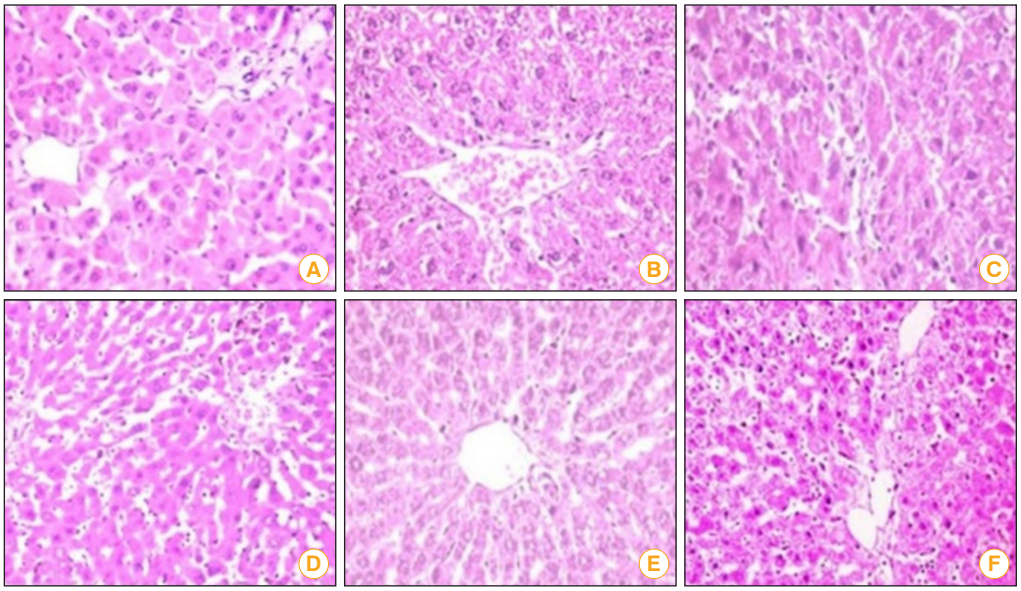

Fig. 5. (A–F) Histopathological changes in liver tissue sections of adult male rats immunized with either Alum, gold nanoparticles (AuNPs) sole, or adjuvant with rabies vaccine after 90 days post-vaccination, stained with hematoxylin and eosin (×200). (A) Photomicrograph of liver from control group showed a normal histological structure of hepatic lobules (H&E, ×200). (B) Photomicrograph of liver from AuNPs group showed swelling and granularity of hepatocytes (H&E, ×200). (C) Photomicrograph of liver from AuNP-Vac group showed degenerative changes of hepatocytes and mononuclear infiltration (H&E, ×200). (D) Photomicrograph of liver from naïve vaccine (Vac) group showed a mild swelling of hepatocytes with vesiculated nucleus (H&E, ×200). (E) Photomicrograph of liver from Alum-Vac group showed swelling hepatocytes with granularity of cytoplasm (H&E, ×200). (F) Photomicrograph of liver from Alum group showed a mild swelling of hepatocytes and hyperplasia of Kupffer cells (H&E, ×200).

