New Approach for Improving Production of *Naja haje* Snake Antivenom

Heba M. Karam*, Esmat A. Shaaban*, Aly F. Mohamed, Hala F. Zaki, and Sanaa A. Kenawy**

Department of Pharmacology and Toxicology − Faculty of Pharmacy − Cairo University, Egypt
[*]Department of Drug Radiation Research−National Center for Radiation Research and Technology −Atomic Energy Authority, Egypt
[**]The Holding Company for Biological Products-Vaccines and Drugs (VACSERA), Egypt

Abstract- Snake-bite is considered a neglected tropical disease that affects thousands of people worldwide. Administration of antivenom is the corner stone in the therapy of snake bite. The study aimed to improve the production of antivenom using calcium phosphate nanoparticles (CPN) as adjuvant and gamma irradiation to detoxify venom. This was carried out by studying the toxicological and immunological properties of the *Naja haje* venom before and after exposure to 2 KGy gamma radiation. Furthermore, the cardiotoxic and hepatotoxic biomarkers of the envenomed rats were examined to compare the effect of native and gamma irradiated venoms. Moreover, in order to achieve the goal of the present study the immune response of immunized rabbits was evaluated through determination of antibody titer using ELISA technique and comparing the neutralizing capacity for lethality and enzyme activities of the serum obtained from rabbits inoculated with *Naja haje* venom in its native and γ irradiated form in presence of CPN as adjuvant or complete Freund's adjuvant. Data revealed that the toxicity of γ irradiated *Naja haje* venom was reduced 6 times as compared to the native venom. There was no change in the antigenic reactivity between both native and γ irradiated *Naja haje* venoms. Furthermore, injection of γ irradiated *Naja haje* venom did not significantly change activities of serum LDH, CPK, CK-MB, ALT and AST as compared to the normal group. In addition, serum titer produced with γ irradiated venom loaded on CPN showed highest titer as compared to other sera. Serum produced from irradiated *Naja haje* showed higher neutralizing capacity than that from native venom. All prepared antivenoms were able to neutralize the cardiotoxic and hepatotoxic biomarkers.

Index Terms- Antivenom production, ELISA, gamma irradiation, *Naja haje* venom, nanoparticles.

I. INTRODUCTION

Venomous snakes are some of the most dangerous poisoning animals in the world. Their bites may be serious depending on the amount of venom injected, the location of the bite, the size of the victim, the species of the snake and the amount of time between the bite and the injection of the right antivenin. Poisoning by snake bite is a real clinical problem, especially in tropical areas, and efficacious treatment should be available. ***(Chippaux, 1991).*** There are many varieties of snakes in Egypt, some of them cause severe damage to snake bite victims. Cobra is one of the major causes of snake bites death in Egypt. ***(Shaaban 2005)***. Cobra venoms cause death by the action of their neurotoxic and cardiotoxic components (***Mebs, 2002).*** Serotherapy is the treatment of choice in snake-bite accidents**.** Clinical investigations have established that generally antivenoms are highly effective in the neutralization of toxins responsible for systemic effects such as hemorrhage, coagulopathy, hemodynamic disturbances and neurotoxicity ***(Warrell, 2003).*** The production of therapeutic antivenoms against venoms from *Elapidae* family has proven to be very difficult where, the low molecular size of the neurotoxins confers low immunogenicity, resulting in the production of antibodies of relatively low potency ***(Ownby and Colberg 1988).*** To improve antisera production and extend the useful life of immunized horse much effort has been devoted to decrease chronic venom toxicity. Several techniques have been used to detoxify venom, for preparing effective toxoid, such as mixing the venom with adjuvant which adsorbs the venom, as aluminum hydroxide gel ***(Christensen, 1955),*** using mixture of the venom with carboxymethyl cellulose ***(Moroz et al., 1963),*** adding chemical agent as formaldehyde ***(Costa et al., 1985)***, controlled iodination of the venom ***(Daniel et al., 1987)*** and encapsulation of purified toxins in liposomes ***(Freitas and Frezard, 1997).*** Towards more effective and safer antivenins, one method that has been shown to be effective for attenuating venom toxicity and maintaining venom immunogenicity is gamma irradiation ***(Nascimento et al., 1996; Shaaban et al., 1996; Clissa et al., 1999; Souza et al., 2002; Oussedik-Oumehdi & Laraba-Djebari, 2011).***

Adjuvants are substances injected along with an antigen that are intended to enhance the immune response to the antigen. The most widely used is Freund's adjuvant but it poses a great problem in commercial antivenom production since they induce inflammation and lesions at the inoculum site leading to shortening the longevity of serum-producing animals ***(Ferreira et al. 2010).*** Many adverse effects were noted in horses used for the production of antivenin, mainly in the form of tissue reaction at the site of injection such as of edema, abscesses, myonecrosis and fibrosis. Micro and nanocarriers such as microspheres, liposomes and nanoparticles have many advantages concerning drug delivery and targeting. These advantages include high drug loading, lack of chemical interaction with drug, which is necessary for encapsulation and considerable protection of the drug molecules ***(Crommelin et al., 2001).***

In this respect, this study aimed to enhance the production of snake antivenoms through the use of gamma irradiation of *Naja haje* venom as detoxifying tool and calcium phosphate nanoparticles as an adjuvant to minimize the adverse reactions during the hyper-immunization process and reduce manufacturing costs.

II. MATERIALS AND METHODS

Animals used in the present study included New Zealand male rabbits (2.5-3 kg), Swiss albino male mice (20-25 g) and Wistar albino male rats (180-200 g). Animals were purchased from the National Research Center (Giza, Egypt). The study was conducted in accordance with the regulations approved by the Ethics Committee at Faculty of Pharmacy, Cairo University.

Venom: Lyophilized crude venom of *Naja haje* (Cobra) snake venom was kindly supplied from the laboratory animal unit of Medical Research Center, Faculty of Medicine, Ain Shams University.

Irradiation of venom: In this study, *Naja haje* venom was dissolved in saline solution (1mg/ml). Samples were subjected to radiation dose level of 2 KGy at the National Center for Radiation Research and Technology (NCRRT) using cobalt-60 Indian gamma cell (GE 4000A). The radiation dose rate was 1.26 Gy/sec at the time of experiment. This dose was selected as it gets rid of venom toxicity while maintaining immunogenicity ***(Clissa et al. 1999; Karam et al., 2010).***

Determination of lethal dose fifty (LD_{50}) of native and γ irradiated venoms. LD_{50} of native and γ irradiated *Naja haje* venoms were determined according to Spearman-karber method by ***Finney (1964).***

Evaluation of the immunological properties of native and γ irradiated venoms. Effect of γ irradiation on the immunological properties of *Naja haje* venom was evaluated using double immunodiffusion technique as described by ***Ouchterlony (1948)***. In immunodiffusion plates saline, native and γ irradiated *Naja haje* venoms solution (20 µl) were placed in peripheral wells (venom concentration were 20 mg/ml), whereas the central well was filled with 20 µl of antivenom. After developing of the precipitation bands (72 h), slides were washed and dried then stained and photographed.

