Research Article

Immunopathological and Comparative Study of Concanavalin- A and *Streptococcus lyophilized* Antigen in Immunized Rats

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Abstract | Background Concanavalin-A is *a plant lectin* neither antibodies, nor enzymes which has four binding sites for glucose and act as an antigen-independent mitogen, frequently used to stimulate proliferation of T-cells and activate the *immune* response. Aims The present study conducted for showing effect of Concanavalin-A, whole killed lyophilized *Streptococcus pyogenes* antigen(WKLA) on immune response of rat. Methods To achieve this purpose, 20 Albino male rats were divided equally into four groups and immunized subcutaneously(s/c), 1st group was immunized with (100 mg/ml) of *S. pyogenes* antigen with added equal volume of Freund's adjuvant, 2 doses, 14 days intervals, 2nd group was immunized s/c with Concanavalin-A Con-A (20 mg/ml), 3rd group was immunized with mixed *S. pyogenes* Ag. and Con-A, 2 doses, 14 days intervals, and 4th group was given Phosphate buffer saline (PBS) as negative control group. Results Analysis of cellular and humeral immunity recorded higher concentration of Immunoglobulin G IgG, Tumer Necrosis Factor alpha TNF-a and Interleukin 10 IL-10 were revealed in 3rd immunized group (40.91 ±0.26, 953.03 ±4.82 and 1223.96 ±10.44) then decline in 2nd and 1st groups as (32.65 ±1.07, 858.42±9.26 and 1223.96±10.44) (21.07 ±0.36, 769.38 ±3.64 and 986.73 ±13.48) respectively. Histopathological findings revealed marked lymphoid hyperplasia with obvious perivascular Mono Nuclear Cells MNCs aggregation composed of lymphocyte and macrophage mainly in hepatic tissue of 3rd group with evidence of reactive lymphoid hyperplasia in splenic tissue of 2nd group and periarteriolar, pericortical lymphoid hyperplasia were reported in spleen of 1st group immunized with *Streptococcus pyogenes* lyophilized antigen. Conclusion This study provide we can conclude that jointly of concanavalin-A and *S.pyogenes* antigen capable to enhance potential immune response butter than antigen alone.

Keywords | Comparative study, *Streptococcus pyogenes*, Concanavalin-A, Lyophilized antigen, Immunized rats, Histopathological

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INTRODUCTION

Streptococcus pyogenes is a gram-positive, Beta B-hemolytic and catalase-negative bacteria (Sadam *et al.*, 2014) produced biofilm formation on mucosal membranes facilitating their prolonged survival (Cinthia *et al.*, 2020) like other gram positive bacteria (Mohammed and Zaid, 2024) as *staphylococcus aureus* (Ali and Alaa, 2022), Group A streptococcus possessed wide variety of virulence factors on their surface such as hyaluronic capsule, M protein, streptolysin O and S, streptococcal pyrogenic exotoxins A and B (Syed *et al.*, 2020; Nikolai and Rudolf, 2021). Some exotoxin act as superantigens and trigger T-lymphocytes excessive stimulation by attaching to class II major

histocompatibility complex molecules, show the way to release of massive mediators of T cell (Akshay, 2019; Anthony *et al*., 2021), depending on virulence and sequalae of bacteria post infection can categorized into two classes: Class I strains cause suppurative inflammation such as meningitis and abscess in both brain and liver while class II strains cause non suppurative inflammation like acute glomerulonephritis and rheumatic fever (Niluni *et al*., 2021; Patience *et al*., 2023).

Concanavalin-A, is defense glycoprotein from Jack bean have mitogenic activity, binding with carbohydrate without modified their biochemical activity (Evandro *et al*., 2017; Abtar *et al*., 2019) and induce the growth, divided of quiescent lymphocytes then activate innate and adaptive immune response by enhancing phagocytic mechanism(Blaise *et al*., 2019), releasing chemical mediators and several cytokines such as Tumer Necrosis Factor alpha TNF-α, Interferon gamma IFN-γ, granulocyte macrophage-colony stimulating factor, and interleukins, that maintain inflammatory and immuno-stimulatory reaction (Sabrin *et al*., 2021) thus improvement the defenses against microbial infection (Batista *et al*., 2017; Jannyson *et al*., 2017; Emadeldin *et al*., 2022) but these phenomena not occur in non-mitogenic lectins (Evandro *et al*., 2017). The present study aimed to investigate the immunomodulator effect of Concanavalin-A Con-A as compare with whole killed lyophilized *Streptococcus pyogenes* antigen in activation of both immune arms including cell mediated and humoral immunity.

