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Hematobiochemical and Immunological Profiles of Horses Used for Preparation of Antitetanic hyperimmune Serum by Different Types of Adjuvants

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ABSTRACT:

This study aimed to characterize the hematobiochemical and immunological profiles following immunization of equine with tetanus toxoid or/and toxin for obtaining antitetanic hyperimmune serum with special reference to the role of adjuvants in boosting immune response to immunization. Forty- Five apparently healthy horses were divided into 9 groups, one control group and other 8 groups were subjected to 8 vaccination protocols including tetanus toxoid or/ and toxin with or without adjuvants according to the experimental design of this study. Blood samples were collected from all horses and were assayed for the selected hematobiochemical and immunological parameters. Hematologically, there was a significant decreasesin the mean values of RBC, Hb, PCV, MCV and platelet in all vaccinated groups compared to control one. MCH and MCHC values showed a significant increases in immunized horses except in group administered tetanus toxoid combined with CFA whereas their values exhibited a significant decreases. The mean values of TLC, neutrophil, lymphocyte, and monocyte counts exhibited a significant increase in all vaccinated groups with or without addition of adjuvants. The results of serum biochemical parameters showed significant reduction in serum albumin levels and marked elevation in the concentrations of total protein, globulin, total bilirubin, direct bilirubin, indirect bilirubin as well as serum enzymatic activities of AST and LDH. Regarding the immunological responses, all vaccinated groups demonstrated a significant increase in the limits of flocculation, IgG and α1, α2 and γ-globulins compared to control particularly in groups injected with a combination tetanus toxoid and toxin. For most tested parameters the effects were more pronounced when CFA was added to the vaccination protocols. In conclusion, the presented results can consider the Freund adjuvants as apotent enhancers of immune response to tetanus vaccines for production of high titer hyperimmune serum with few side effects and thus high safety margin. However, the repeated use of horses for preparation of antitetanic hyperimmune serum should be

avoided as it can result in chronic inflammatory stimulus leading to various health hazards particularly on liver.

Keywords: Equine, Freund Adjuvants, Hyperimmune serum, Tetanus and Toxoid.

INTRODUCTION

Tetanus is a potentially fatal bacterial disease that can affect most animals and humans and caused by Clostridium tetani, a well-known Gram-positive spore forming motile, anaerobic bacterium (Brook, 2018). The bacterium is an obligatory anaerobe, and its spores can be found in a variety of substrates, including dirt, rust, and dust.

Because the disease's spore can survive in soil for long periods of time, it has a good chance of entering the body through any wound, particularly deep puncture lacerated wounds (Brüggemann et al., 2003).In contaminated wounds, in the presence of necrotic tissue with lower oxygen potential, the spores germinate into metabolically active vegetative rod-shaped bacterium that generates a potent metalloprotease neurotoxin known as tetanospasmin (Thwaites et al., 2015; Yen and Thwaites, 2019 and Cardinal et al., 2020).

Most mammals are susceptible to tetanus, but horses and humans appear to be the most sensitive of all species because of their environment and tendency to suffer injuries (Immunization handbook, 2020).

Despite tetanus eradication is difficult, but fortunately, it is an easily preventable disease and vaccination programs are now reducing the occurrence and prevalence of tetanus around the world. Immunization to tetanus is antibody-mediated and can be accomplished with tetanus toxoid which is introduced as vaccine to animals and humans to elicit an active immunization by stimulating theindividual's own immune system to produce anti-tetanus antibodies (antitoxin) to cure or fight against toxin-based diseases (Bae and Bourge, 2020).

Injection of antitetanic hyperimmune serum containing tetanus antitoxin provides passive immunity in situations where there is risk of tetanus to protect against infection or to treat a disease the individuals have been exposed to and there is no sufficient time for the body to develop its own immune response (Lasocka et al., 2021).

Specifically, antitetanic hyperimmune serum prepared in equine has evolved into essential, effective therapeutic medicines that are widely acknowledged by most equine practitioners and used in equine clinics throughout the last few decades (Roberts et al., 2012).

Adjuvants are important components of vaccinations that have proven to be particularly efficient in increasing protective humoral immunity against infectious illnesses and reducing mortality and morbidity (Wu and Liu, 2021). Adjuvants come in a variety of forms, each with its own mechanism of action, therefore the selection of appropriate adjuvant is influenced by its mechanism of action, animal species, specific pathogens, vaccine antigens, vaccination technique, and type of immunity required (Spickler and Roth, 2003).

Freund's adjuvant is one of the most effective adjuvants currently available, it is essential for the protocols of production of high titer antibodies and also for research, but it has its own advantages and disadvantages (Nanishi et al., 2020).

Many earlier studies gave a comprehensive description of the tetanus's etiology, diagnostic processes, pathogen checklists, and treatment and prevention strategies. However, there is limited information on some features of using Freund's adjuvant in eliciting an immune response to tetanus antigenicity in relation to changes in clinicopathological parameters.

Therefore, the aim of the current study was to characterize the hematobiochemical and immunological profiles of horses used for obtaining antitetanic hyperimmune serum in response to toxoid and/or toxin immunizationwith more light shedding on the role of Freund's adjuvants in boosting immune response to vaccination to explore their clinical importance and application. Some indicators were also measured to evaluate how these adjuvants affected equine health.

MATERIALS AND METHODS

Experimental animals:

This study was conducted on forty-five male native horses of 5-8 years oldobtained from Egyptian Company for Production of Vaccines, Sera & Drugs (VACSERA). The newly purchased horses were quarantined for at least 2 weeks in the quarantined area. During quarantined period, horses were continuously monitored for health condition, lameness, bad habits and infectious diseases. The horses were apparently healthy and free from internal and external parasites based on physical and laboratory examinations. The experiment was carried out under a strict hygienic condition.Each horse was immunized with 2 ml of tetanus toxoid (40LF) (VACSERA), Giza, Egypt as a self-vaccination against tetanus. All horses were housed in barn 2 in Helwan farm, supplied with ration containing barley, alfalfa and hay and received ad-libitum access to clean tap water throughout the experimental period.

Vaccines

Tetanus toxoid (20 Lf /ml) and tetanus toxin (20 Lf /ml) were supplied by the Tetanus Department, Holding Company for Biological Products and Vaccines (VACSERA), Giza, Egypt.Doses of vaccines were approved according to the WHO manual for the production and control of tetanus toxoid vaccines (1977) and WHO guidelines for the production, control, and regulation of the snake antivenomimmunoglobulins (2010).

Adjuvants

Two forms of Freund adjuvant were used in this study, the incomplete Freund adjuvant (IFA) and complete Freund adjuvant (CFA). Freund adjuvant is a solution of antigen emulsified in mineral oil and used as an immunopotentiator (booster). The CFA is composed of inactivated and dried mycobacteria (usually M. tuberculosis) (Each ml of CFA contains 1mg of heat killed dried mycobacterium tuberculosis, 0.85 ml paraffin oil and 0.15 ml of mannidemonooleate). The IFA contains just water in oil emulsion including 0.85 ml paraffin oil and 0.15 ml of mannidemonooleate without the mycobacterial components (Chotwiwatthanakun et al., 2001). These adjuvants were supplied by Sigma-Aldrich® Brand (St. Louis, Missouri, USA).