Evaluation of the biochemical activities of native and γ irradiated venoms. Toxic effects of native and γ irradiated *Naja haje* venoms were evaluated through determination of the cardiotoxic and hepatotoxic biomarkers in rats. Since, the LD_{50} of native venom was measured in mice; the equivalent rat dose was calculated according to ***Paget and Barnes (1964)***. Rats were classified into three groups, each consisting of seven rats that were treated as follows:

Group I**:** received 0.1 ml saline i.p. and served as normal control.
Group II**:** received native *Naja haje* venom (0.163 mg/kg; i.p.).
Group III**:** received γ irradiated *Naja haje* venom (0.163 mg/kg; i.p.).

After 4 h of envenomation (***Mohamed et al., 1981***), rats were anesthetized by i.p. injection of urethane (1.2 g/kg) ***(Flecknell, 1987)***. Blood samples were withdrawn via the retro-orbital vein using heparinized capillary tubes (***Cocchetto and Bjornsoon, 1983***) for serum separation.

Lactate dehydrogenase (LDH) activity was measured using a test reagent kit according to the method of ***Stentz (2010),*** creatine phosphokinase (CPK) activity was measured using a test reagent kit according to the method of ***Szasz et al. (1976),*** and creatine kinase isoenzyme (CK-MB) activity was measured using a test reagent kit according to the method of ***Lott and Stang (1980).*** Moreover, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined using a test reagent kit according to the method of ***Retiman and Frankel (1957).***

Preparation and characterization of calcium phosphate nanoparticles (CPN) were prepared to be used as adjuvant for *Naja haje* venom in antivenoms preparation**.** This was performed according to the method of ***He et al. (2000).*** Particle size and morphological feature of prepared CPN was observed using transmission electron microscope TEM (JEOL JEM-1230, Japan) (Vacsera) according to the method described by ***Van der et al. (2003).*** The structure features of venom and venom loaded nanoparticles were estimated by Fourier transform infrared (FTIR) (3600 JASCO, Colchester United Kingdom) (Vacsera) at room temperature. For comparison, venom solution was measured by the same process.

Preparation of antivenoms

Rabbits were used as antivenom producing animals. They were classified into four groups each included 3 rabbits that were treated as follows:

Group 1**:** was injected s.c. with native venom emulsified in 0.5 ml complete Freund's adjuvant (CFA).
Group 2**:** was injected s.c. with 2KGy irradiated venom emulsified in 0.5 ml CFA.
Group 3: was injected s.c. with native venom loaded on calcium phosphate nanoparticles (CPN).
Group 4: was injected s.c. with 2KGy irradiated venom loaded on CPN.

Immunization was carried out as described by ***WHO (2010)***. Ten days after the final dose, rabbits were injected with a booster dose without adjuvants 500 µg/ml of native venom for groups 1 and 3 meanwhile 500 µg/ml of irradiated venom were used for groups 2 and 4. Blood samples were collected ten days thereafter. Serum was distributed in small tubes and kept at -20 °C until the moment of use for evaluation.

Evaluation of immune response post immunization using enzyme-linked immunosorbent assay (ELISA) Blood samples were withdrawn post immunization. The levels of specific antibodies in serum samples were compared using ELISA according to the method of ***Nascimento (1996)***.

Neutralization of lethality

1- *In-vitro* neutralization (Pre-incubation type assay)

This was done according to the method of ***Gutiearrez et al. (1990***). Mixtures containing a constant amount of venom (10 LD_{50}) and varying dilutions of antivenoms were incubated at 37ºC for 1h. Aliquots of the mixtures (0.5 ml) were i.p. injected into groups of six mice. Control included venom alone. Deaths were recorded after 24 h. Median effective dose (ED_{50}) was calculated by spearman-karber analysis and defined as the ratio of antivenom (µl): venom (mg) at which 50% of mice were protected

2- *In-vivo* neutralization (independent type assay)

This assay is considered as *in vivo* neutralization where independent injection of venom and antivenom attempted to simulate the natural route of envenomation. In these assays, a challenge dose of venom (2 LD_{50}) was i.p. injected first and then, at various time intervals, antivenom is administered i.v. according to the method of ***Leon et al. (2001*)**.

Neutralization of biochemical activities

It was assessed as described by ***Ghazal, et al. (1975).*** Venom was incubated with each of the prepared antivenom for 1 h at 37°C in a ratio of 1: 4 (1 mg venom to 4 ml serum). Then, aliquots of the mixtures containing a challenge dose equivalent to 1 LD_{50} of venom were i.p. injected to rats that were classified into five groups, each consisting of seven rats that were treated as follows:

***Group I*:** received native *Naja haje* venom (0.163 mg/kg; i.p.) and served as control.

***Group II*:** received a mixture of venom and the antivenom raised against native venom emulsified in complete Freund's adjuvant (CFA) (0.815 ml/kg; i.p.).

***Group III*:** received a mixture of venom and the antivenom raised against 2 KGy irradiated venom emulsified in CFA (0.815 ml/kg; i.p.).

***Group IV*:** received a mixture of venom and the antivenom raised against native venom loaded on calcium phosphate nanoparticles (CPN) (0.815 ml/kg; i.p.).

***Group V*:** received a mixture of venom and the antivenom raised against 2 KGy irradiated venom loaded on CPN (0.815 ml/kg; i.p.).

After 4 h of envenomation, rats were anesthetized by i.p. injection of urethane (1.2 g/kg). Blood was collected and used for estimation of LDH, CPK, CK-MB, ALT and AST activities.

Statistical analysis

Values were calculated as mean ± standard error (S.E) of the mean. Comparisons between different groups were carried out by one way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. The *p* value was set at ≤ 0.05.

III. RESULTS AND DISCUSSION

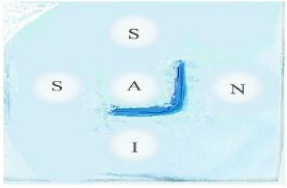
Lethal dose fifty (LD_{50}) of native and 2 KGy irradiated venoms LD_{50} for native *Naja haje* venom was 0.233 mg/kg (i.e. 4.66 µg/20 g mice) with 95% confidence limits of 0.198 to 0.273 mg/kg. Meanwhile, LD_{50} for γ irradiated *Naja haje* venom was 1.39 mg/kg (i.e. 27.8 µg/20 g mice) with 95% confidence limits of 1.276 to 1.513 mg/kg.