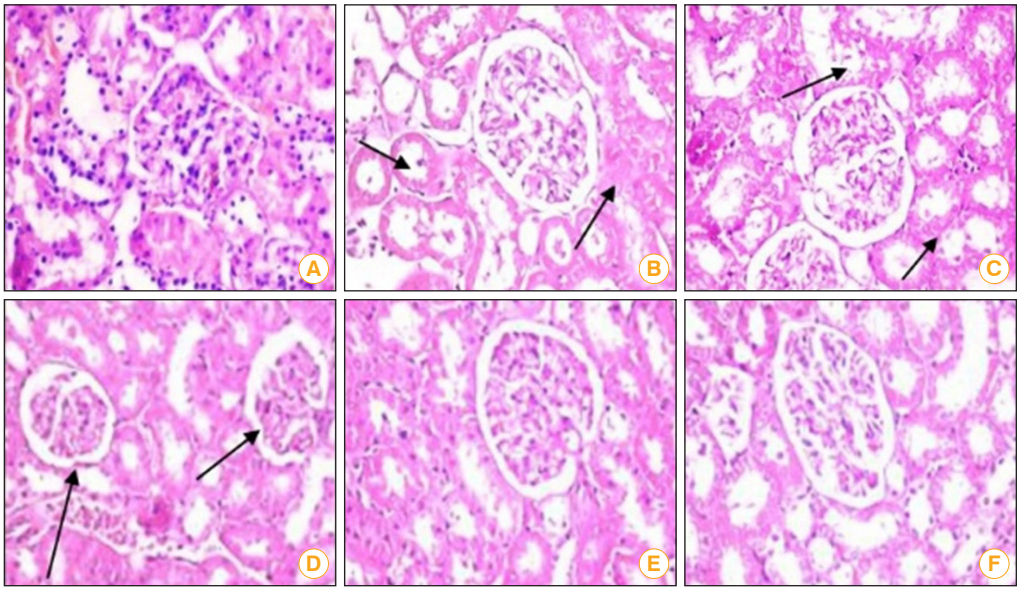

Fig. 6. (A–F) Histopathological changes in kidney tissue sections of adult male rats immunized with either Alum, gold nanoparticles (AuNPs) sole, or adjuvant with rabies vaccine after 90 days post-vaccination, stained with hematoxylin and eosin (×200). (A) Photomicrograph of kidney from the control group showing a normal histological structure of both renal glomerulus and tubules (H&E, ×200). (B) Photomicrograph of kidney from AuNPs group showing degenerative changes and necrosis of tubular epithelial lining arrows (H&E, ×200). (C) Photomicrograph of kidney from AuNP-Vac group showing necrosis of tubular epithelial lining arrows (H&E, ×200). (D) Photomicrograph of kidney from vaccine (Vac) group showing a mild shrinkage of glomerular tufts (arrows) (H&E, ×200). (E) Photomicrograph of kidney from Alum-Vac group showing mild degenerative changes of the tubular epithelial lining (H&E, ×200). (F) Photomicrograph of kidney from Alum group showing intact tubular epithelial lining with a clear lumen (H&E, ×200).

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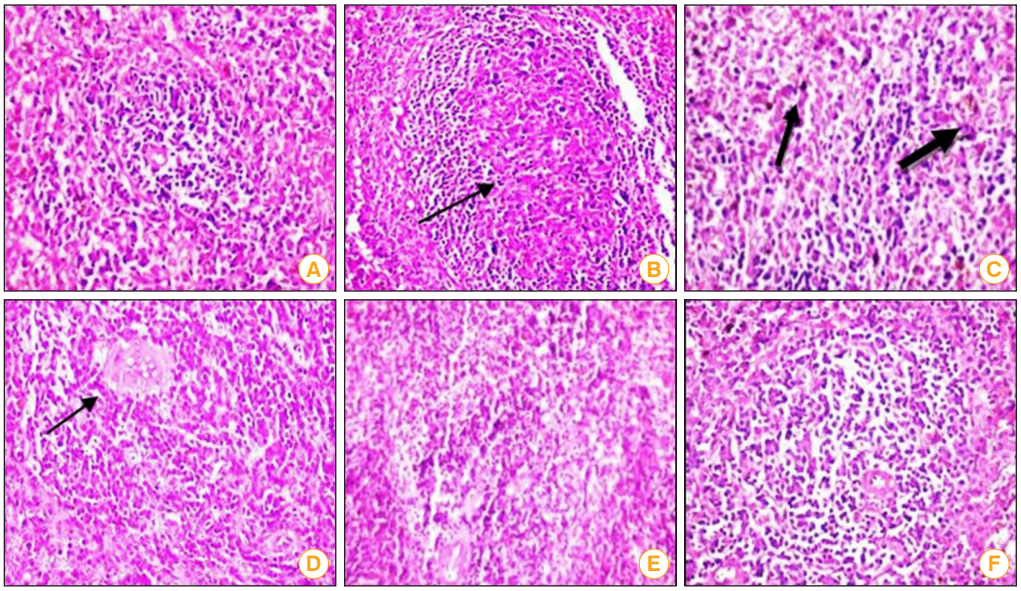