MATERIALS AND METHODS

Sample collection and bacterial identification

About 50 throat swabs were collected from sheep showing obvious respiratory signs during the duration from January to May 2022, and these isolates identified by routine culturing, gram stain and confirmatory by biochemical indole test (vitek-2 system) to recognize the isolated bacteria to the genus and species level according to (Alaa *et al*., 2022).

Preparation of *Streptococcus pyogenes* antigen

The *S.pyogenes* antigen was prepared according to (Nguyen *et al*., 2022), then lyophilized and measured the protein concentration by Biuret method (Randox Lab). It was kept under 4 °C in a refrigerator until use as antigen in experimental rats, when used re-suspended by adding 2 ml of Phosphate buffer saline PBS to 2 gm of dry lyophilized antigen to make stock solution according to (Rafeek *et al*., 2021).

Preparation of con-A

Each vial contained 100 mg of lyophilized Concanavalin-A Con-A powder was dissolve in 20 ml of sterile phosphate buffer saline then kept as stock solution of (1.5) mg/ml at 4°C in dark vial and each animal received two doses, 14 days intervals(1.5mg/kg B.W.) with dose volume was (0.1) ml per (100)gm of rat weight by intraperitoneal rout, which is representing the ideal therapeutic dose according to (Xu *et al*., 2006; Anroop and Shery, 2016; Kathem *et al*., 2022).

Animal immunization

Twenty male rat were distributed randomly into four groups and immunized subcutaneously, 1st group was immunized with (100) µl of *S. pyogenes* antigen (100 mg/ml) mixed with equal volume of Freund's adjuvant, 2nd group was immunized with (1.5) ml of Concanavalin-A Con-A(20 mg/ml), 2 doses and 3rd group was immunized with mixed *S. pyogenes* Antigen Ag. and Concanavalin-A Con-A, 4th group was inoculated with Phosphate buffer saline PBS as negative control group, each 1st and 3rd groups administrated booster dose after 14 days with equal volume of Freund's adjuvant (First immunization in Freund's complete adjuvant at 0 day and followed with booster immunization in Freund's incomplete adjuvant after 14 day of immunization) according to (Yang *et al*., 2011).

At 28 day post immunization, blood samples were obtained for check cellular and humoral immune response by cytokines and Immunoglobulin G IgG measurement, with taken tissue sampling involving spleen and liver for histopathological examination.

Histopathological examination

Experimental rats were euthanized and a full necropsy was performed to detect any abnormalities and gross changes in liver and spleen that dissected after that 1c.m^3 from organs were taken then fixed in 10% neutral buffered formalin for (2-3) days, then routinely processed in histokinette (ATP-1000\HESTION), tissue sections were embedded in paraffin (Eman *et al*., 2016) after that paraffin blocks with implanted tissue slices were sectioned at 5 µm thickness by microtome) (Yidi) (Ahmed and Bushra, 2013), and staining by routine hematoxylin and eosin stain then examined under light microscope(Olympus) according to (Steven and Bancroft, 1997).

Statistical analysis

SAS program was used to analyze the data and find the influence of various factors on the study's parameters. The Least Significant Difference (LSD) test was used for analysis of variance by using Analysis of variance ANOVA. Two ways to identify the results' significant differences for the result were determined at ($P \leq 0.05$) according to (SAS, 2018).

Table 1: Mean and standard error of (IgG, TNF-a and IL-10) titers of the 1st , 2nd and 3rd immunized groups and 4th control negative groups at 28 day post immunization.