Lethality is the most important activity in the study of the toxicity of snake venoms ***(WHO, 1981).*** In the present study, the toxicity of *Naja haje* venom was reduced 6 times following exposure to 2 KGy gamma radiation compared to its native venom. These results were in accordance with results of ***Shaaban, (2003)*** who reported that irradiated *Naja haje* venom at dose level of 15 KGy was at least 28.1 % less toxic than non-irradiated one. Furthermore, ***Clissa et al., (1999)*** showed that the 2 KGy dose showed to be the best radiation dose to promote venom detoxification with maintenance of its immunogenicity. Moreover, ***Bennacef-Heffar and Laraba-Djebari (2003)*** showed that when, *Vipera lebtina* venom was irradiated with 1 KGy and 2 KGy, there was significant decrease in the toxicity four and nine times, respectively. Furthermore, the study of ***Caproni et al., (2009)*** added that, gamma irradiation of bothrops toxin protein leads to significant structural modifications. There is qualitative difference in the protein composition of *snake* venom as a result of gamma irradiation as, both chromatographic and electrophoretic profiles of the gamma irradiated venom were drastically changed as compared with that of the native venom ***(Shaaban et al., 2010).*** Effects of gamma irradiation on venom solution could be attributed to its known effects on protein molecules, as venoms are mainly protein in nature, as well as, ionizing radiation can change the molecular structure and the biological properties of protein molecules ***(Boni-Mitake et al., 2001).*** This can occur by two forms: direct process by which ionizing radiation interacts directly on target molecules and an indirect process by which the product generated by water radiolysis, like e-, O_2^-, H° and OH° interact with target molecules and can modify the biological activity of protein and peptides by reacting with certain sites or groups in the molecule ***(Garrison, 1987; Casare et al., 2006).*** These radicals act by removing hydrogen, breaking disulfide bonds, promoting deamination as well as inducing the formation of intramolecular and intermolecular covalent bonds (***Alexander & Hamilton 1962; Halliwell & Gutteridge, 1989).*** These structural changes result in a decrease or loss of the enzymatic and biological activities of the proteins ***(Gallacci et al., 2000).***

Immunological properties of native and γ irradiated venoms

Results of the double immunodiffusion test showed that, there was no change in the antigenic reactivity of native and 2 KGy *Naja haje* venom. The visible lines obtained were identical, continuous and joined smoothly at the corner, indicating that there was no change in antigenic determinants i.e. the antivenom cannot distinguish between the native and γ irradiated *Naja haje* venoms as they are immunologically identical (Figure 1).

This finding is in harmony with that of ***Rogero & Nascimento, (1995)*** who reported that, the part of protein responsible for toxicity of the venom was associated with the radio-labile group while, the immunogenic part of the venom was located in a confined portion, which was either resistant to gamma radiation or was structurally shielded from it. Radiation is able to induce changes in the structural and antigenic properties of egg albumin and bovine serum albumin. This finding is attributed to that, the main part of conformation dependent antigenic structures (conformational epitopes) is easily lost by radiation, but some antigenicity, which is mostly due to the amino acid sequence-dependent antigenic structures (sequential epitopes) remain, even at high doses ***(Kume & Matsuda, 1995)***.



S = Saline

A = Antivenom

N = Native *Naja haje* venom.

I = Irradiated *Naja haje* venom

Figure (1): Immunodiffusion reaction of commercial polyvalent antivenom with native and γ irradiated *Naja haje* venoms.

Biochemical activities of native and γ irradiated venoms

Activities of serum LDH, CPK and CK-MB of normal group were 626.40 ± 44.01 U/l, 410.80 ± 37.17 U/l and 278.76 ± 25.08 U/l, respectively. Injection of native *Naja haje* venom significantly elevated activities of serum LDH, CPK and CK-MB by 49.82%, 267.87% and 80.15%, respectively as compared to the normal group. In addition, activities of serum ALT and AST of normal group were 70.60 ± 1.51 U/l and 132.40 ± 1.69 U/l, respectively. Injection of native *Naja haje* venom significantly elevated activities of serum ALT and AST by 208.16% and 99.98%, respectively as compared to the normal group. On the other hand, injection of γ irradiated *Naja haje* venom did not significantly change activities of serum LDH, CPK, CK-MB, ALT and AST as compared to the normal group (Table 1).

Thus, the results of this study clearly demonstrated that a single injection of native *Naja haje* venom at a dose equal its LD_{50} caused a significant elevation in activities of serum LDH, CPK, CK-MB, ALT and AST as compared to the normal group**.** The present increase in enzymes activities due to *Naja haje* envenomations was in accordance with the results of the studies carried out by ***Fernando et al., (1989)*** who reported that *B. asper* venom caused serum AST, LDH and CPK to increase significantly, the highest peak being observed at 3h in the cases of AST and CPK, and at 6 h in the case of LDH. Furthermore, in a study by ***Aguiyi et al., (2001),*** the effect of lethal *Echis carinatus* venom on serum enzyme levels and blood plasma coagulation parameters in rats subjected to (i.p.) venom injection was investigated. Measurements of the enzyme and coagulation parameter levels 4 h after venom administration showed an increase in the level of enzymes; creatinine phosphokinase (CPK), lactate dehydrogenase (LDH) and glutamic pyruvic transaminase (ALT) as well as a change in the level of coagulation parameters due to envenomation. ***Mebs et al., (1983)*** suggested that, the increase in enzymatic activities of CPK and CPK-MB in the serum could be explained by an increase of the permeability of the cell membrane. Elevation of creatine phosphokinase is an indication of damage to muscle, therefore indicative of injury, myocardial infarction, muscular dystrophy and myocarditis ***(Wallimann & Hemmer, 1994).***

In addition, ***Shaaban & Hafez (2003)*** reported that, tissue destruction occurs in most of the organs secondary to venom injections. The increase in enzymatic activity of the serum attributed to the release of enzymes from liver, kidney and heart. Organ damage is followed by an increase in levels of ALT, AST and ALP.