Animal grouping and experimental design:

Horses were randomly divided into nine groups, each of 5. Horses were subjected to different vaccinations protocols as shown in the following table (1).

Table 1: Animal grouping and experimental design:

Groups	Description of vaccination protocol
G1	Control untreated group
G2	Injected with 2 ml of incomplete Freund adjuvant subcutaneously twice with 2 weeks interval.
G3	Injected with 2 ml of complete Freund adjuvant subcutaneously twice with 2 weeks interval.
G4	Injected with gradually increased doses oftetanus toxoid (150 Lf, 300 Lf, 450 Lf and 750 Lf/ml) (equivalent to7.5, 15, 22.5 and 37.5-ml tetanus toxoid respectively) subcutaneously with 5 days interval between doses.
G5	Similar to protocol 3 with the addition of 2 ml of incomplete Freund adjuvant to each toxoid dose.
G6	Similar to protocol 3 with the addition of 2 ml of complete Freund adjuvant to each toxoid dose.
G7	Administered protocol 3 (tetanus toxoid injection) then 2 weeks after the last toxoid dose, gradually increased doses of tetanus toxin (150 Lf, 300 Lf, 450 Lf and 750 Lf/ml) (equivalent to7.5, 15, 22.5 and 37.5ml tetanus toxin respectively) were injected subcutaneously with 5 days interval between doses.
G8	Similar to protocol 6 with the addition of 2ml of incomplete Freund adjuvant to each toxoid and toxin dose.
G9	Similar to protocol 6 with the addition of 2ml of complete Freund adjuvant to each toxoid and toxin dose.

Samples:

Blood samples were collected from the jugular veins of all horses 2 weeks following the last dose of treatments. Blood samples were divided into two portions. The first portion was collected on disodium ethylene diaminetetracetic acid (EDTA) for hematological assays. The second portion was placed in plain centrifuge tubes, centrifuged at 3000 rpm for separation of serum. Serum samples were divided into 0.5 ml aliquots and were stored at –20°C until assayed for further biochemical and immunological tests.

Analytical methods:

Limit of Flocculation (Lf) for titration of epsilon antitoxin in test serum from rabbits and immunized sheep (Brazilian Pharmacopoeia, 2001). It was defined as unit of flocculation (Fl) the quantity of toxin that flocculates in the shortest time with a unit of antitoxin in the time it takes for floccules to appear, the constant of flocculation denominated Kf.

The NIBSC epsilontoxicoid was previously titrated with a standard antitoxin containing 100 Lf/mL. In this manner, it was possible to quantify the toxicoid in the Fl unit. The toxicoid was diluted to contain 100 Fl/mL. Next, Ramon's (1922) recommendation for the titration of epsilon antitoxin present in serum of immunized animals was followed. Varied volumes of test serum of immunized sheep were dispensed in a series of seven assay tubes followed by an addition of standard

toxicoid containing 100 Fl/mL in each of the tubes. The tubes were immersed in water at 45°C and observed, with the help of magnifying lenses and lighting, in regular intervals up to the appearance of the first floccule.

Hematological studies:

The evaluated hematological parameters in this study included estimation of red blood cell count (RBCs), hemoglobin concentration (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), platelet count and totalleukocytic count (TLC) and differential leukocytic counts. Assessment of these parameters were accomplished using an Automatic cell counter (MICROS ANALYSER, ABC VET, France), Model: ABC VET. Serial No: 907 AB 6012), and according to the hematological procedures adopted by Grindem (2011).

Serum biochemical studies:

Serum samples were assayed for the concentrations of total proteins (TP) by colorimetric method using commercial kits of Diamond diagnostics Company as described by Cannon et al., (1974) while serum albumin levels (Alb) were determined using commercial kits supplied by Bio diagnostics Company (Egypt) following the method of Doumas et al., (1971). Serum Globulins were determined by subtracting albumin from serum total protein and then A/G ratio was estimated.

Assay of serum concentrations of total and direct bilirubin was carried out by colorimetric method using commercial kits of Biodiagnostics Company (Egypt) according to the method described by Walter and Gerade, (1970). Serum indirect bilirubin was calculated by subtracting direct fraction from total bilirubin.

Serum enzymatic activities of aspartate aminotransferase (AST) (Breuer, 1996) and lactate dehydrogenase (LDH) (Van der Heiden et al., 1994) were determined colorimetrically using commercial kits supplied by Spectrum Diagnostics Company (Germany) and following manufacture instructions.

Monitoring the enzyme activities of total creatine Kinase (CK) and creatine kinase-MB isoenzyme (CK-MB) was performed using colorimetric method and test kits provided by Beckman Coulter inc. (USA) following the method of Hughs (1962) while serum creatine kinase- MM isoenzyme (CK-MM) activity was evaluated according to Argiroudis et al. (1982).

Immunological studies:

Flocculation test for determination of vaccine titer:

This assay, also known as Ramon assay, is based on the observation of a macroscopic flocculation complex for detection of antibody titer. Positive result depends on the combination of an antigen and antibody to produce a flocculent precipitate. Limit of flocculation (Lf) means the amount of a toxin or toxoid that causes the most rapid flocculation when combined with its antitoxin (Ramon, 1956).

Immunoglobulins:

Serum levels of immunoglobulin G (IgG) was measured by IgG/ELISA kit (Genesis Co., UK) and serum immunoglobulin M (IgM) was detected by IgM/ELISA kit (Anthos, Austria) according to the manufacturer's protocol.

Serum protein electrophoresis:

Serum protein electrophoretic fractionation profile was carried out by a Polyacrylamide Gel Electrophoresis according to Lewis et al. (2006).

Statistical analysis procedures:

Data were expressed as mean values ± SE. The statistical analyses were performed by using one-way ANOVA according to Snedecorand Cochran, (1980) followed by Duncan's post hocusing SPSS® (Statistical Package for Social Sciences) Version 26, IBM Inc. (Chicago, IL, USA.) to determine the statistically significant differences among the experimental groups. The differences were considered statistically significant at $p < 0.05$.

RESULTS

I. The effects of tetanus toxoid and toxin with or without freund adjuvants on hematological parameters of horse:

Red blood cell indices:

Data shown in Table 2, clarified that no significant differences were recorded in the mean values of RBC, Hb, PCV, MCH, and MCHC, between the control and IFA and CFA groups. On the other hand, the mean values of RBC, Hb and PCV showed significant (*P≤0.05*) decreases in animals injected with toxoid vaccine with or without adjuvants. The combination of toxoid and toxin vaccines also resulted in significant reduction in these parameters particularly when CFA was added.

Mean cell volume was significantly reduced (*P≤0.05*) in groups administered toxoid vaccine or combination of toxoid and toxin and the lowest values were reported by addition of CFA to both vaccination protocols. The results of MCH and MCHC values presented in Table 3 showed significant (*P≤0.05*) increases in all vaccinated groups except in G5 and G6 which administered tetanus toxoid combined Freund adjuvants whereas their values exhibited significant decreases (Table 2).

Total and differential white blood cell counts:

Data shown in Table 3, clarified that the mean values of TLC, neutrophil, lymphocyte, and monocyte counts showed significant (*P≤0.05*) increases in animals injected with IFA and CFA groups compared to control group. The mean values of TLC and neutrophil counts were significantly increased in all vaccinated groups with or without adjuvants, but the highest increase was observed when horses were injected with a combination of toxoid and toxin and provided with CFA.