Groups	Mean ± SE of IgG, TNF-a and IL-10 (nm)		
	IgG	TNF-a	IL-10
1st group immunized with WKLAg	21.07 ±0.36 C	769.38 ±3.64 C	986.73 ±13.48 B
2nd group immunized with Con-A	32.65 ±1.07 B	858.42 ±9.26 B	1223.96 ±10.44 A
3rd group immunized with Mixed Antigene	40.91 ±0.26 A	953.03 ±4.82 A	1275.35 ±7.26 A
4th group non- immunized Control negative	12.22 ±0.23 D	364.79 ±4.91 D	372.83 ±14.96 C

Capital different letters denoted that significant difference between groups (P≤0.05).

RESULTS AND DISCUSSION

Biochemical identification of bacteria

The isolate was identified by using Gram stain based on colony morphology under the light microscope with evidence of gram-positive cocci, arranged in single or pairs, long or short chains as Figure 1.

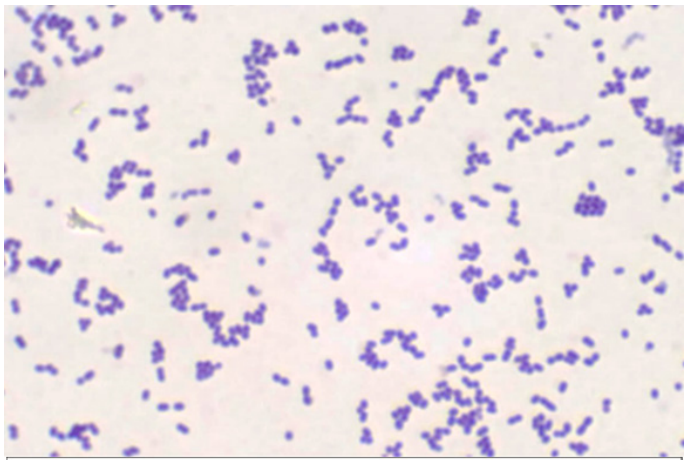

Figure 1: Gram positive *S. pyogenes* cocci (X100).

Another biochemical analysis used Vitek-2 system as speed and accuracy test for proof identification of Group A *streptococcus pyogenes* GAS bacteria with a probability ratio (97%) depended on the results of 64 biochemical tests as shown in microbiological chart report Figure 2.

شركة المصنر العلمي للتدريب و التطوير و الخدمات
bioMérieux Customer: Microbiology Chart Report Printed May 25, 2022 5:43:41 PM CDT

Patient Name: مترى Sample Patient ID: 32239
Location: Physician:
Lab ID: 32239 Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes

Source: **Collected:**

Comments:	

Identification Information	Analysis Time:	4.85 hours	Status:	Final
Selected Organism	97% Probability	Streptococcus pyogenes		
	Bionumber:	051432320711231		
ID Analysis Messages				

Biochemical Details																
2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	-	11	AGLU	+
13	APPA	+	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	+
20	LeuA	+	23	ProA	+	24	BGURr	-	25	AGAL	-	26	PyrA	+	27	BGUR	-
28	AlaA	+	29	TyrA	+	30	dSOR	-	31	URE	-	32	POLYB	+	37	dGAL	-
38	dRIB	-	39	ILATk	-	42	LAC	-	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	+	50	NC6.5	-	52	dMAN	-	53	dMNE	+	54	MBdG	-	56	PUL	(-)
57	dRAF	-	58	O129R	+	59	SAL	-	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

Figure 2: Microbiological chart report of vitek-2 test.

Humoral and cellular response in immunized rat (ELISA technique)

The serological finding of 3rd immunized group recorded significantly elevator in Abs titer (40.91±0.26) and both Tumer Necrosis Factor TNF and Interleukin 10 IL10 (953.03±4.82, 1275.35±7.26), respectively when performed at 28 day post immunization, with noticeable decreased in 2nd and 1st groups for Immunoglobulin G IgG concentration (32.65±1.07 and 21.07±0.36) consecutive and for mention's cytokines as (858.42±9.26, 769.38±3.64 and 1223.96±10.44, 986.73±13.48), respectively compared with negative control group as above (12.22±0.23, 364.79± 4.91 and 372.83±14.96), with significant difference P≤0.05 as in Table 1 and Figure 3.