Fig. 7. (A–F) Histopathological changes in spleen tissue sections of adult male rats immunized with either Alum, gold nanoparticles (AuNPs) sole, or adjuvant with rabies vaccine after 90 days post-vaccination, stained with hematoxylin and eosin (×200). (A) Photomicrograph of spleen from control group showing a normal histological structure (H&E, ×200). (B) Photomicrograph of spleen from AuNPs group showing hyperplasia of lymphoid follicles and numerous numbers of macrophages arrow (H&E, ×200). (C) Photomicrograph of spleen from AuNP-Vac group showing hyperplasia of lymphoid follicles and deposition of hemosiderin granules arrows (H&E, ×200). (D) Photomicrograph of spleen from vaccine (Vac) group showing proliferation of lymphocytes with thickening of arterioles vein wall arrow (H&E, ×200). (E) Photomicrograph of spleen from Alum-Vac group showing moderate hyperplasia of lymphoid follicle (H&E, ×200). (F) Photomicrograph of spleen from Alum group showing mild hyperplasia of lymphoid follicle (H&E, ×200).

ized by clear red and white pulps with distinct central arterioles (Fig. 7A). Spleen of animals exposed to rabies non-adjuvant vaccine showed proliferation of lymphocytes (Fig. 7B). Spleen of sole Alum and Alum adjuvant rabies vaccine groups showed mild-to-moderate hyperplasia of lymphoid follicles (Fig. 7C, D). Examination of sections from the AuNPs group showed hyperplasia of lymphoid follicles (Fig. 7E). Meanwhile, animals inoculated with AuNPs adjuvant rabies vaccine group revealed hyperplasia of lymphoid follicles (Fig. 7F).

Discussion

Understanding how an adjuvant activates the immune response is important for rational vaccine design to tailor the response based on the antigen and type of immunity necessary to protect against infection [11]. Therefore, the present work was planned to evaluate the possible chance of using of AuNPs as an adjuvant for rabies virus vaccine against the nowadays used Alum adjuvanted rabies vaccine. ALT and AST were assessed as the determinants for liver diseases, especially ALT which is the most reliable biochemical value to show hepatocytes injury. Liver and kidney function indices of adult rats were variable within the normal ranges. These elevations could be attributed to the little damage of hepatocytes which is reflected by increased ALT levels more than AST, as ALT is densely located in the cytosol of liver cells [12]. A previous study on AuNPs indicated that 3 days post-injection of male rats had no adverse effect on serum ALT, AST, ALP activities, urea, and creatinine levels, but documented elevated ALT, AST, and urea levels 7 days post-injection and were returned to normal levels on 60th-day post-injection [13]. Although rabies vaccination is considered safe, a rare case in humans was reported to induce glomerular and renal tubular disorders of glomerulonephritis after immunization with the anti-rabies vaccine, which in alignment with the present results indicating different variations in serum levels of urea and creatinine after 3, 7, and 14 days, and despite the values were increased, they were still within the normal ranges of kidney functions compared to control. These variations could be attributed to hypersensitivity, resulting from excessive immune stimulation which led to the production of a pathogenic immune complex [14].

In accordance with the present results, it was reported that creatinine level depends on the speed of renal glomeruli func-

tion, which indicates renal performance [15]. An elevated creatinine level above the normal value revealed a chronic kidney disease. Moreover, a lower concentration of AuNPs did not show severe toxicity in terms of creatinine and blood urea levels. Meanwhile, higher concentrations of AuNPs caused significant changes in organ indices [16], and the distribution of AuNPs in tissues is shape-dependent and the most affected organs are the heart, lungs, liver, spleen, kidney, thymus, brain, reproductive organs, and blood as well [17].