However, the 2KGy gamma irradiated *Naje haje* venom, showed non-significant changes in the rats' serum LDH, CPK, CK-MB, ALT and AST compared to the normal rats. These findings were attributed to loss of the myotoxic activity of snake venoms as a secondary event following the exposure to gamma radiation. Previous studies have emphasized that irradiation of protein has been shown to cause several chemical changes and alterations of the physico-chemical properties and of the secondary and tertiary structure of the proteins, all these changes are closely connected with the loss of enzymatic, hormonal and toxic activity of venom after irradiation ***(Skalka & Antoni, 1970; Souza-Filho et al., 1992).*** This is attributed to the disorganization of the molecular structure of venom after exposure to gamma radiation, resulting in a change in its biological activity ***(Shaaban et al., 1996; Hayes, 2001).*** In this respect, radiation is able to detoxify snake venoms and decrease its harmful effects. In this context, gamma radiation has showed to be a promising tool for snake venom detoxification without affecting their immunogenic properties.

Table (1): **Effect of native and γ irradiated *Naja haje* snake venoms on serum lactate dehydrogenase (LDH), creatine phosphokinase (CPK), creatine kinase isoenzyme (CK-MB), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in rats**

Groups / Parameters	Normal (Saline) (0.1 ml; i.p.)	Native *Naja haje* (0.163 mg/kg; i.p.)	Irradiated *Naja haje* (0.163 mg/kg; i.p.)
LDH (U/l)	626.40 ± 44.01	938.50^{*} ± 33.57	$726.22^{\#}$ ± 24.27
CPK (U/l)	410.80 ± 37.17	1510.46^{*} ± 78.82	$709.57^{\#}$ ± 50.64
CK-MB (U/l)	278.76 ± 25.08	502.20^{*} ± 9.58	$330.21^{\#}$ ± 29.88
ALT (U/l)	70.60 ± 1.51	217.56* ±1.32	$83.71^{\#}$ ± 2.96
AST (U/l)	132.40 ± 1.69	264.77* ± 1.14	$149.72^{\#}$ ± 2.55

Native and γ irradiated *Naja haje* were injected as single doses (a dose equivalent to native LD50). Blood samples were collected 4 h thereafter.
Each value represents the mean ± S.E (n=7).
Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer multiple comparison test.
*Significantly different from the normal group at $p \leq 0.05$.
#Significantly different from native *Naja haje* group at $p \leq 0.05$.

Characterization of calcium phosphate nanoparticles

Electron microscope scanning was used to determine the adjuvant particle characteristics (shape, size). The particles are spherical in shape and uniformly distributed (mono dispersed) without significant agglomeration. The particles size ranges from 155 to 274 nm and possess an average size of ≈ 225 nm although very tiny particles have also been observed that may be due to vigorous shaking (Figure 2).

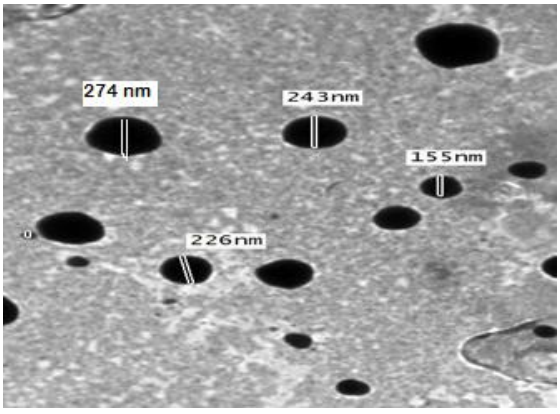


Figure (2): The morphological characteristics of nanoparticles were investigated using transmission electron microscope (TEM).

Fourier transform infrared (FTIR) measurements

On the basis of the FTIR spectra, a slight difference in the width and frequency of the peaks can be observed between venom loaded and unloaded nanoparticles.

In figure (3a) strong and broad peaks at the 3459 cm^{-1} correspond to O–H stretching and intermolecular hydrogen bonding. The peak at 2078 cm^{-1} belong to N-H stretching vibration. At wave numbers of 1639 cm^{-1} and 1442 cm^{-1} observed peaks belong to the C=O stretching (amide) and, N-H bending in primary amine with carbonyl group (C-N) stretching vibration in amide, respectively. The peak at 559 cm^{-1} belong to C=O bending (amide). Meanwhile, in figure (3b) appearance of P=O peak at 1014 cm^{-1} due to interaction of venom and calcium phosphate nanoparticles. Moreover, the peak in N-H stretching vibration shifted to 1388 cm^{-1} after interaction with CPN.

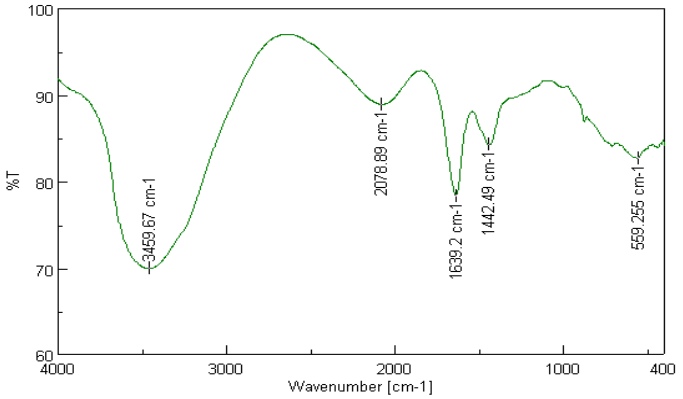


Figure (3a): Fourier transform infrared spectrum of *Naja haje* venom.

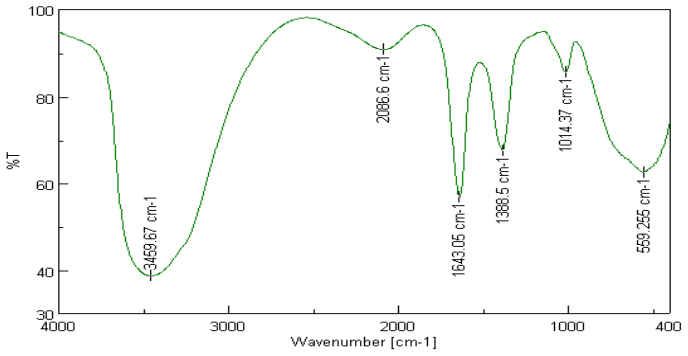


Figure (3b): Fourier transform infrared spectrum of calcium phosphate nanoparticles (CPN) loaded venom.

This study was extend to compare the prepared antivenoms, analysis of ELISA results found that, the sera raised against irradiated venom loaded on calcium phosphate nanoparticles (CPN) showed highest titer as compared to other sera. This was in accordance with ***Abde el-razek et al., (2011)*** who reported that, CPN formulated vaccine showed a highly significant and long durative antibodies level than that detected post immunization with alum formulated vaccine.