The results presented in Table 3 showed a significant (*P≤0.05*) increase of lymphocytic count in all groups, but interestingly, this increase was more prominent in groups G4 and G7 that were not supplemented with adjuvants. The groups administered toxoid or combination of toxoid and toxin vaccines provided with adjuvants demonstrated the clearer (*P≤0.05*) increase in monocytic count particularly when CFA was added to the vaccine. No significant differences in eosinophil and basophil numbers were reported between the control and all treated groups (Table 3).

Platelets:

The data of platelet count as presented in Table 4 implicated significant (*P≤0.05*) decreases in animals injected with toxoid vaccine especially in combination of adjuvants.

The combination of toxoid and toxin vaccines also resulted in a significant (*P≤0.05*) reduction in platelet count particularly after addition of CFA. On the other hand, no significant differences were recorded in the mean values of the platelets count between the control and IFA and CFA groups (Table 4).

II. The effects of tetanus toxoid and toxin with or without freund adjuvants on serum biochemical parameters of horse:

Serum proteins:

Data shown in Table 5, clarified that the mean values of total proteins, albumin, globulins, and A\G ratio showed non-significant changes in animals injected with IFA and CFA groups compared to the control one.With respect to the effects of vaccination on serum TP, the results presented in Table 5 showed a significant (*P≤0.05*) increase of TP in all vaccinated groups noticeably when IFA was added. On the other hand, horses administered a combination of toxoid and toxin vaccines did not exhibit significant changes in TP compared to control. The average values of serum albumin levels were significantly decreased only in groups injected with a combination of toxoid and toxin vaccines whether adjuvants were added or not (Table 5).

Compared to control, all vaccinated horses demonstrated significantly (*P≤0.05*) higher serum globulin concentrations particularly when IFA was provided to the vaccination protocols, but the increase was greater in the toxoid-toxin group when compared to the group received only toxoid vaccine. A\G ratio showed a significant (*P≤0.05*) decrease in groups either injected with tetanus toxoid or a combination of toxoid and toxin with or without Freund adjuvants specifically in toxoid-toxin groups but there were no significant changes between adjuvant groups (Table 5).

Serum concentrations of total, direct and indirect bilirubin:

Data shown in Table 6, implicated that the mean values of total and direct bilirubin were significantly (*P≤0.05*) increased in vaccinated groups particularly when IFA was added to the combined toxoid-toxin vaccine. On the other hand, the elevation in indirect bilirubin was noticed in toxoid-administered groups supplemented with either incomplete or complete Freund adjuvants when compared to control. In groups administered a combination of toxoid and toxin, the highest increase in U-BILLI was observed in G7 which injected with the combined toxoid toxin vaccine as well as G9 when CFA was added to the combination (Table 6).

Serum enzymatic activities:

Data shown in Table 7, clarified that no significant differences were recorded in the mean values of serum activities of AST and LDH between the control and IFA and CFA groups when compared to control. Vaccination of horse by different vaccination protocols resulted in significant (*P≤0.05*) increase in serum AST and LDH activities particularly when a combination of toxoid and toxin vaccines was administered with or without adjuvants. With regard to the muscle enzymatic biomarkers, the results presented in Table 7 showed that in comparison to control group, serum activity of total CK as well as serum enzymatic concentrations of CK-MM and CK-MB were not significantly changed in all groups whether vaccines were administered or not.

III. The effects of tetanus toxoid and toxin with or without freund adjuvants on immunological parameters of horse:

Vaccine titer:

The vaccine titer was calculated as limit of flocculation and the data presented in Table 8 implicated non-significant changes in animals injected with IFA and CFA compared to the control group. All vaccinated groups demonstrated significant (*P≤0.05*) increases in the limits of flocculation compared to control. In the animals injected with tetanus toxoid, the highest values were reported when CFA was administered with toxoid compared to the other 2 groups. Vaccination of horses with a combination of toxoid and toxin resulted in a greater increase (*P≤0.05*) in the limit of

flocculation compared to the toxoid groups (G4, G5 and G6) with the most prominent increase was noticed when adjuvants were added to this combination (Table 8).

Immunoglobulins:

The mean values of serum concentrations of IgG in the control group and adjuvant groups as well as group injected with toxoid alone were similar as clarified in Table 8.Otherwise, other groups (G5-G9) exhibited significant *(P≤0.05)* increases in serum levels of IgG with no differences recorded between these groups and each other. Serum levels of IgM showed non-significant changes in all vaccinated groups with or without adjuvants when compared to control (Table 8).

Serum protein electrophoresis:

The results presented in Table 9 implied that there was no effect for the administration of IFA and CFA on serum protein electrophoretic fractionation profile of horse. Serum albumin concentrations showed significant *(P≤0.05)* decreases in horses injected with the toxoid-toxin combination with or without adjuvants. With respect to the globulin fractions, groups treated with a combination of toxoid and toxin exhibited significant *(P≤0.05)* increases in both α1 and α2-globulins compared to control as well as when compared to toxoid groups whether Freund adjuvants were included in the vaccination protocol or not. No significant variations were detected in serum levels of β-globulin in all treated groups (Table 9).

In comparison to control group, the mean values of serum gamma globulin concentrations were significantly *(P≤0.05)* higher in all vaccinated groups with or without addition of adjuvants particularly when a combination of toxoid and toxin was applied as a vaccination protocol compared to groups administered the toxoid vaccine (Table 9).

Table (2): Effects of tetanus toxoid and toxin used for preparation of equine antitetanic hyperimmune serum on red blood cell parameters with or without addition of adjuvants (values mean± SE):

Group No = 5	Parameters					
	RBCs (×106 /µl)	**Hb (g/dl)**	**PCV (%)**	**MCV (fl)**	**MCH (pg)**	**MCHC (%)**
G1	9.50±0.52 a	14.98 ±0.31 a	44.42 ±0.88 a	46.68± 2.66 b	15.62 ±0.84 b	33.7±1.11 b
G2	9.37±0.27 **a**	13.76 ±0.53 **a**	40.62 ±1.26 **a**	43.55 ±1.46 **c**	14.78 ±0.94 **b**	33.86 ±2.37 **b**
G3	9.17 ±0.21 **a**	14.04 ±0.24 **a**	44.46 ±0.73 **a**	49.36 ±0.81 **b**	15.40 ±0.54 **b**	31.55 ±1.63 **b**
G4	7.85 ±0.31 **bc**	12.70 ±0.41 **b**	33.18 ±2.46 **b**	42.48 ±2.72 **c**	16.68 ±0.92 **a**	38.57 ±3.34 **a**
G5	5.31±0.62 **cd**	8.25±0.56 **c**	26.25 ±3.34 **c**	49.31 ±3.92 **b**	16.43 ±2.07 **a**	31.48 ±9.64 **b**
G6	6.59±0.71 **c**	7.62±0.70 **c**	26.85 ±2.53 **c**	40.59 ±3.88 **d**	11.70 ±0.95 **c**	28.34 ±4.31 **c**
G7	7.68±0.27 **bc**	12.52±0.13 **b**	32.36 ±0.52 **b**	42.14 ±0.86 **c**	16.35 ±0.50 **a**	38.61 ±1.62 **a**
G8	7.72±0.46 **bc**	12.22±0.39 **b**	33.81 ±4.82 **b**	43.76 ±6.94 **c**	16.01 ±1.01 **a**	36.15 ±5.00 **a**
G9	5.35 ±0.56 **cd**	8.78 ±0.74 **c**	23.56 ±2.40 **c**	44.15 ±4.46 **c**	16.76 ±1.39 **a**	37.34 ±2.41 **a**

Means in the column without a common letter differ significantly at (*P≤0.05*).