Concerning the cellular immune markers (IL-6 and IFN-γ) data recorded was in agreement with those who indicated that effective delivery of antigens to antigen-presenting cells eliciting a prolonged immune response still remains a significant challenge for vaccination [4]. In the same way, enhanced eosinophil activation, post-administration of Alum was reported [18], promoting an influx of neutrophils and enhancing the secretion of pro-inflammatory cytokines and chemokines. The viral infection produces pro-inflammatory cytokines (various pro-inflammatory cytokines such as tumor necrosis factor [TNF]-α, IL-1, and IL-6) causing a hyperinflammatory response by recruiting macrophages, T and B cells, enhancing the transcription or translation of IL-6 from cells (fibroblast, mesenchymal, endothelial, and many other cells) and binding of TNF-α, IL-6, and IFN-β to their receptors, and leading to activation of the JAK/STAT pathway which initiate IFN transcription via hundreds of genes. IFN-γ (a pleiotropic cytokine) has a variety of biological responses including protection from viral and bacterial infections, anti-tumor effects, and as a key link and regulator of effector cells in innate and adaptive immunity. IFN-γ is produced by natural killer cells and other specialized cells of the immune system. JAK signal transducer/STAT transcription activator pathway activates IFN-γ signals via its binding to its receptor which is expressed in most cells. It is considered a master switch of cytokine cascade containing large numbers of separate molecules operating through different receptors. Moreover, IFN-γ, inhibits intracellular viral replication via potentiation of nitric oxide induction, resulting in efficient viral clearance [19].

Moreover, Alum was found to induce endogenous danger signals via cellular necrosis which elicits inflammation-associated cytokines resulting in humoral immunity [11]. Furthermore, the aggregated forms of aluminum salts present in Alum based adjuvants caused an oxidative or inflammatory response in brain-derived astrocytes in comparison to peripherally-derived macrophages immune-competent cells were reported, suggesting a decreased cell viability accompanied with a specific profile of cytokine secretion [20]. The use of nanoparticles as vaccine delivery platforms showed significant progress, playing an essential role in novel vaccine design and a significant role, in improving vaccines efficacy, and offering unique advantageous properties as vaccine carriers, as these nanoparticles prevent premature antigen release while prolonging antigen presentation for potent immunity against infectious diseases [4]. Moreover, nanotechnology was incorporated into vaccine development due to the low immunogenicity, toxicity, and instability of some vaccines. These advantages result from their comparable size giving nanoparticles the ability to attach to biological entities without changing their functions, while the high surface-area-to-volume ratio of nanoparticles permits strong bonds with surfactant molecules [21]. In the present study, 40 nm spherical AuNPs as an adjuvant for rabies vaccine induced an elevation of IL-6 levels on the 3rd- and 7th-day post-vaccination but continued to increase till the 14th day of AuNPs adjuvant vaccine immunization exceeding that detected post-Alum adjuvanted vaccine. Similarly, 40 nm spherical AuNPs injections were found to induce inflammatory cytokine production [22], including TNF-α, IL-6, IL-12, IL-10, and granulocyte-macrophage colony-stimulating factors, suggesting that AuNPs are effective vaccine adjuvant can enhance the immune response via different cytokine pathways depending on their sizes and shapes.

AuNPs activate the production of proinflammatory cytokines via penetrating various immune cells [23]. INF-γ is mainly secreted by T helper (Th)1 cells that play a central role in many immunoregulatory processes, including the activation of mononuclear phagocytes, and Th1 signature cytokines (INF-γ and IL-2) were detected in sera samples at an early time during infection [24], suggesting that Th1 responses were facilitated following infection. It was observed, in the current study, that an extreme elevation of INF-γ level in rats' sera during the 3rd, 7th, and 14th days post-immunization Alum adjuvanted vaccine compared to control and sole vaccine group (non-adjuvant vaccine), which may reflect the important role of INF-γ as an antiviral agent that protects rats against virus challenge, in agreement with who reported that enhanced Th1-cell responses *in vivo* increased IFN-γ induction by Alum salts [25]. TNF-γ levels in rat sera immunized with AuNPs adjuvanted vaccine showed a high elevation compared with Alum adjuvanted vaccine, these results are in accordance with Zhou et al. [26] who indicated enhanced elevated secretion of Th1 cytokines, such as IFN-γ with sphere