In addition, ***He et al. (2000)*** reported that, CPN was more potent as an adjuvant than alum, elicited little or no inflammation at the site of administration and induced high titers of immunoglobulin antibody for herpes simplex virus as compared to the commonly used aluminum adjuvants. Additional benefits that CPN is a natural constituent of the human body. Thus, CPN is very well tolerated and absorbed. There is support for this concept in recent studies by ***Maughan et al. (2015)*** and ***Olmedo et al. (2013)*** who recorded that, the comparison of the adjuvant activity of aluminum hydroxide and calcium phosphate on the antibody response towards *Bothrops asper* snake venom revealed that, venom adsorbed on calcium phosphate induced a higher antibody response towards all tested fractions of the venom.

The mechanism by which CPN act as adjuvant was explained by ***He et al. (2000)*** who reported that, CPN is believed to act similarly to alum by releasing the antigen slowly over an extended period of time. This is a technique in which some antigens are entrapped in the core of the final CPN formulation, which helps to boost immune responses over an extended time and which has the potential to reduce the antigen dose required for immunization. Although there was no way to show the kinetics of antigen release from CPN, it is reasonable to assume that the surface antigens are released from CPN immediately after injection. Conceivably, then, the antigens from the "core" of the CAP would continue to be released as CPN dissolves, thus making antigen available to stimulate the host's immune system over an extended period of time.

Enzyme linked immunosorbent assay (ELISA)

Analysis of ELISA showed that, the sera pool from hyper-immunized rabbits with irradiated venom had titers higher than those of sera pool from animals hyper immunized with native venom whatever the used adjuvant (Figure 4).

This was confirmed by the findings of ***Caproni et al., (2009)***, who reported that, irradiated toxins were immunogenic and antibodies elicited by them were able to recognize the native toxin in ELISA. Other studies had already proven that, when *Vipera lebetina* venom was detoxified by gamma rays and used for active immunization, presence of antibody in the immune sera was detected by ELISA. Thus, immunogenic properties were preserved and the antisera obtained with the irradiated venoms could cross-react and were able to neutralize the toxic effect of native *Vipera lebetina* venom ***(Bennacef-Heffar and Laraba-Djebari, 2003).*** This occurs because the irradiation promotes molecule oxidation, facilitating its phagocytosis due to the presence of scavenger receptors in the macrophages surface. Moreover, it is known that antigens, as they enter the organism, suffer an oxidation process by the defensive cells to facilitate phagocytosis. In irradiated samples, macrophages already find these molecules oxidized, and therefore, they eliminate this step of the process. A better processing, associated to a faster antigen presentation, makes the immune system produce more complete antibodies against a higher number of antigen epitopes ***(Rogero and Nascimento 1995).***

It must be pointed out that, in this study, five rabbits died after the first inoculation with native *Naja haje* venom emulsified in CFA and one rabbit after the first inoculation with native *Naja haje* venom loaded on CPN, meanwhile there was no mortality in other groups. It was attributed to the high toxicity of native venom which was reduced 6 times following exposure to 2 KGy gamma radiation. This is in agreement with the previous study of

Souza et al. (2002) who concluded that the radiation is able to abolish paralysis caused by the venom. According to the same authors, these facts support the hypothesis that gamma radiation may be an important tool to improve sera production, since it reduces venoms toxicity, preserving immunogenicity. Taken together, these data indicate that CPN as adjuvant induces very

little inflammation at the site of entry, and CPN loaded irradiated venom induce a higher titer relative to the responses induced by CFA, On the basis of these observations, we suggest that CPN present an improved alternative to CFA, especially for snake antivenoms production.

This was in harmony with ***Shaaban et al., (2010)*** who reported that, the antivenom raised against 1.5 kGy γ irradiated *Echis pyramidum* venom is quite as effective as the antivenom raised against the native venom in neutralizing the lethality induced by native venom.

In this respect ***Guittrez et al. (1990)*** reported that, when standardizing the neutralizing ability of an antivenom, pre-incubation type of experiments are used more extensively, since results do not depend on pharmacokinetics of venom and antivenom, but instead on concentration and neutralizing ability of antibodies present in the antivenom. Meanwhile, this study