G1: Control non-treated group.

G2: Injected with 2 ml of IFA S/C twice at 2 weeks interval.

G3: Injected with 2 ml of CFA S/C twice at 2 weeks interval.

G4: Injected with gradually increased doses of tetanus toxoid S/C with 5 days interval between doses.

G5: Similar to group 4 with the addition of 2 ml of IFA to each toxoid dose.

G6: Similar to group 4 with the addition of 2 ml of CFA to each toxoid dose.

G7: Injected with gradually increased doses of tetanus toxoid S/C with 5 days interval then, injected with gradually increased doses of tetanus toxin S/C 2 weeks after the last toxoid dose with 5 days interval between doses.

G8: Similar to group 7 with the injection of 2 ml of IFA S/C with each toxoid or toxin dose.
G9: Similar to group 7 with the injection of 2 ml of CFA S/C with each toxoid or toxin dose.

Table (3): Effects of tetanus toxoid and toxin used for preparation of equine antitetanic hyperimmune serum on total and differential white blood cell counts with or without addition of adjuvants (Values are mean ± SE):

Groups No = 5	Parameters($\times 10^3$/µl)					
	TLC	**Neutrophils**	**Lymphocytes**	**Monocytes**	**Eosinophils**	**Basophils**
G1	8.17± 0.09 **d**	4.33 ± 0.14 **d**	3.07 ± 0.06 **c**	0.34 ± 0.047 **d**	0.35 ± 0.09 **a**	0.07 ± 0.01 **a**
G2	12.46± 0.15 **c**	6.74 ± 0.27 **c**	4.81 ± 0.16 **b**	0.71 ± 0.081 **c**	0.14 ± 0.03 **a**	0.05 ± 0.03 **a**
G3	12.28± 0.10 **c**	6.50 ± 0.33 **c**	4.94 ± 0.04 **b**	0.72 ± 0.25 **c**	0.10 ± 0.02 **a**	0.01 ± 0.01 **a**
G4	13.56± 0.17 **b**	7.15 ± 0.48 **b**	5.53 ± 0.14 **a**	0.75 ± 0.27 **c**	0.13 ± 0.02 **a**	0.00 ± 0.00 **a**
G5	12.63± 0.29 **c**	6.48 ± 0.42 **c**	4.65 ± 0.14 **b**	1.36 ± 0.19 **a**	0.13 ± 0.02 **a**	0.00 ±0.00 **a**
G6	12.90± 0.05 **bc**	6.53 ± 0.36 **c**	4.66 ± 0.01 **b**	1.38 ± 0.13 **a**	0.28 ± 0.04 **a**	0.03 ± 0.03 **a**
G7	14.30± 0.20 **a**	7.58 ± 0.32 **b**	5.60 ± 0.35 **a**	0.97 ± 0.32 **b**	0.03 ± 0.02 **a**	0.11 ± 0.03 **a**
G8	12.58± 0.13 **c**	7.09 ± 0.13 **b**	4.07 ± 0.04 **b**	0.94 ± 0.01 **b**	0.13 ± 0.12 **a**	0.08 ± 0.03 **a**
G9	14.36± 0.28 **a**	8.06 ± 0.29 **a**	4.73 ± 0.21 **b**	1.28 ± 0.25 **a**	0.24 ± 0.04 **a**	0.05 ± 0.05 **a**

TLC: Total Leukocyte Count.

Means in the column without a common letter differ significantly at ($P \leq 0.05$).

G1: Control non-treated group.
G2: Injected with 2 ml of IFA S/C twice with 2 weeks interval.
G3: Injected with 2 ml of CFA S/C twice with 2 weeks interval.
G4: Injected with gradually increased doses of tetanus toxoid S/C with 5 days interval between doses.
G5: Similar to group 4 with the addition of 2 ml of IFA to each toxoid dose.
G6: Similar to group 4 with the addition of 2 ml of CFA to each toxoid dose.

G7: Injected with gradually increased doses of tetanus toxoid S/C with 5 days interval then, injected with gradually increased doses of tetanus toxin S/C 2 weeks after the last toxoid dose with 5 days interval between doses.
G8: Similar to group 7 with the injection of 2 ml of IFA S/C with each toxoid or toxin dose.
G9: Similar to group 7 with the injection of 2 ml of CFA S/C with each toxoid or toxin dose.

Table (4): Effects of tetanus toxoid and toxin used for preparation of equine antitetanic hyperimmune serum on platelets count with or without addition of adjuvants (Values are mean ± SE):

Groups No = 5	Parameters
	Platelets
G1	138.00 ± 3.49 **a**
G2	135.80 ± 2.33 **a**
G3	132.80 ± 3.21 **a**
G4	105.20 ± 3.39 **b**
G5	68.60 ± 4.64 **c**
G6	74.0 ± 6.69 **c**
G7	118.00 ± 1.92 **b**
G8	109.80 ± 0.35 **b**
G9	68.40 ± 5.06 **c**

Means in the column without a common letter differ significantly at ($P \leq 0.05$).

G1: Control non-treated group.
G2: Injected with 2 ml of IFA S/C twice with 2 weeks interval.
G3: Injected with 2 ml of CFA S/C twice with 2 weeks interval.
G4: Injected with gradually increased doses of tetanus toxoid S/C with 5 days interval between doses.
G5: Similar to group 4 with the addition of 2 ml of IFA to each toxoid dose.
G6: Similar to group 4 with the addition of 2 ml of CFA to each toxoid dose.

G7: Injected with gradually increased doses of tetanus toxoid S/C with 5 days interval then, injected with gradually increased doses of tetanus toxin S/C 2 weeks after the last toxoid dose with 5 days interval between doses.
G8: Similar to group 7 with the injection of 2 ml of IFA S/C with each toxoid or toxin dose.
G9: Similar to group 7 with the injection of 2 ml of CFA S/C with each toxoid or toxin dose.

Table (5): Effects of tetanus toxoid and toxin used for preparation of equine antitetanic hyperimmune serum on concentrations of serum proteins with or without addition of adjuvants (Values are mean ± SE):

Groups No = 5	Parameters (g/dl)			
	TP	**Alb**	**Glob**	**A/G**
G1	6.40± 0.24 **c**	3.18± 0.19 **a**	3.24± 0.10 **d**	0.98± 0.05 **a**
G2	6.48± 0.23 **c**	3.20± 0.19 **a**	3.30± 0.10 **d**	0.97± 0.06 **a**
G3	6.42± 0.13 **c**	3.30± 0.13 **a**	3.14± 0.02 **d**	1.05± 0.04 **a**
G4	7.20± 0.06 **b**	3.30± 0.10 **a**	3.92± 0.11 **c**	0.84± 0.05 **b**
G5	7.72± 0.14 **a**	3.52± 0.09 **a**	4.20± 0.07 **b**	0.83± 0.01 **c**
G6	7.03± 0.15 **b**	3.38± 0.08 **a**	3.65± 0.14 **c**	0.93± 0.04 **b**
G7	6.22± 0.15 **c**	2.02± 0.20 **b**	4.20± 0.07 **b**	0.48± 0.05 **d**
G8	7.71± 0.15 **a**	2.30± 0.22 **b**	5.41± 0.20 **a**	0.43± 0.05 **d**
G9	7.36± 0.25 **b**	2.46± 0.18 **b**	4.90± 0.17 **b**	0.50± 0.03 **d**

TP: Total Protein, Alb: Albumin, Glob: globulins, A/G: Albumin/Globulin ratio.
Means in the column without a common letter differ significantly at (*P≤0.05*).