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AuNPs, and exaggerated increased production of IL-1, IL-6, and IFN-γ by AuNPs, as animal immunization with AuNPs displayed a Th1/Th2 (IFN γ and IL-10, respectively) immune response indicating the ability of these animals to develop memory T cells for protection against subsequent challenges in the future, suggesting the effectiveness of AuNPs as vaccine adjuvants in enhancing the immune response. Generally, data herein concerning AuNPs suggesting that efficient AuNPs based vaccines should be a mixture of particles of varying sizes and shapes designed to induce more broad and robust immune responses. As specific antibodies are important for protection against viral infection. The most effective type of antibody is the neutralizing one which binds to the virus, either the viral envelope or capsid proteins and blocks the virus from binding and gaining entry to the host cell [27]. The humoral immune response against rabies virus antigen as the increased humoral immune response induced by Alum-βPL inactivated rabies vaccine was time dependent, and the Alum adjuvant selectively stimulate Th2 immune response and other immunomodulatory molecule which lead to increased production of B cells that secrete Th2-cell-associated antibody isotypes IgG and immunoglobulin E (IgE) [28].

Although aluminum-based adjuvants may provide a very important and useful tool in eliciting the immunogenicity of vaccines, there may be a select group of individuals who may be predisposed to adverse consequences of Alum-containing vaccines. These consequences include its ability to exert neurological responses by only low concentrations [20], as Alum-based adjuvants were found to have minimal induction of cell-mediated immunity and elicit undesirable IgE responses. Moreover, an *in-vivo* study revealed that AuNPs activate B cells stimulating antibody synthesis and enhancing IgG secretion which is in agreement with the presented data [29]. *In-vivo* and *in-vitro* studies, the immune response by AuNPs was indicated to be shape and size-dependent, as the highest level of antibodies was found to induce after the use of spherical AuNPs [22], suggesting that AuNPs cause a greater total antigen-specific antibody response and considered AuNPs is an ideal adjuvant enhancing long-term protective immunity [30].

Several mechanisms enable any mammalian organism to defend itself against oxidative stress, including the small molecular antioxidants, such as glutathione, and antioxidant scavenging enzymes, such as GPx, SOD, and catalase [31]. In the present study, TAC level, SOD, and GPx activities increased in AuNPs adjuvanted rabies vaccine group compared to control values, while MDA levels (indicating lipid peroxidation) were decreased. These results agreed partially with Abdelhalim et al. [32] who found an increase in the GPx, TAC, glutathione, and MDA in rat kidney, liver, and lung after intraperitoneal administration of 10 nm AuNPs for 3 and 7 days, while the SOD levels significantly decreased and the level of MDA could be organ dependent. The GPx plays a crucial role in protecting cells from the damage induced by the free radicals formed by peroxide decomposition. The lipid components of the cell are especially susceptible to reactions with free radicals, resulting in lipid peroxidation. The GPx enzymes reduce peroxides to alcohols using glutathione as a substrate, thus preventing the formation of free radicals. Under physiological conditions, glutathione reductase rapidly reduces any oxidized glutathione to its thiol form (glutathione) [32]. The current results suggest an antioxidant effect of AuNPs, reflected by an increase in TAC, SOD, and GPx. As SOD is one of the most important antioxidant enzymes, it plays a central role in the defense against oxidative stress by catalyzing the dismutation of superoxide to hydrogen peroxide and oxygen [31].

MDA, the main product of fatty acid peroxidation, was also confirmed to act as a mutagen and carcinogen and causes DNA damage by the production of deoxyguanosine, deoxyadenosine, and deoxycytidine by triggering the signal transduction pathways leading to cell death or apoptosis [33]. Herein, the MDA levels were decreased by AuNPs injection which suggests increased GPx levels. The potential antioxidant ability of AuNPs and its effectiveness in quenching reactive oxygen species (ROS) included H_2O_2 and the superoxide anion radical (O_2-) in a dose-dependent manner [34].

