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INFLUENCE IMMUNIZATION BY CULTURE FILTRATE STAPHYLOCOCCUS AUREUS ANTIGEN CARRIED BY SILVER NANOPARTICLES AS ADJUVANT AGAINST *STAPHYLOCOCCUS AUREUS* INFECTION IN MICE

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ABSTRACT
In order to investigate the action of silver nanoparticles as immune stimulator adjuvants against *Staphylococcus aureus*, fourty white rats both sex ,average age 8 to 10 weeks were randomly divided into five group and treatment as following.1st group (n=10) was immunized with o,3ml of CFSAgs (protein concentration 5.6 mg/ml) S/C two dose for two weeks intervals, 2nd group (N=10) was immunized as 1st group but mixed CFSAg with AgNPs (1:1 ratio). 3rd group (n=5) was inoculated S/C with 0.1ml of AgNPs, 4th group (n=5) was served as control positive group and 5th group (n=10) was served as control negative group, at 28-30 day post immunization skin test and passive hemagglutination test were done than animals of 1st, 2nd, and 4th groups were inoculated I/P with 1*10 cfu of virulent *S. aureus* and the 5 th group was inoculated I/P with 0.3ml of sterile normal saline the result revealed high cellular and humeral immune response in 2nd group as compared with 1st group and 1,3 animals were died from 1st and 3rd group, respectively, during 2 weeks post infection with heavy bacterial load. The result expressed severe supportive reaction abscess formation, thrombus in blood vessels degenerative changes, necrosis and fibrosis in examined organs of 3rd group while mild to absent to granulomatous lesions were seen in immunized animals particularly in 2nd group, it concluded that immunized animals with CFSAgs carried with AgNPs provide a good immune response that completely protective immunity against *S. aureus* infection.

KEYWORDS: silver nanoparticles, CFSAgs, granulomatous lesions, immunity, *S. aureus*

INTRODUCTION

food borne disease is considered important healthy and economic problem worldwide ,these disease caused by bacteria or their toxins ,viruses or parasites that induced human infection post consumption of contaminated food by these pathogens such as gastrointestinal infections[1] food can contaminated by these pathogens through food processing or from environment and animal feces at slaughterhouse [2,3] the important bacterial species isolated from animals meat are *Staphylococcus aureus* and other pathogens[4,5]. *S. aureus* was considered a third essential cause of food borne disease worldwide [6]. it was reported that the most common outbreaks of food poisoning occur by enterotoxin A of *S. aureus* in USA that form (77.8% of all food poisoning outbreaks [7] . *S. aureus* characterized by resistance to beta lactam antibiotic particularly MIRSA strains therefore the treatment of these pathogen particularly MIRSA required prolong time of treatment with high costs and high percentage of patient hospitalizing [8]. in addition to difficult treatment because MIRSA stain has ability to form biofilm and induced chronic infection[9], *S. aureus* cause wide range of human and animal infections due to these pathogen has broad spectrum virulence factors including secreted and cell surface associated factors, these factors play role in bacterial adhesion to target cells, proliferation, avoid host defense mechanism, invasion, and damage host tissue and induced disease[10] therefore augument immune system may be play a role in the control *S. aureus* infection, several attempts were done to find vaccine against *S. aureus* infection in humans and animals but these trials are failed and till day, no effective vaccine development against these pathogen[11].

In order to improve vaccine immunogenicity, varies naturally and synthetic materials were used as adjuvant with vaccine antigen such as salts of aluminum to activated innate and acquired immune response[12] but these salts has disadvange such as induced local reaction with prolong inflammatory reaction in addition to weak cellular immune response[13] therefore the researches focus on new adjuvant that increase immunogenicity of weak antigens and prolong stimulation of immune response with less side effects, new science field development is nanotechnology that applied in different fields such medical field which used as drug deliver system in addition in activate immune system due to their physicochemical features such as size, shape, surface area and their charges, these features facilitated trapping or capturing of proteins and nucleic acids with nanoparticles and deliver antigens to antigen presenting cells and improve the immune response[14] there were several nanoparticles usage as adjuvant such as silver, gold, polylactide co glycolide that enhance immune response against numerous pathogens [15-17] .

In Iraq, there are few studies about the application silver nanoparticles as adjuvant vaccine against *S. aureus*, therefore the aim of the present study was to determine the

role silver nanoparticle adjuvant to improve immune response against *S. aureus* infection.

MATERIALS & METHOD

Source of silver nanoparticles

Pure commercial nanoparticles (AgNPs) with certain concentration 25µg were obtained from department of chemistry, college of science, University of Al-Nahrin.

Isolation and confirmation of *S. aureus*

S. aureus was isolated from bovine meat samples, using bacteriological identification and biochemical test according to[18] and molecular study to determine sea gene of *Staphylococcal enterotoxin A.* Culture filtrated *Staphylococcus aureus* antigen were prepared according to [19, 20].

Experimental designs

Fourty healthy white rats, both sex, age ranged between 8-10 weeks. Were randomly divided into four groups and treated as the following:

1.1st group (n=10) was immunized with (0.3) ml of *S. aureus* CFSAgs concentration of protein (5.6mg/ml), S/C two doses, 14 days intervals.