G1: Control non-treated group.
G2: Injected with 2 ml of IFA S/C twice with 2 weeks interval.
G3: Injected with 2 ml of CFA S/C twice with 2 weeks interval.
G4: Injected with gradually increased doses of tetanus toxoid S/C with 5 days interval between doses.
G5: Similar to group 4 with the addition of 2 ml of IFA to each toxoid dose.
G6: Similar to group 4 with the addition of 2 ml of CFA to each toxoid dose.

G7: Injected with gradually increased doses of tetanus toxoid S/C with 5 days interval then, injected with gradually increased doses of tetanus toxin S/C 2 weeks after the last toxoid dose with 5 days interval between doses.
G8: Similar to group 7 with the injection of 2 ml of IFA S/C with each toxoid or toxin dose.
G9: Similar to group 7 with the injection of 2 ml of CFA S/C with each toxoid or toxin dose.

Table (6): Effects of tetanus toxoid and toxin used for preparation of equine antitetanic hyperimmune serum on serum levels of total, direct (C-BILLI) and indirect (U-BILLI) bilirubin with or without addition of adjuvants (Values are mean ± SE):

Groups No = 5	Parameters (mg/dl)		
	T-BILI	C-BILI	U-BILI
G1	1.56 ± 0.09 **e**	0.70 ± 0.03 **c**	0.85 ± 0.09 **b**
G2	1.52 ± 0.13 **e**	0.66 ± 0.03 **c**	0.85 ± 0.13 **b**
G3	1.56 ± 0.18 **e**	0.74 ± 0.05 **c**	0.79 ± 0.15 **b**
G4	1.86 ± 0.05 **e**	0.96± 0.04 **b**	0.90± 0.04 **b**
G5	2.22 ± 0.05 **c**	0.80± 0.08 **b**	1.44± 0.10 **a**
G6	2.32 ± 0.01 **c**	0.90± 0.13 **b**	1.42± 0.11 **a**
G7	2.78 ± 0.07 **b**	0.98± 0.05 **b**	1.86± 0.06 **a**
G8	2.98 ± 0.11 **a**	1.99± 0.13 **a**	1.00± 0.14 **b**
G9	2.62 ± 0.10 **b**	1.04± 0.12 **b**	1.68± 0.11 **a**

T-BILI: Total bilirubin, C-BILI: Conjugated bilirubin, U-BILI: Unconjugated bilirubin.
Means in the column without a common letter differ significantly at ($P \leq 0.05$).

G1: Control non-treated group.
G2: Injected with 2 ml of IFA S/C twice with 2 weeks interval.
G3: Injected with 2 ml of CFA S/C twice with 2 weeks interval.
G4: Injected with gradually increased doses of tetanus toxoid S/C with 5 days interval between doses.
G5: Similar to group 4 with the addition of 2 ml of IFA to each toxoid dose.

G6: Similar to group 4 with the addition of 2 ml of CFA to each toxoid dose.
G7: Injected with gradually increased doses of tetanus toxoid S/C with 5 days interval then, injected with gradually increased doses of tetanus toxin S/C 2 weeks after the last toxoid dose with 5 days interval between doses.
G8: Similar to group 7 with the injection of 2 ml of IFA S/C with each toxoid or toxin dose.
G9: Similar to group 7 with the injection of 2 ml of CFA S/C with each toxoid or toxin dose.

Table (7): Effects of tetanus toxoid and toxin used for preparation of equine antitetanic hyperimmune serum on serum enzymes with or without addition of adjuvants:

Groups No = 5	Enzymes				
	AST (U/L)	**LDH (U/ L)**	**CK (U/ L)**	**CK-MB (ng / mL)**	**CK-MM (ng / mL)**
G1	306.80± 3.36 **c**	372.66± 3.48 **e**	195.66 ± 13.86 **a**	87.00± 4.041 **a**	84.33± 2.96 **a**
G2	309.80± 3.30 **c**	380.33± 10.58 **e**	202.00 ± 5.56 **a**	80.00± 3.05 **a**	83.66± 3.28 **a**
G3	305.40± 5.15 **c**	367.66± 9.20 **e**	179.33 ± 2.72 **a**	83.66± 1.20 **a**	75.33± 2.90 **a**
G4	358.80± 3.24 **b**	525.33± 11.06 **c**	212.00 ± 6.55 **a**	82.66± 1.45 **a**	87.66± 3.84 **a**
G5	362.0± 2.0 **b**	497.33± 10.56 **d**	199.66 ± 7.83 **a**	83.33± 0.88 **a**	85.00± 1.00 **a**
G6	361.20± 0.86 **b**	501.33± 4.91 **d**	175.33 ± 7.44 **a**	83.66± 3.28 **a**	77.33± 3.84 **a**
G7	387.0± 2.09 **a**	593.66± 1.85 **b**	186.33 ± 12.41 **a**	80.66± 2.84 **a**	80.66± 4.63 **a**
G8	390.60± 2.11 **a**	605.00± 9.07 **a**	205.66 ± 9.76 **a**	85.66± 0.88 **a**	85.66± 6.69 **a**
G9	385.60± 2.63 **a**	620.33± 22.34 **a**	206.66 ± 8.81 **a**	87.00± 1.52 **a**	86.66± 6.93 **a**

AST: Aspartate aminotransferase , LDH: Lactate dehydrogenase , CK: Creatine kinase , CK-MB: Creatine kinase-myocardial band , CK-MM: Creatine kinase MM isoenzyme.

Values are mean ± SE.Means in the column without a common letter differ significantly at (*P≤0.05*).

G1: Control non-treated group.

G2: Injected with 2 ml of IFA S/C twice with 2 weeks interval.
G3: Injected with 2 ml of CFA S/C twice with 2 weeks interval.
G4: Injected with gradually increased doses of tetanus toxoid S/C with 5 days interval between doses.
G5: Similar to group 4 with the addition of 2 ml of IFA to each toxoid dose.
G6: Similar to group 4 with the addition of 2 ml of CFA to each toxoid dose.
G7: Injected with gradually increased doses of tetanus toxoid S/C with 5 days interval then, injected with gradually increased doses of tetanus toxin S/C 2 weeks after the last toxoid dose with 5 days interval between doses.
G8: Similar to group 7 with the injection of 2 ml of IFA S/C with each toxoid or toxin dose.
G9: Similar to group 7 with the injection of 2 ml of CFA S/C with each toxoid or toxin dose.