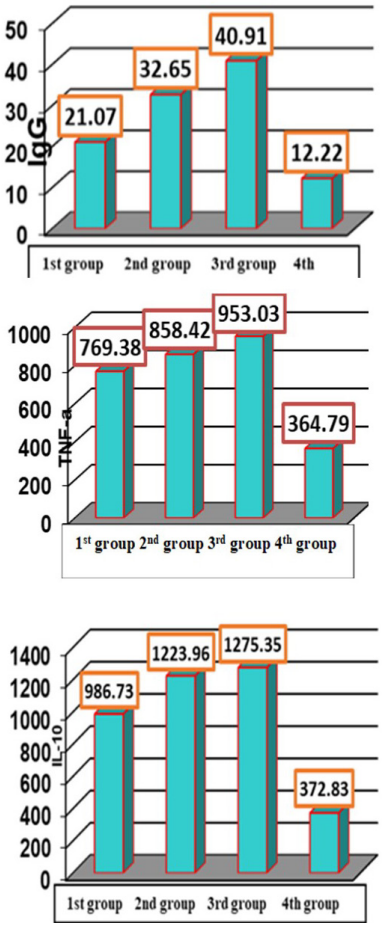

Figure 3: Mean and standard error of (IgG, TNF-a and IL-10) titers of the 1st, 2nd and 3rd immunized groups and 4th control negative groups at 28 day postimmunization.

Postmortem examination

Estimation of any gross and histopathological lesions associated with immunization was carried out. No gross changes associated with immunization in any organ, except various degree of hepato- splenomegaly were recorded mainly in 3rd mixed immunized group and 2nd group immunized with concanavalin-A in comparison with other groups as Figure 4A and B.

Microscopical examination

Histopathological changes in liver tissue of 1st group immunized with whole killed lyophilized antigen of *S.pyogenic* exhibited focal Mono Nuclear Cells MNCs infiltration with obvious binucleated hepatocyte Figure 5A, another section show hepatic edema with hepatocyte anisonucleosis Figure 5B, while splenic observation showed reactive lymphoid hyperplasia with slight red pulp congestion Figure 5C, together with marked paracortical lymphoid hyperplasia as Figure 5D.

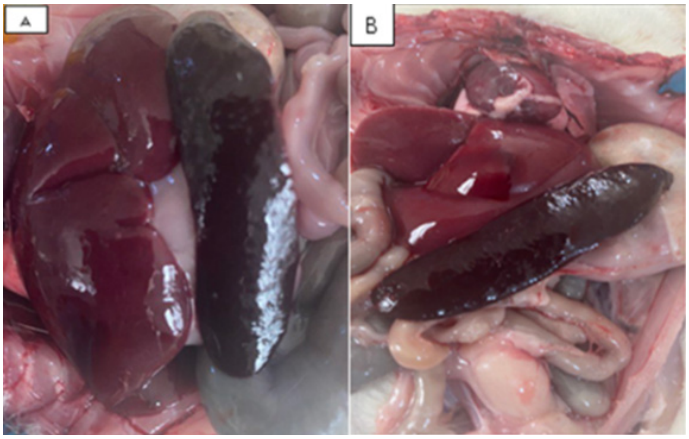

Figure 4: Gross appearance of hepatosplenomegaly in 3rd group (A) and 2nd group (B).