The presented results indicated no change in TAC level, decreased MDA content, and increased SOD and GPx activities, after Alum injection, and the decline in MDA level and elevation of SOD and GPx activities were more pronounced in the AuNPs group than in Alum immunized group. In consistent with the present results, increased glutathione reductase, GPx, and SOD activities after treating rats with aluminum sulfate (1,000 ppb) in drinking water were reported [35]. They attributed these changes to Al concentration and route of administration which lead to oxidative stress and lipid peroxidation in tissues causing their damage [35], as it circulates in the blood and interferes with Fe homeostasis by displacing it from transferrin, resulting in the release of Fe into the bloodstream which interacts with molecular oxygen and generates the superoxide anion, generating highly reactive hydroxyl radical.

Alum and AuNPs adjuvanted rabies vaccine administration, induced elevated TAC levels, SOD, and GPx activities while the MDA levels declined 90 days post-vaccination compared to control and sole vaccine groups. The SOD activity increased post-AuNPs administration than post-Alum administration indicating its higher ability to eliminate free radicals. On the other hand, non-specific ROS formation after Alum-based adjuvants *in vitro* was measured and an increased ROS accompanied by decreased cell viability after 72 hours post-Alum administration was reported [20].

The histopathological profile of the liver, kidney, and spleen was examined in all groups post-vaccination. Liver sections post-Alum administration illustrated mild swelling of hepatocytes and hyperplasia of Kupffer cells [36]. In the mean times, administration of AuNPs induced mild swelling, hyperplasia of Kupffer cells, and dilation of the central vein. A cloudy swelling in the rat liver post-treatment with AuNPs was observed which might attribute to the defect in liver cell membrane function [32]. Meanwhile, rats' liver tissue examined post-administration of rabies vaccine, Alum-adsorbed vaccine, and AuNPs adjuvanted rabies vaccine showed swelling of hepatocytes. The swelling observed in all groups might be accompanied by leakage of lysosomal hydrolytic enzymes that lead to cytoplasmic degeneration and macromolecular crowding [32]. The histopathological picture of the liver in all treated groups of this study, as well as the liver function parameters, indicated a hepatotoxicity that may be induced 8 weeks later which could be attributed to accumulation of Alum or AuNPs in liver tissue depending on particle size and the duration [32].

The histopathological changes detected in the kidney revealed that both AuNPs and AuNPs adjuvanted rabies vaccine groups showed signs of kidney damage than other groups which may be attributed to the irreversible accumulation of AuNPs that differ from in the case of Alum as reported before. Renal injury represented by shrinkage of glomerular tufts and degenerative changes of the epithelial lining of the proximal convoluted tubule appeared in form of swelling and sometimes necrosis. The distribution of AuNPs in the kidneys depends on their sizes and concentration [32]. The increase in concentration and aggregation of nanoparticles can cause irreparable damage to the function and tissues of the kidneys. So, the application of small quantities of AuNPs at low concentrations in the medical field does not create serious hazards [37].

The present study revealed that the splenic rat tissues of Alum, Alum-adsorbed vaccine, and AuNPs groups showed hyperplasia of lymphoid follicles. Their germinal center contained numerous numbers of "tingible body" macrophages. The splenic tissue of the AuNPs vaccine mix group showed hyperplasia of lymphoid follicles and deposition of golden yellow hemosiderin granules. The result observed post-administration of AuNPs groups was in agreement with Fu et al. [38] who found an increase in the proliferation of lymphocytes of the splenic tissue post-treatment with AuNPs which was dose-dependent. It was confirmed that the spleen develops and produces mature immune cells which can identify and destroy pathogens [39]. Therefore, the hyperplasia of lymphoid tissues observed in this study enhanced by Alum, AuNPs sole administration or adjuvanted to rabies vaccine stimulated the immune response.

From the present data, it could be concluded that both Alum and AuNPs as adjuvants are good enhancers of the immune responses manifested by the elevation of IL-6, INF-γ, and total anti-rabies IgG levels. The AuNPs-based adjuvant elicits more immune responses which are indicated by a high increase in the INF-γ and IL-6 levels. In addition, AuNPs showed antioxidant properties evidenced by the increase of TAC and very low levels of MDA. Furthermore, the SOD activities were more increased in the AuNPs rabies vaccine mix group than in Alum adsorbed vaccine. Also, both Alum and AuNPs-based adjuvants caused alterations around the border of liver and kidney functions despite the little degeneration in liver and kidney tissues. Finally, the greater immunological responses showed that AuNPs as an immune enhancer make it better for long-term immune-enhancing potential. The undesirable effects of AuNPs may be overcome by choosing more suitable sizes, shapes, and concentrations that make them more appropriate for use in vaccine delivery.