Table (8): Limit of flocculation (Lf) and serum levels of IgG and IgM following injection of tetanus toxoid or/and toxin used for preparation of equine antitetanic hyperimmune with or without addition of adjuvants (Values are mean ± SE):

Groups No = 5	Limit of flocculation (Lf)	IgG (mg/dl)	IgM (mg/dl)
G1	0.00± 0.00 **e**	741.00± 17.89 **b**	71.04± 9.97 **a**
G2	0.00± 0.00 **e**	744.60± 6.35 **b**	74.76± 1.53 **a**
G3	0.00± 0.00 **e**	745.30± 9.59 **b**	82.66± 1.76 **a**
G4	200± 57.73 **d**	767.88± 5.51 **b**	74.65± 2.85 **a**
G5	600± 115.47 **c**	794.33± 12.17 **a**	84.98± 7.35 **a**
G6	800± 0.05 **b**	796.00± 5.00 **a**	76.66± 6.76 **a**
G7	800± 0.01 **b**	809.93± 6.86 **a**	87.00± 2.08 **a**
G8	1000± 80.47 **a**	840.60± 13.24 **a**	75.00± 4.93 **a**
G9	1100± 57.73 **a**	841.00± 15.56 **a**	74.00± 5.29 **a**

Lf: Limit of flocculation, IgG: Immunoglobulin G, IgM: Immunoglobulin M.

Means in the column without a common letter differ significantly at ($P \leq 0.05$).

G1: Control non-treated group.
G2: Injected with 2 ml of IFA S/C twice with 2 weeks interval.
G3: Injected with 2 ml of CFA S/C twice with 2 weeks interval.

G4: Injected with gradually increased doses of tetanus toxoid S/C with 5 days interval between doses.
G5: Similar to group 4 with the addition of 2 ml of IFA to each toxoid dose.
G6: Similar to group 4 with the addition of 2 ml of CFA to each toxoid dose.
G7: Injected with gradually increased doses of tetanus toxoid S/C with 5 days interval then, injected with gradually increased doses of tetanus toxin S/C 2 weeks after the last toxoid dose with 5 days interval between doses.
G8: Similar to group 7 with the injection of 2 ml of IFA S/C with each toxoid or toxin dose.
G9: Similar to group 7 with the injection of 2 ml of CFA S/C with each toxoid or toxin dose.

Table (9): Effects of tetanus toxoid and toxin used for preparation of equine antitetanic hyperimmune serum on serum protein electrophoretic fractionation profile with or without addition of adjuvants (Values are mean ± SE):

Groups No = 5	Protein (%)				
	Albumin	**α1-globulin**	**α2-globulin**	**β-globulin**	**γ-globulin**
G1	49.7±1.00 **a**	11.16±0.78 **b**	11.10±0.78 **b**	7.98±0.08 **a**	20.06±0.53 **c**
G2	49.38±1.01 **a**	10.10 ±1.36 **b**	10.86±0.90 **b**	8.76±0.218 **a**	20.90±2.15 **c**
G3	51.40±1.96 **a**	10.06 ±0.79 **b**	11.01±1.58 **b**	7.50±0.185 **a**	20.03±1.085 **c**
G4	45.8±3.30 **a**	9.63±0.03 **b**	10.01±0.46 **b**	8.13±0.233 **a**	26.43±1.07 **b**
G5	45.6±1.76 **a**	0.32 10.26± **b**	10.10±0.76 **b**	8.08±0.44 **a**	25.96±0.31 **b**
G6	48.08±2.12 **a**	9.46±1.50 **b**	9.70±2.10 **b**	7.56±0.29 **a**	25.20±1.21 **b**
G7	32.47±0.43 **b**	15.16±0.24 **a**	14.30±0.05 **a**	8.66±0.23 **a**	29.41±1.78 **a**
G8	29.83±1.84 **b**	14.86±0.545 **a**	14.40±0.721 **a**	8.56±0.17 **a**	32.35±2.44 **a**
G9	33.42±1.21 **b**	14.46±0.874 **a**	14.40±0.40 **a**	8.68±0.15 **a**	29.04±3.72 **a**

Means in the column without a common letter differ significantly at ($P \leq 0.05$).

G1: Control non-treated group.
G2: Injected with 2 ml of IFA S/C twice with 2 weeks interval.
G3: Injected with 2 ml of CFA S/C twice with 2 weeks interval.
G4: Injected with gradually increased doses of tetanus toxoid S/C with 5 days interval between doses.

G5: Similar to group 4 with the addition of 2 ml of IFA to each toxoid dose.
G6: Similar to group 4 with the addition of 2 ml of CFA to each toxoid dose.
G7: Injected with gradually increased doses of tetanus toxoid S/C with 5 days interval then, injected with gradually increased doses of tetanus toxin S/C 2 weeks after the last toxoid dose with 5 days interval between doses.
G8: Similar to group 7 with the injection of 2 ml of IFA S/C with each toxoid or toxin dose.
G9: Similar to group 7 with the injection of 2 ml of CFA S/C with each toxoid or toxin dose.

DISCUSSION

Tetanus is one of the widespread and deadliest diseases that affect both humans and animals, and because the causal agent may create spores, it cannot be completely eradicated and thus prevention is crucial for managing this disease (Brüggemann et al., 2003 and Brook, 2018). Tetanus is a vaccine-avoidable illness for which both tetanus toxin and toxoid were introduced as vaccines to animals and humans to elicit an active immunization by forming tetanus antitoxins or to fight against toxin-based diseases (Sonobe et al., 2007; Martinović et al., 2016; Forstner et al., 2018 and Bae and Bourge, 2020).

Recently, producing hyperimmune serum in animals has become an essential activity of many research projects. Injection of hyperimmune serum is considered a type of passive immunization that includes transferring high levels of antibodies specific to a pathogen or toxin to individuals who are at high risk of infection and insufficient time for the body to develop its own immune response (Lasocka et al., 2021).

Additionally, these specific antibodies in hyperimmune serum could be used to treat and control diseases in the event of an outbreak (Zhang et al., 2015 and Putri et al., 2022).

Specifically, tetanus hyperimmune serum was crucial in treating tetanus because it was effective within hours of administration and could be used in animals in which the potential development of tetanus could be anticipated (Motamedi et al., 2011). The hyperimmune serum can be produced by repeated immunizations (repeated injections of antigens). However, there are several concerns about the repeated use of horse for production of hyperimmune sera. One of the most important objectives during hyperimmune serum production is to achieve a balance between safety of immunized horses and effectiveness of the product. Therefore, earlier research has attempted to link the presence of clinical, hematological, and biochemical alterations with the usage of hyperimmune serum-producing horses to evaluate how these vaccination protocols affected equine health.

In this regard, assessment of the hematological effects of injection of tetanus toxoid or/and toxin with or without adjuvants implicated that RBC, Hb, PCV and MCV values were significantly decreased in all vaccinated groups particularly with addition of CFA to the vaccination protocol. On the other hand, the MCH and MCHC values exhibited significant increase in immunized horses except for groups G5 and G6. These results provide evidence of a typical secondary microcytic anemia. This anemic condition may be attributed to the impaired iron storage in the reticuloendothelial system following inflammation elicited by vaccination (Arfuso et al., 2021).