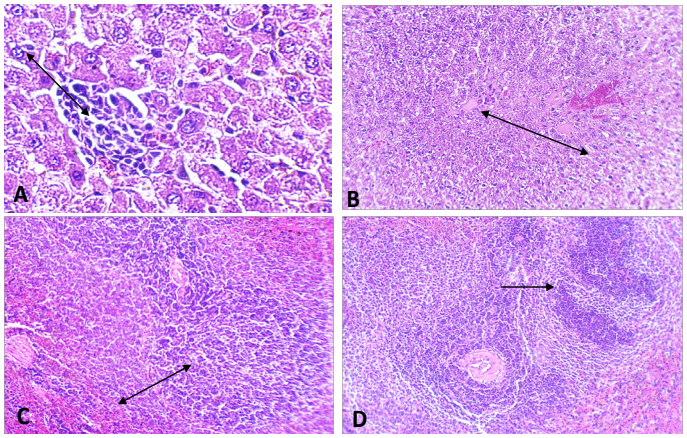

Figure 5: A histopathological slices in rat immunized with WKL Ags. at 28 day post immunization. (A) Liver showed focal MNCs infiltration in liver tissue with obvious binucleated hepatocyte. (B) Liver showed edema in hepatic tissue with anisonucleosis. (C) Spleen showed reactive lymphoid hyperplasia with slight red pulp congestion. (D) Spleen showed obvious paracortical lymphoid hyperplasia.

The pathological picture of liver in 2nd group immunized with Concanavalin-A Con-A perceive perivenular Mono Nuclear Cells MNCs infiltration with hepatic vasodilation Figure 6A, another section represents slight portal MNCs infiltration with ductal dilation Figure 6B, while the splenic tissue appeared with reactive lymphoid hyperplasia with slight splenic vessels congestion Figure 6C, another section clarified lymphoid hyperplasia with vascular fibromuscular hypertrophy Figure 6D.

The liver tissue manifestation of 3rd mixed immunized group referred perivascular and periductal Mono Nuclear Cells MNCs infiltration with nuclear pyknosis and the cytoplasm of hepatocyte appear hyper eosinophilic Figure 7A, while another section showed mild periductal Mono Nuclear Cells MNCs infiltration with obvious ductal dilation Figure 7B, as well as evidence of periarteriolar lymphoid hyperplasia were recorded in splenic tissue Figure 7C, accompanied with great lymphoid hyperplasia and red pulp congestion Figure 7D.

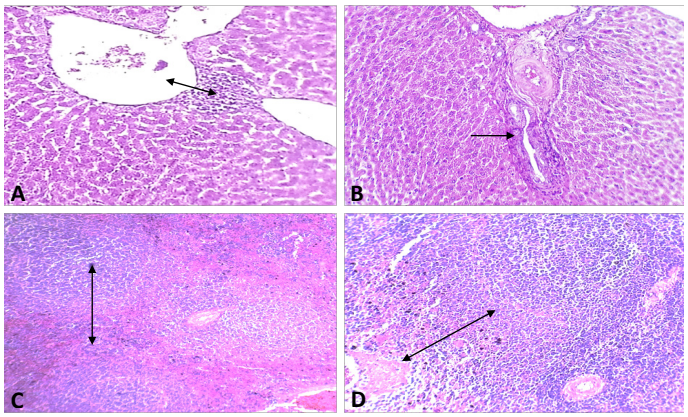

Figure 6: A histopathological slices in rat immunized with Con-A at 28 day post immunization. (A) Liver showed perivenular MNCs infiltration with ductal dilation. (B) Liver showed slight portal MNCs infiltration with ductal dilation. (C) Spleen showed reactive lymphoid hyperplasia with slight splenic vessels congestion. (D) Spleen showed lymphoid hyperplasia with vascular fibromuscular hypertrophy.

Streptococci are pathogenic bacteria affect humans and animals cause invasive infection and produced many virulence factors and toxins help escaping the host immune response (Simone *et al*., 2022) such as M related surface protein as primary antigenic factor have critical role in phagocytic survival and providing antiphagocytic functions also given continuous ability for *Streptococcus pyogenes* to diversity and variable for that no safe and effective vaccine only slight known about anti- Group A *streptococcus pyogenes* GAS protective immunity (Douglas *et al*., 2022).

As the first step toward biochemical identification of Beta

B-hemolytic Group A *streptococcus pyogenes* GAS bacteria used gram stain as analysis method to detect the presence of single or pair gram positive streptococci (Abdur Rehman *et al*., 2021) when culture in blood agar under (5-10%) Co_2 condition at 37°C for 24 hours show the B-hemolytic (Mais *et al*., 2018; Minas *et al*., 2022), these cocci stained dark purple color because their peptidoglycan cell wall that absorbed crystal violet while (Amity *et al*., 2012; Al-Ogaidi *et al*., 2020) indicated staining of microcolonies by blue -violet color revealed investigative of biofilms in specimens that give positive result for present of *streptococcus pyogenes*, However in current study a specific selective media for *S. pyogenes* were used and the bacterial colonies appeared as small white colonies.