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References

1. World Health Organization. WHO expert consultation on rabies [Internet]. Geneva: World Health Organization; 2016 [cited 2021 Nov 5]. Available from: http://www.who.int/mediacentre/factsheets/fs099/en/.
2. Francois G, Duclos P, Margolis H, et al. Vaccine safety controversies and the future of vaccination programs. Pediatr Infect Dis J 2005;24:953-61.
3. Mohan T, Verma P, Rao DN. Novel adjuvants & delivery vehicles for vaccines development: a road ahead. Indian J Med Res 2013;138:779-95.
4. Poon C, Patel AA. Organic and inorganic nanoparticle vaccines for prevention of infectious diseases. Nano Express 2020;1:012001.
5. Gupta RK. Aluminum compounds as vaccine adjuvants. Adv Drug Deliv Rev 1998;32:155-72.
6. el-Karamany RM. Production in Vero cells of an inactivated rabies vaccine from strain FRV/K for animal and human use. Acta Virol 1987;31:321-8.
7. Mohamed FA, El-Kaschef AH, Reda IM, Tantawey HM, Hassen RA. Biological studies for the importance of Rift Valley fever vaccine [dissertation]. Ismailia: Suez Canal University; 1997.
8. Engvall E, Perlmann P. Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. Immunochemistry 1971;8:871-4.
9. Drury RA, Wallington EA. Preparation and fixation of tissues. In: Carleton HM, Drury RA, Wallington EA, editors. Carleton's histological technique. 5th ed. Oxford: Oxford University Press; 1980. p. 41-54.
10. Gomez KA, Gomez AA. Statistical procedure for agriculture research. 2nd ed. Singapore: John Wiley & Sons Inc.; 1984. p. 680-8.
11. Rose WA, Okragly AJ, Patel CN, Benschop RJ. IL-33 released by alum is responsible for early cytokine production and has adjuvant properties. Sci Rep 2015;5:13146.
12. Pincus MR, Schaffer JA. Assessment of liver function. In: Henry JB, editor. Clinical diagnosis and management by laboratory methods. 20th ed. Philadelphia (PA): W.B. Saunders Company; 2001. p. 253-67.
13. Orabi SH, Mansour DA, Fathalla SI, Gadallah SM, ElDin AA, Abdoon AS. Effects of administration of 10 nm or 50 nm gold nanoparticles (AuNPs) on blood profile, liver and kidney functions in male albino rats. Indian J Biochem Biophys 2020;57:486-93.
14. Sriwijitalai W, Wiwanitkit V. Rabies, rabies vaccine, and renal failure: clinical issues. Saudi J Kidney Dis Transpl 2019;30:560-3.
15. Abdelhalim MA, Abdelmottaleb Moussa SA. The gold nanoparticle size and exposure duration effect on the liver and kidney function of rats: in vivo. Saudi J Biol Sci 2013;20:177-81.
16. Lasagna-Reeves C, Gonzalez-Romero D, Barria MA, et al. Bioaccumulation and toxicity of gold nanoparticles after repeated administration in mice. Biochem Biophys Res Commun 2010;393:649-55.
17. De Jong WH, Hagens WI, Krystek P, Burger MC, Sips AJ, Geertsma RE. Particle size-dependent organ distribution of gold nanoparticles after intravenous administration. Biomaterials 2008;29:1912-9.
18. Cruz-Tapias P, Agmon-Levin N, Israeli E, Anaya JM, Shoenfeld Y. Autoimmune (auto-inflammatory) syndrome induced by adjuvants (ASIA): animal models as a proof of concept. Curr Med Chem 2013;20:4030-6.
19. Todorovic-Rakovic N, Whitfield JR. Between immunomodulation and immunotolerance: the role of IFNγ in SARS-CoV-2 disease. Cytokine 2021;146:155637.
20. Nies I, Hidalgo K, Bondy SC, Campbell A. Distinctive cellular response to aluminum based adjuvants. Environ Toxicol Pharmacol 2020;78:103404.
21. Nam NH, Luong NH. Nanoparticles: synthesis and applications. In: Grumezescu V, Grumezescu AM, editors. Materials for biomedical engineering: inorganic micro and nanostructures. Amsterdam: Elsevier Inc.; 2019. p. 211-40.
22. Niikura K, Matsunaga T, Suzuki T, et al. Gold nanoparticles as a vaccine platform: influence of size and shape on immunological responses in vitro and in vivo. ACS Nano 2013;7:3926-38.
23. Dykman LA, Khlebtsov NG. Immunological properties of gold nanoparticles. Chem Sci 2017;8:1719-35.
24. Howe RC, Dhiman N, Ovsyannikova IG, Poland GA. Induction of CD4 T cell proliferation and in vitro Th1-like cytokine responses to measles virus. Clin Exp Immunol 2005;140:333-42.
25. McKee AS, Munks MW, MacLeod MK, et al. 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;183:4403-14.
26. Zhou Q, Zhang Y, Du J, et al. Different-sized gold nanoparticle activator/antigen increases dendritic cells accumula-