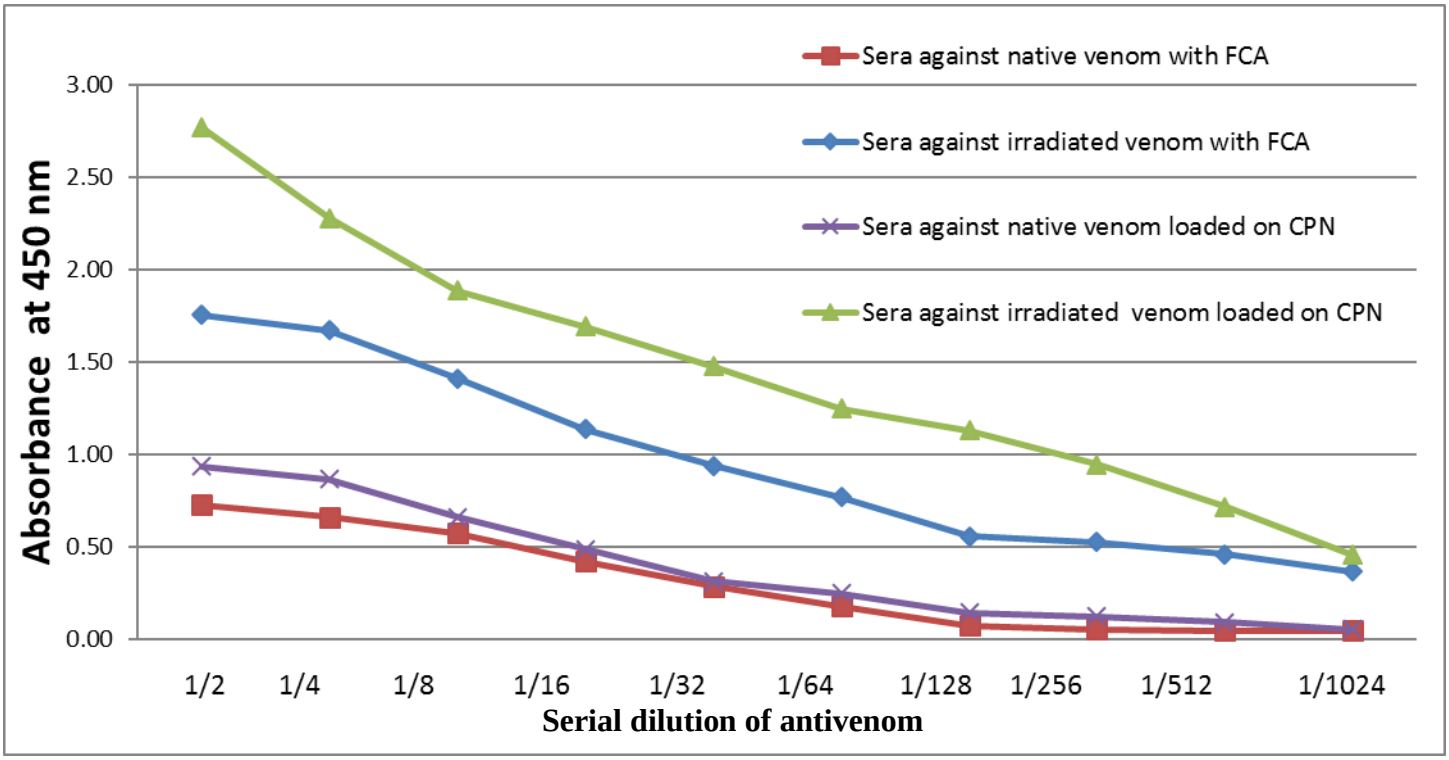


Figure (4): Comparative evaluation of immune response post immunization with native and 2 KGy irradiated *Naja haje* venoms with complete Freund's adjuvant (CFA) or loaded on calcium phosphate nanoparticles (CPN) using ELISA.

Neutralization of lethality

1- *In-vitro* neutralization (Pre-incubation type)

All the prepared antivenoms neutralized the lethal effects of *Naja haje* venom in pre-incubation type experiments as, ED_{50} for antivenom raised against native venom emulsified in complete Freund's adjuvant (CFA) was 38.02 µg/ml with 95% confidence limits of 33.60 to 43.01 µg/ml. ED_{50} for antivenom raised against irradiated venom emulsified in CFA was 60.26 µg/ml with 95% confidence limits of 46.95 to 77.34 µg/ml. ED_{50} for antivenom raised against native venom loaded on calcium phosphate nanoparticles (CPN) was 39.81 µg/ml with 95% confidence limits of 30.14 to 52.54 µg/ml. ED_{50} for antivenom raised against irradiated venom loaded on CPN was 56.23 µg/ml with 95% confidence limits of 49.70 to 63.62 µg/ml.

This results were in accordance with ***Ferrier et al. (2006)*** who reported that neutralizing capacity of antivenoms raised against irradiated *Crotalus durisss* venom was higher than that of antivenom raised against native venom.

observed that, antivenoms prepared using complete Freund's adjuvant were equipotent to that prepared using calcium phosphate nanoparticles as adjuvant.

This was in harmony with ***Soares (2012)*** who mentioned that, when the chitosan nanoparticles applied as an adjuvant in for venom immunization, it can provide considerably effective immune response and may promote production of antibody equivalent to aluminum hydroxide, but with the added advantage of being less or non-inflammatory and it can provide a modified release of antigen, which can promote antibody titers in serum with the administration of a smaller amount of antigen.

2- *In-vivo* neutralization (independent type)

Results of *in vivo* neutralization assay revealed that, when 2 LD_{50} of venom was injected i.p. all mice dead at 40 ± 3 min. on the other hand, when antivenom (0.2 ml) was administered i.v. immediately after envenomation, lethality was reduced markedly. However, when antivenom administration was delayed or 15 min. neutralization was partial, while when antivenom

administration was delayed for 30 min. all the mice died (Table 2). The antivenom produced against irradiated venom loaded on CPN showed significant differences compared to the antivenom produced from immunization with native venom and complete Freund's adjuvant. The efficacy of antivenom for reversal of cytotoxic manifestations occurring after envenomation is time limited. This was in accordance with the finding of ***Leon et al. (2001)*** who showed that there was no significant differences between antivenoms concerning neutralization of lethality by either i.v. or i.m. administration of antivenoms. Moreover, results indicated that, the sooner antivenoms was injected, the higher is its neutralizing activity. In agreement with the results of ***Gutierrez et al. (1991)*** antivenoms were effective only when administered immediately after envenomation, and this is probably due to the rapid action of *Nigrocinctus* myotoxins on muscle cells. The principle by which independent injection type assay was described by ***Lomonte et al. (2009)*** who reported that, a number of factors that are extrinsic to the neutralizing ability of an antivenoms may have a major influence upon its effectiveness and final outcome. Among these factors stand pharmacodynamics and pharmacokinetic parameters for the distribution of both venom components and antivenom, as well as the velocity by which the particular toxic effects developed. Therefore, it is recommended to use antivenom as early as possible whether the systemic sign and symptoms is present or not i.e. even before the occurrence of serious cytotoxic effect on various organs ***(Zayerzadeh et al. 2011)***.

Table (2): Neutralization of lethality of *Naja haje* venom by the prepared antivenoms using independent injection type assay.

Antivenom	Mortality (%)		
	Delay in antivenom administration (min)		
	0	15	30
Antivenom raised against native venom emulsified in CFA	60 %	40 %	100 %
Antivenom raised against irradiated venom emulsified in CFA	40 %	60 %	100 %
Antivenom raised against native venom loaded on CPN	40 %	60 %	100 %
Antivenom raised against irradiated venom loaded on CPN	20 %	40 %	100 %

Groups of five mice were injected i.p. with a challenge dose (2 LD_{50}) then at various time intervals, mice received 0.2ml of each of the prepared antivenoms by i.v. route, death were recorded during 24 h.
CFA: Complete Freund's adjuvant.
CPN: Calcium phosphate adjuvant.

Neutralization of biochemical activities

Effect of native *Naja haje* venom and mixtures of the prepared antivenoms on serum lactate dehydrogenase (LDH), creatine phosphokinase (CPK), creatine kinase isoenzyme (CK-MB), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in rats.

Injection of the incubated mixture of venom and antivenom prepared from native venom emulsified in CFA (in a dose equivalent to native LD_{50}) significantly reduced the activities of serum LDH, CPK, CK-MB, ALT and AST by 17.88%, 58.92%, 26.84%, 60.54% and 47.39%, respectively as compared to the native venom (control) group.

Injection of the incubated mixture of venom and antivenom prepared from irradiated venom emulsified in CFA (in a dose equivalent to native LD_{50}) significantly reduced the activities of serum LDH, CPK, CK-MB, ALT and AST by 16.97%, 53.65%, 35.42%, 62.78% and 41.45%, respectively, as compared to the native venom (control) group.

Injection of the incubated mixture of venom and antivenom prepared from native venom loaded on CPN (in a dose equivalent to native LD_{50}) significantly reduced the activities of serum LDH, CPK, CK-MB, ALT and AST 16.18%, 52.80%, 31.73%, 59.61% and 42.68%, respectively as compared to the native venom (control) group.