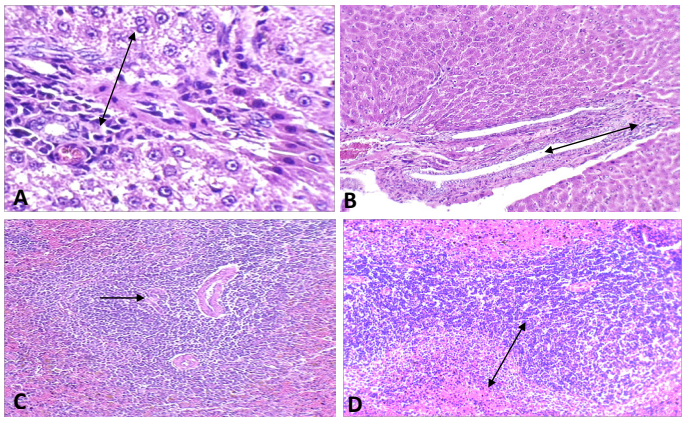

Figure 7: A histopathological slices in rat immunized with mixed Ag. at 28 day post immunization. (A) Liver showed perivascular and periductiolar MNCs infiltration with nuclear pyknosis and hyper eosinophilic of hepatocyte cytoplasm. (B) Liver showed mild periductal MNCs infiltration with obvious ductal dilation. (C) Spleen showed periarteriolar lymphoid hyperplasia. (D) Spleen showed great lymphoid hyperplasia with red pulp congestion.

The result of vitek-2 test agent with 10 samples encountered with present isolates that obtained from sheep suffering from respiratory symptoms and explain by (Kadhum and Hussain, 2020; Abbas *et al*., 2022) who referred that vitek-2 anew automated and exactness technique used for rapid identification of *S. pyogenes*. In order to activate immune system some mitogenic substance was used in current study like Concanavalin-A con-A can recruit lymphocyte and elicit cytokine production together with increased Antibody Ab titers in immunized groups compared with control group, these observation supported by study of (Huldani *et al*., 2022) whose revealed that administration of non-toxic dose of Concanavalin-A con-A has essential role in activation of immune cells including neutrophils, kupffer and B cells distinguished in liver and production massive amount of lymphokines as result from their mitogenic activity in murine, Also study by (Beilei *et al*., 2022) explain the Concanavalin-A Con-A ability to binding with several receptors like Membrane Type Matrix Metallo Proteinase-1 (MT1-MMP) in normal and cancer cells which accountable for lectin's modulations, oscillating from triggering immune cells to kill of tumor cell moreover to study consequence of Concanavalin-A Con-A on signaling ability to induce autophagy and apoptosis together with modulate related signaling cascades these finding mainly recognized in hepatic tissue of 3rd immunized group.

The analysis of Immunoglobulin IgG, Tumor Necrosis Factor TNF-a and Interleukin 10 IL-10 in the present work revealed a significant elevator in all immunized groups at 28 day post immunization specially in 3rd group and this result accorded with (Roua and Ikram, 2021) who showed substantial increasing in antibody concentration with significant difference between groups ($P<0.05$) at 28 days post immunization compared with the negative control group, for record these elevate in cytokines used Enzyme-Linked Immunosorbent Assay ELISA technique according to (Agharid *et al*., 2022).

According to above histopathological evidence, perivenular and periductular Mono Nuclear Cells MNCs infiltration mainly in hepatic tissue of mixed immunized group because the liver is an chief fence between us and openair world and have ability to mount a rapid and robust immune response under appropriate conditions, also has capability to screen and immunological filtrate the blood and designer to reveal, catch, and visible bacteria, virus, macromolecules and great gathering of phagocytic cells in body however immune suppression can occur due to absenteeism of costimulatory molecules, to the relative low expression of *Major Histocompatibility Complex* MHC, and expressing of anti-inflammatory cytokines such as IL-10 (Paul and Craig, 2018).