tion in liver-draining lymph nodes and CD8+ T cell responses. ACS Nano 2016;10:2678-92.
27. Paweska JT, Burt FJ, Swanepoel R. Validation of IgG-sandwich and IgM-capture ELISA for the detection of antibody to Rift Valley fever virus in humans. J Virol Methods 2005; 124:173-81.
28. Hogenesch H. Mechanism of immunopotentiation and safety of aluminum adjuvants. Front Immunol 2013;3:406.
29. Lee CH, Syu SH, Chen YS, et al. Gold nanoparticles regulate the blimp1/pax5 pathway and enhance antibody secretion in B-cells. Nanotechnology 2014;25:125103.
30. Soliman MG, Mohamed AF, El sayed RA, Elqasem AA. Immunohistochemical and histological changes in the spleen induced by gold nanoparticles as alternative adjuvant against Rift Vally fever virus. Eur J Biomed Pharm Sci 2017;4: 537-42.
31. Kakarla P, Vadluri G, Reddy Kesireddy S. Response of hepatic antioxidant system to exercise training in aging female rat. J Exp Zool A Comp Exp Biol 2005;303:203-8.
32. Abdelhalim MA, Al-Ayed MS, Moussa SA. The effects of intraperitoneal administration of gold nanoparticles size and exposure duration on oxidative and antioxidants levels in various rat organs. Pak J Pharm Sci 2015;28(2 Suppl):705-12.
33. Niedernhofer LJ, Daniels JS, Rouzer CA, Greene RE, Marnett LJ. Malondialdehyde, a product of lipid peroxidation, is mutagenic in human cells. J Biol Chem 2003;278:31426-33.
34. Barathmanikanth S, Kalishwaralal K, Sriram M, et al. Antioxidant effect of gold nanoparticles restrains hyperglycemic conditions in diabetic mice. J Nanobiotechnology 2010; 8:16.
35. Muselin F, Garban Z, Cristina RT, et al. Homeostatic changes of some trace elements in geriatric rats in the condition of oxidative stress induced by aluminum and the beneficial role of resveratrol. J Trace Elem Med Biol 2019;55:136-42.
36. Bogdanovic M, Janeva AB, Bulat P. Histopathological changes in rat liver after a single high dose of aluminium. Arh Hig Rada Toksikol 2008;59:97-101.
37. Doudi M, Setorki M. The effect of gold nanoparticle on renal function in rats. Nanomed J 2014;1:171-9.
38. Fu Y, Zhang Y, Chang X, et al. Systemic immune effects of titanium dioxide nanoparticles after repeated intratracheal instillation in rat. Int J Mol Sci 2014;15:6961-73.
39. Crane GM, Liu YC, Chadburn A. Spleen: development, anatomy and reactive lymphoid proliferations. Semin Diagn Pathol 2021;38:112-24.