Injection of the incubated mixture of venom and antivenom prepared from γ irradiated venom loaded on CPN (in a dose equivalent to native LD_{50}) significantly reduced the activities of serum LDH, CPK, CK-MB, ALT and AST by 17.07%, 62.43%, 41.16%, 62.68% and 46.61%, respectively as compared to the native venom (control) group. The percentage inhibition was calculated by considering the effect induced by venom alone as 100% activity. It was observed that, there was a significant change in the activity of serum CK-MB after the injection of the incubated mixture of venom and the antivenom prepared from γ irradiated venom loaded on CPN as compared to the group injected with the incubated mixture of venom and antivenom prepared from native venom emulsified in CFA (Figures 5 & 6).

Data of the present study indicates that, all the prepared antivenoms have a protective action against cardiotoxicity and hepatotoxicity venom almost to the same extent. It is interesting to note that, although much work was published about the protection against venom induce lethality, little and scattered work was published concerning the neutralization efficacy of antivenoms against the pharmacological and biochemical action of venoms despite, the study of the neutralization of other clinically relevant effects is highly important to gain a more comprehensive picture of the efficacy of an antivenom.

The study of ***Chaves et al. (1995)*** added that, antivenom neutralized venom-induced increases in serum enzyme levels following pre-incubation with venom, indicating that antivenoms contains antibodies against tissue-damaging toxins. ***Gutierrez et al. (1987)*** has been suggested that, the antivenoms contains antibodies capable of preventing and neutralizing the toxic and enzymatic activities of the venom**.**

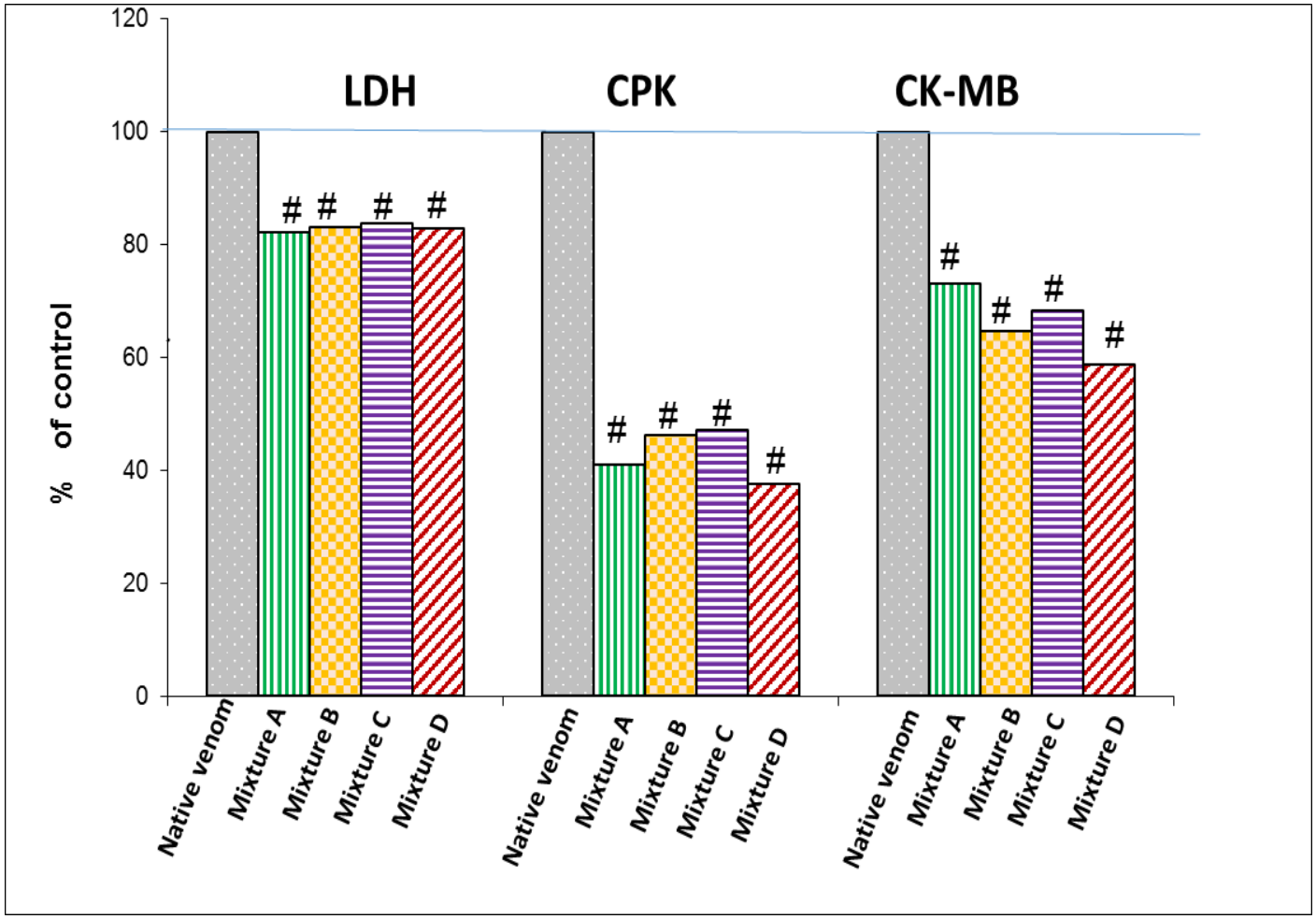


Figure (5): Effect of native *Naja haje* venom and mixtures of the prepared antivenoms on serum lactate dehydrogenase (LDH), creatine phosphokinase (CPK) and creatine kinase isoenzyme (CK-MB) activities in rats.

Native *Naja haje* venom (0.163 mg/kg; i.p.) and mixtures of the prepared antivenoms (0.815 mg/kg; i.p.) were injected as single doses. Blood samples were collected 4 h thereafter.
Mixture A: incubated mixture of venom and antivenom prepared from native venom emulsified in complete Freund's adjuvant (CFA) in a ratio of 1: 4.
Mixture B: incubated mixture of venom and antivenom prepared from irradiated venom emulsified in CFA in a ratio of 1: 4.
Mixture C: incubated mixture of venom and antivenom prepared from native venom loaded on calcium phosphate nanoparticles (CPN) in a ratio of 1: 4.
Mixture D: incubated mixture of venom and antivenom prepared from irradiated venom loaded on CPN in a ratio of 1: 4.