The elevation in hemoglobin indices including MCH and MCHC could be related

to the fact that the tetanus toxoids being easily bound to erythrocytes leading to autohemolysis as suggested by Haneberg et al. 1978; Sahal et al. (2004) and Bowen et al. (2019). Similarly, the diphtheria-pertussis-tetanus (DPT) vaccination was found to be a possible trigger for autoimmune hemolytic anemia attributed to hemolytic activity of Tetanolysin toxin (Johnson et al., 2012 and Samaila et al., 2018). Injection of Freund adjuvants alone did not result in significant differences in RBC parameters which is consistent with the results of Arguedaset al. (2022).

With respect of leukogram, it was found that administration of both Freund adjuvants elicited significant increase in TLC, neutrophil, lymphocyte, and monocyte counts when compared to control (Vitoriano-Souza et al., 2012). Noh et al. (2021) reported a significant leukocytosis following the initial administration of CFA compared to baseline values which was explained in the term of the ability of CFA to function as stimulator of the immune system to generate antibodies against an antigen (Halliday et al., 2004). Furthermore, vaccine adjuvants might increase the local inflammation by inducing the release of inflammatory cytokines, promote the growth of non-specific lymphocytes, and prolong the antigen's persistence to prod uce improved immunogenicity (Medzhitov and Janeway, 1997; Singh and O'Hagan, 1999 and Kensil et al., 2004).

In comparison to the values measured in control group, all vaccinated groups with or without addition of adjuvants in this study demonstrated a significant increase in TLC indicated by the marked neutrophilia, lymphocytosis and monocytosis with the highest increase was observed when horses were injected with a combination of toxoid and toxin and provided with CFA (Sahal et al., 2004). These effects were suggested to be due to the release of the biologically active lymphocytosis promoting factor (LPF) which was thought to be the major responsible for the large increase in leukocyte, neutrophil, and lymphocyte counts (Mink et al., 1990).Further, vaccine is known to behave as mitogens or superantigens on some lymphocyte subpopulations leading to lymphocyte proliferation and this is likely a factor in the immunological activation following vaccination (Monaco et al., 2019 and Arfuso et al., 2021).

Arfuso et al., (2021) added that following vaccination, several leukocyte populations, such as lymphocytes, monocytes, and neutrophils, changed which likely reflected the animals' systemic immune-biological adaptation to the vaccine, and in particular to the second dosage of injection. The increase in monocyte count could indicate the presence of sub-acute or chronic inflammation after the second dose of the vaccine. The antigen presentation, phagocytosis, and immunomodulation are at least three of the primary roles played by monocytes (Weiss and Wardrop, 2010). No significant differences in eosinophil and basophil numbers were reported between the control and all treated groups as agree with the results of Sahal et al. (2004).

Tetanus vaccine has been linked to i mmune thrombocytopenia (ITP) and an acquired coagulopathy brought by antibody-mediated platelet destruction which could explain the significant reduction in platelet count observed in vaccinated groups in this work (Halperin et al., 2018 and Banerjee et al., 2021). No significant differences were

recorded in the mean values of the platelets count between the control and IFA and CFA groups as reported by Arguedas et al., (2022).

With respect to the serum biochemical studies, the findings of the present investigation showed that serum total protein levels were greater following vaccination compared to the horses in the control group which could be attributed to increased serum globulin levels. Increases in serum proteins have also been observed in horses used for production of polyvalent snake antivenom (Waghmare et al., 2014 and Ramos et al., 2022).

In the current investigation the increase in globulin concentrations were greater when a combination of toxoid and toxin was injected compared to the group received only toxoid vaccine.Moreover, the electrophoretic fractions of serum globulin, such as α1 and α2 and gamma globulins, also increased along with the rising trend of serum globulinparticularly in the groups administered the combined toxoid-toxin vaccines (Arfuso et al., 2021 and Ramos et al., 2022).

The results of this work are consistent with those of earlier research on animals that had received vaccinations. Overall, it has been reported that vaccination causes a considerable inflammatory response regardless of the vaccine type and the microorganism the animal is to be inoculated against (Andersen, 2012).Additionally, the inflammatory conditions were documented to be reflected in a modification in the serum protein electrophoretic pattern as some protein fractions that include acute phase proteins (Kaneko et al., 1989).

Because most important proteins of the acute-phase response to inflammation are in α and β globulins (Duncan et al., 1994), it is probable that the rise in α1 and α2 globulins seen in the present study was brought on by an inflammatory trigger in the hyperimmunized horses and implicates a systemic acute phase response to an inflammatory condition elicited by immunization (Andersen, 2012). Even in the absence of systemic clinical symptoms of inflammation, such as a fever, vaccination seemed to be quite an effective inducer of the acute phase response (Jacobsen et al., 2006).

In particular, Jacobsen et al., (2006) stated that horses that received vaccinations against the influenza virus stains, and tetanus toxoid showed signs of inflammation similar to those seen in horses who had highly inflammatory arthritis brought on by intra-articular injection of lipopolysaccharide or horses who had undergone surgery.Further, vaccination of horses against West Nile virus was associated with modulation of serum protein electrophoretic pattern including elevation of all globulin fractions (Arfuso et al., 2021).

The average values of serum albumin levels in the present study were significantly decreased in groups injected with a combination of toxoid and toxin vaccines whether adjuvants were added or not which agree with Ramaiah et al. (2003) and Waghmare et al. (2014).Albumin is a negative acute-phase systemic protein that migrates to inflamed tissues through the increased vascular permeability and is considered an immune-inflammatory biomarker (Kaneko et al., 1989 and Singh, 2000). Sadeket al. (2017) added that during the acute phase response, albumin synthesis is downregulated, and amino acids in liver are shunted into synthesis of acute phase proteins. Another possible explanation for decreased serum levels of albumin is the

decreased protein synthesis in the liver as a result of hepatic injury.

Hepatic injury caused by tetanus toxin injection was found to account for the majority of problems seen in hyperimmune serum-producing horses (Barton and Morris, 1998 and Sahal et al., 2004). Also, hepatic disorders can proceed in horse after administration of tetanus antitoxin even if no clinical indications of hepatic insufficiency are present (Guglick et al., 1995).

Rather, Abdelkader et al. (1991) reported that horses that have been used for serum production for a long time were frequently found to die unexpectedly of hepatic insufficiency and hepatic coma with no accompanying clinical signs.

Tetanus toxin exposure could lead to equine hepatitis probably because the antigenic overload results in a primary antibody response and formation of tiny immune complexes. Consequently, intermediate-sized immune complexes, or microprecipitates, develop and deposit in vascular endothelium and basement membranes during the subsequent phase of unremarkable antigen overload, causing a widespread vasculitis and activating complement (Guglick et al., 1995 and Alissa and Adams, 2003). Following that, proinflammatory cytokines are released by macrophages and monocytes and adhesion molecules are accelerated by endothelial cells. More inflammatory cells are also loaded, and tiny vascular necrosis takes place (Sahal et al., 2004).

In the present study, the occurrence of hepatic injury was further strengthened by the results of the other hepatic biomarkers including total, indirect, and direct bilirubin as well as serum enzymatic activities of AST and LDH which showed significant increases in vaccinated horses specifically in groups injected with a combination of toxoid and toxin vaccines with no differences reported between the two types of adjuvants. These changes might be probably related to the toxic effects of tetanus toxoid and toxin on liver cells leading to hepatocyte damage or increase hepatic cells permeability (Divers, 2015).