Various forms of lymphoid hyperplasia were main finding in 2nd and 3rd groups related with elevated of bacterial load and proliferated of B and T-lymphocyte, This phenomena has been associated with many bacterial types including *Staphylococcus aureus*, *Haemophilus influenzae, S. pneumoniae* and Group A *streptococcus pyogenes* GAS (Amity *et al*., 2012). *In vitro*, streptococcal pyrogenic exotoxin A (SPEA) stimulate murine T-lymphocyte result by serial of varied seen in lymphoid tissues after streptococcal sepsis were consistent with an *in vivo* superantigen response as well as proinflammatory streptococcal pyrogenic exotoxin A (SPEA) induced responses can provide for protecting innate responses through aggressive sepsis (Donlan, 2001), our result concluded that display to bacterial superantigen in sepsis performed directly to infiltrate lymphoblastic tissues mainly in splenic immunized group and confirmed best correlation between immunological and pathological finding.

Subcutaneous immunization with streptococcus antigen accompanied with adjuvant enhancement potential effect of immune response as compared with negative control group and this observation consistence with several authors idea who mentioned that subcutaneous (Edilberto *et al*., 2013) intramuscular (Tan *et al*., 2021) and intranasal (Aniela *et al*., 2018) vaccination with effective adjuvanted protein prevent even fatal invasion infections in mice, but great improvement in immune response reported in mixed immunized group and that may indicate passive transfer of immunity used antisera and collective human immunoglobulin when it's highly concentration successful to induce immunity in animal models (Reglinski *et al*., 2015). Obvious reduction in the Immunoglobulin G IgG levels were recorded in the streptococcus antigen immunized group as compared with other immunized groups this evidence may be due to regulation activity of streptococcal cysteine protease, Streptococcal pyrogenic exotoxin B Spe B, by cleaving bacterial and host protein also may be explained that Strep A strategic for inhibiting antibody function include specific proteases of Immunoglobulin G IgG, Ides and EndoS, that split antibodies at Fc region (Ulrich *et al*., 2002). There are many theories explained the ability of Strep A antibodies confer protection one of them Strep A innate immunity is type-specific and focused against M-protein, builds specific responses accountable for safeguard and another theory mention that immune responses accumulate with recurrent exposure to conserved Strep A antigens resulted in elevation threshold of protection against following infection (Pandey *et al*., 2012). Streptococcus pyogenes has developed numerous mechanisms to alter the structure and function of Immunoglobulin G IgG to avid Antibody Ab mediated immune response (Toledo *et al*., 2023).

CONCLUSIONS AND RECOMMENDATION

The result concluded that Concanavalin-A Con-A have immunological effect by enhancing both pro and anti-inflammatory cytokine when given in non-toxic dose, also Concanavalin-A have the best immunomodulatory effect against *S. pyogenes* infection when mixed with bacterial antigen. The current study recommended for used of farther technique like immunohistochemistry and immunocytochemistry by using several markers together with study the effect of Concanavalin-A on the expression of genes related with virulence and biofilm formation of *S. pyogenes*.

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Many thanks go to the Veterinary Medicine College, University of Baghdad, for providing resources to accomplish this study.

NOVELTY STATEMENT

This report provide A novel mechanism in which Concanavalin-A used as immunomodulator and give supporting for *S. pyogenes* antigens which act as anti-microbial agent.

AUTHOR'S CONTRIBUTION

Sura Ayed Radam and Inam Bader Falih: Designed and Performed the experiments, analyzed the data, contributed reagents, materials, analysis tools and wrote the paper.

Ethical approval

All procedures used in this study were approved by the local Scientific Research Committee of veterinary medicine College, Baghdad University consent with the ethical principles guidelines on the care and use of animals in research of animal welfare (Approval number P.G 380 in 19-2-2024).

Data availability

Data are available upon reasonable request.

Conflict of interest

The authors have declared no conflict of interest.

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