Each value represents the mean ± S.E. (n=7).
Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer multiple comparison test.
[#]Significantly different from native *Naja haje* group at $p \leq 0.05$.
[@]Significantly different from mixture A group at $p \leq 0.05$.

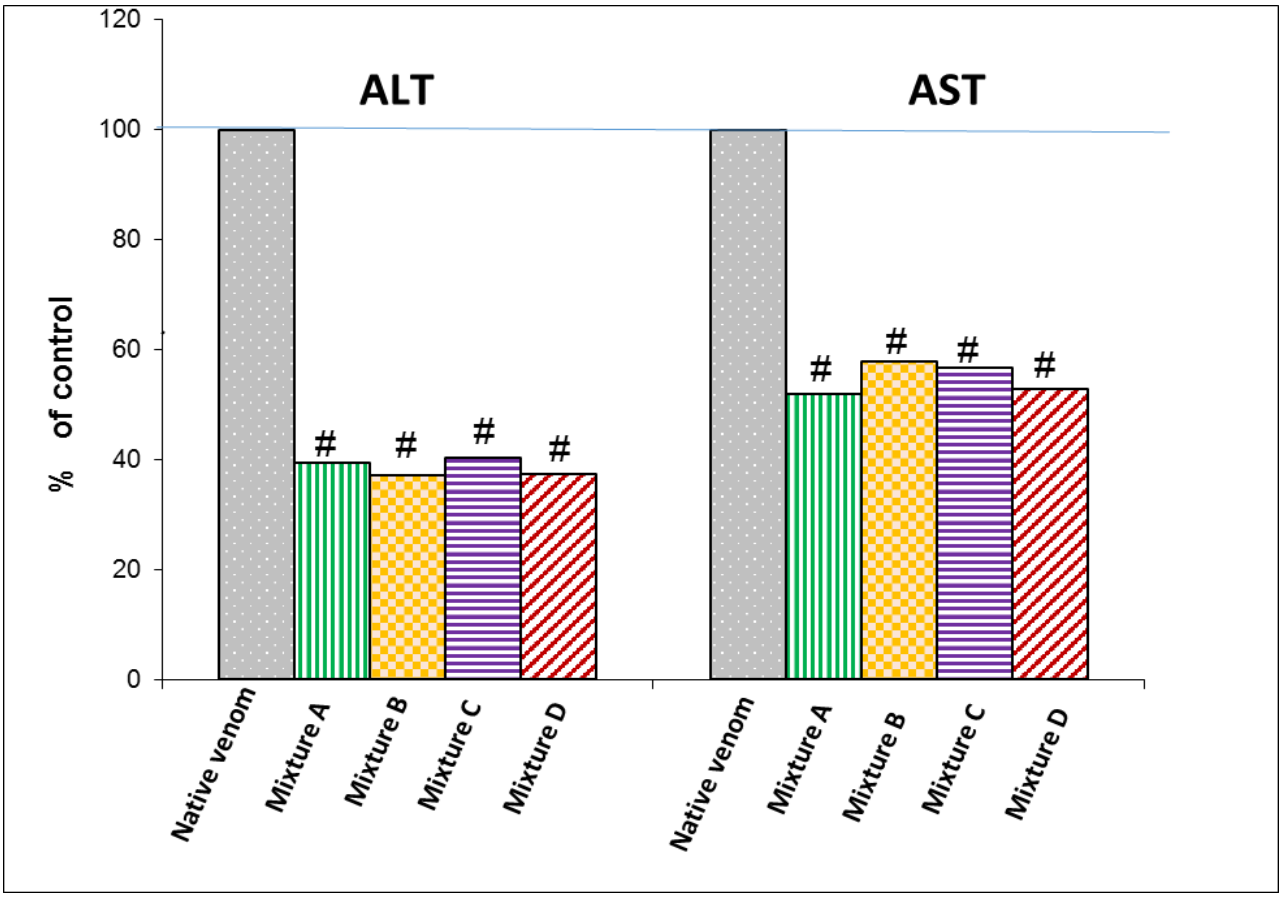


Figure (6): Effect of native *Naja haje* venom and mixtures of the prepared antivenoms on serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in rats.

Native *Naja haje* venom (0.163 mg/kg; i.p.) and mixtures of the prepared antivenoms (0.815 mg/kg; i.p.) were injected as single doses. Blood samples were collected 4 h thereafter.

Mixture A: incubated mixture of venom and antivenom prepared from native venom emulsified in complete Freund's adjuvant (CFA) in a ratio of 1: 4.
Mixture B: incubated mixture of venom and antivenom prepared from irradiated venom emulsified in CFA in a ratio of 1: 4.
Mixture C: incubated mixture of venom and antivenom prepared from native venom loaded on calcium phosphate nanoparticles (CPN) in a ratio of 1: 4.
Mixture D: incubated mixture of venom and antivenom prepared from irradiated venom loaded on CPN in a ratio of 1: 4.

Each value represents the mean ± S.E. (n=7).
Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer multiple comparison test.
[#]Significantly different from native *Naja haje* group at $p \leq 0.05$.

IV. CONCLUSION

In conclusion, based on the experimental findings, irradiation was found to be a reliable tool in detoxification of venoms, minimizing the toxic effect while maintaining the immunogenicity. In addition, calcium phosphate nanoparticles when applied as adjuvant, provide enhancement of immune response with the adjuvant of being less or non-inflammatory and it can provide a modified release of antigen, which can promote obtaining antibody titers in serum with the administration of a smaller amount of antigen. Taken together, this study showed an immunization adjuvant system for *Naja haje* snake venom that should be tested with venom of other snakes. Thus, this approach allows achieving new biotechnological antivenoms to be used in the future.

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AUTHORS

Heba M. Karam- Assistant Lecturer in Drug Radiation Research Department − National Center for Radiation Research and Technology − Atomic Energy Authority, Egypt. Heba.karam.mohmed@gmail.com,

Esmat A. Shaaban- Professor of Pharmacology & Toxicology − National Center for Radiation Research and Technology − Atomic Energy Authority**,** Egypt. Esmat_shaaban@yahoo.com

Aly F. Mohamed −Head of Applied Research Sector − Holding Company for Biological Products, Vaccines and Drug (VACSERA), Egypt**.** Fahmy.aly@gmail.com

Hala F. Zaki − Professor of Pharmacology & Toxicology Faculty of Pharmacy − Cairo University. Halafzaki@gmail.com

Sanaa A. Kenawy − Professor of Pharmacology & Toxicology Faculty of Pharmacy − Cairo University. Sanaa_kenawy@hotmail.com

Correspondence Author – Heba M. Karam, Heba.karam.mohmed@gmail.com, or dr_heba2005@yahoo.com, +201114786345