AST has been described to be more sensitive as a hepatic marker than ALT in horses, however although the enzyme is quite sensitive for liver it lacks specificity for the hepatic dysfunction (Stockham and Scott, 2002; Davoudi et al., 2013 and Yoon et al., 2021). Meanwhile in the study of Sahal et al. (2004), horses treated with tetanus toxin for serum production exhibited acute hepatic insufficiency indicated by elevated serum total bilirubin as well as serum activities of AST, ALT and LDH. Even in case of subclinical hepatitis, AST could be considered a good indicator for diagnosis of hepatic insufficiency in horses (Guglick et al., 1995).

Elfiky et al., (2021) recorded significant increases in serum AST activity with normal ALT activity when CFA administered in combination with snake venom to rabbits for snake antisera production.Because high concentrations of AST come from both liver and skeletal muscle (Davoudi et al., 2013) and to rule out the possibility that the increase in AST was due to muscle involvement, serum activities of the muscle specific enzymes as total CK, CK-MB and CK3 were assayed, and the results demonstrated normal serum activities of these muscle specific enzymes in vaccinated horses(Purcell et al., 2012and Bautista et al., 2015).

Therefore, taking together, the increase in serum activity of AST with the concomitant increase in serum total, direct and indirect bilirubin concurrently with the

normal serum activities of muscle specific enzymes seen in the present study could reflect the occurrence of hepatic insufficiency in immunized horses (Satué et al., 2013 and 2022).

The production of hyperimmune serum that contains exceptionally large quantities of specific antibodies provides an unusual degree of immunization and can be produced by repeated immunizations i.e., repeated injections of antigens and can be frequently used as a successful treatment for various types of bacterial and viral diseases (Zhang et al., 2015 and Putri et al., 2022).The potency of the antitetanic serum is usually measured by the amount of antibodies it contains (Waghmare et al., 2014). In the current investigation the antibody titer was calculated as a limit of flocculation. The flocculation value (Lf units) of tetanus vaccine could be used to express the antigenic potency, purity, and amount of antigens in a sample (WHO, 2007).

Overall, the results of this work are consistent with those of earlier research on animals that had received vaccinations. The results presented here showed that all vaccinated groups demonstrated significant increases in the limits of flocculation compared to control.

The tetanus toxoid was good at boosting the humoral immune response and inducing antibody production which agrees with the report of Manivannan et al. (2008) and Li et al. (2012). The highest values of flocculation limit were reported when CFA was administered with toxoid compared to the IFA group. Similarly, EL-Helw et al. (2010) suggested that tetanus toxoid was a potent immunogenic in combination with adjuvants and gave higher flocculation units.

In the present work, combination of toxoid and toxin provided the greater antibody titer compared to the groups administered toxoid vaccines only with no differences were recorded between the two types of Freund adjuvants. These results are in accordance with Xiang et al., (2011) who reported that tetanus vaccine which contain a combination of tetanus toxoid and recombinant tetanus toxin C fragment was of good quality and provided satisfied protection and was on par with or better than that of imported products, offering technical support for improving equine antiserum of the same kind.

Consistently, Seida and Soliman (2017) found that production of satisfied tetanus antibodies titer was better accomplished by injection of different doses of tetanus toxoid and toxin in combination with incomplete and complete freund adjuvants.

The evaluated serum proteins electrophoretic profile of hyperimmunized horses in this study revealed a significant increase in the concentrations of α1, α2 and γ-globulins in the vaccinated groups. The increase in α1 and α2 globulins particularly in groups administered the combined toxoid-toxin vaccine could implicate as mentioned previously the development of systemic acute phase response to an inflammatory condition induced by vaccination, a finding which is consistent with previous studies carried out on immunized animals (Andersen, 2012; Arfuso et al., 2021 and Ramos et al., 2022).

Notably, the γ-globulin fraction showed a significant elevation in all vaccinated groups particularly when a combination of toxoid and toxin was applied as a vaccination protocol compared to groups administered the toxoid only which appears to suggest the creation of

immunoglobulins, a response expected after vaccination. This finding was further supported by the results of IgG which demonstrated significant increases in the vaccinated groups with no differences recorded between groups and each other. This increase in IgG along with the observed increase in lymphocytes seems to be the result of antigenic stimulation of antibody-forming cells of the immune system and, consequently, increased antibody production and might reflect the occurrence of strong humoral immune reaction following vaccination (Halassy et al., 2019; Fehér et al., 2020 and Zhuang et al., 2021).

Xu-Amano et al. (1997) provided evidence that tetanus toxoid selectively causes antigen-specific T-helper 2 (Th2) responses that induce antigen-specific IgG in serum. Pathogen-specific IgGisotypes could also be employed as markers of immunity and disease prevention (Keggan et al., 2013). Additionally, passive immunotherapy with animal-derived antivenoms including either IgG or its Fab fragment was regarded as the only effective and specific treatment method (Lukacevic et al., 2020).

Adjuvants are elements that capable of enhancing the antigen-specific immune responses and have proven to be key components in vaccines (Wu and Liu, 2021).

Including adjuvants in vaccination protocols appeared to improve the effectiveness of weak antigens and trigger suitable immune responses that would not have been properly triggered in their absence. Additionally, adjuvants were found to boost functional antibodies, antibodies with higher affinity for vaccination antigens, or both, as well as the overall antibody titer (Reed et al., 2013). Elfiky et al. (2021) added thatadjuvants generally permit the use of a smaller antigen dose and may modulate immune response to the antigen.

As the production of hyperimmune serum as a biological reagent continued to be developed, the use of adjuvants also continued to be required with continuing concerns about them. Therefore, adjuvants have been extensively investigated for their adjuvanticity potential, safety tolerability, and efficacy as well as their adverse side effects (Elfiky et al., 2021).

In this study, injection of Freund adjuvants alone did not result in significant differences in the most selected hematobiochemical parameters probably due to the low dose of adjuvants used in the present work. Wauben et al., (1994) reported that the CFA dose ranging from 1.0 to 5.0 mg/ml might produce less severe effects while higher doses may potentially produce more chronic systemic effects which are consistent with the findings of this study.

Besides, Marwah and Wafaa (2013) recorded that using adjuvants pre immunization with tetanus antigen showed no significant change in limit of flocculation of the tetanus titer. Kim et al., (2016) and Alavala et al., (2020) found that CFA at a dose of 5 mg/ml produced mini local reactions and arthritis in rats. Additionally,various CFA doses ranged from 5.0 mg/ml to 10 mg/ml were tested to establish arthritis in rat models and the results showed that CFA at the dose of 10 mg/ml had the most potential and reliable dosage to develop polyarthritis in a rat model (Noh et al., 2021).

CONCLUSION

In conclusion, the results obtained from this study could provide evidence of the efficacy of Freund adjuvants as potent enhancers of immune response to tetanus vaccines for production of high titer

hyperimmune serum with few side effects and thus high safety margin. However, tetanus vaccines might result in some harmful health effects as indicated by the hematobiochemical alterations shown in this study and therefore, the repeated use of horses for preparation of antitetanichyperimmune serum should be avoided as it can result in chronic inflammatory stimulus leading to various health hazards particularly on liver.

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