2. 2nd group (n=10) was immunized as in the 1st group but was used Ag NP with bacterial antigen as adjuvant.

3. 3rd group (n=5) was inoculated with 0.2ml of S/C AgNP.

4. 4th group (n=10) was saved as control positive group.

5. 5th group (n=5) was served as control negative group.

Cellular immune response was detected at 28 days post immunization with skin test and at day 30 post immunization, 5 animals from 1st, 2nd groups were sacrificed for the collection of blood and study the humeral immune response, then 1st, 2nd, and 4th groups were challenge I/P with $1*10^9$ cfu/ml of viable virulent *S. aureus* and 5th group was inoculated with 0.3 ml of sterile normal saline I/P.

All animals of each group were sacrificed at 3 weeks post challenge and post-mortem examination was done, pieces from internal organs were taken for bacterial isolation and other pieces were fixed in 10% neutrals buffer formaldehyde (72 hrs) for histopathological examination [21].

RESULTS

Immune response

At 24 hr post injection soluble sonicated *Staphylococcus aureus* antigen, the mean thickness of skin in the CFSAg + adjuvant (1.26) was higher than those value in immunized animals with CFSAg (0.74), at 48h post examination both mean value were decreased (0.49 and 0.43) respectively, Table :1

TABLE 1: Mean an standard error of skin test in differencs immunized groups against (SCFSAg) and (SCFSAg+ adjuvant) at 24hr ,48hr (measured in mm)

Group	24 (mean ±SE)	48hr (mean ±SE)
CFSAg + adjuvant	1.26 ±0.11	0.49 ±0.05
CFSAg	0.74 ±0.08	0.43 ±0.15
Control positive	0	0

also passive hemagglutination test revealed high antibodies titer in the serum of animals immunized with CFSAg–AgNPs (768hemagglutination units) as comparing with those values in immunized animals with CFSAgs alone (112 hemagglutination units) (Table: 2).

TABLE 2: mean and standard error of titer Abs in passive hemagglutination test in immunized groups against (SCFSAg) and (SCFSAg + adjuvant) at 30 days

Group	Titer of Ab(mean ±SE)
CFSAg + adjuvant	768±147.8
CFSAg	112±16
Control positive	0

Bacterial isolation and clinical signs

The results showed that 3 out 5 of non-immunized infected animals and 1 out 5 of immunized animals with CFSAgs were died during the first 2 weeks post infection with heavy bacterial isolated from examined organs while no mortality were recorded in immunized animals with CFSAg –AgNPs adjuvant with mild or no bacterial isolated from internal organs of these group and remain life animals of other groups

TABLE 3: - show bacterial loaded in the examine organ

Animal group	Liver	kidney	Spleen	Lung
Control	+++	++++	++++	+++
CFS Ag	+	++	_	_
CFS Ag+ adjuvant	_	_	_	_

-= absent, +=mild, ++=moderate, +++=heavy, ++++=very heavy

Histopathological examination

Non immunized infected animals

The main lesions in the lung characterized by fibrin deposition and neutrophils infiltration in the intestitial tsissue in addition to thrombus formation, abscess formation was recorded in the lung tissue (Fig:1)The heart showed neutrophils infiltration in the pericardium and around congested blood vessels between cardiac muscle fiber and fragment cardiac muscle fiber (Fig: 2), severe congested of blood vessels and dilated sinusoids, thrombus

formation were seen in the liver in addition to necrosis and pyogranulomatous lesions in liver parenchyma (Fig: 3), it was recorded neutroiphils around and in the lumen of congested blood vessels in pia pai matter, degenerative changes in the neuron, in addition to central chromatolysis of purkinje cells (Fig: 4) and proliferation of astrocytes, oligodentrocytes and microglial cells, the spleen expressed severe hemorrhage of red pulp with atrophy of white pulp (Fig: 5), fibrosis of interstitial tissuewith atrophy of glomerular tufts were the main lesions in kidney (Fig: 6). Animals inoculated with AgNPs Histopathological section of the examined organs revealed no clear lesions except mononuclear cells aggregation around blood vessels ,hyperplasia of white pulp of the spleen in addition mononuclear cells infiltration around glomerula (Fig:7).
Immunized animals with CFSAg T3 weeks post infection, the main lesions in the lung characterized by marked lymphoid tissue hyperplasia with granulomatous lesions consisting from aggregation of macrophages and lymphocytes (Fig:8), also proliferation of kupffer cells of the liver with mononuclear cells aggregation around blood vessels, in other section, it was found granulomatous lesions in liver parenchyma (Fig:9), however, there were proliferation of lymphocytes in the periarterioler sheath of the spleen and between rental tubules of the kidney ,in addition to congested blood vessels in pia matter of the brain were seen (Fig:10)
Immunized animals with CFSAg-AgNPs post infection
It found no clear lesions in the examined organs such as lung (Fig:11) with mononuclear cells aggregation in portal area of the liver and small granulomatous lesions in the parenchyma and around glomerula (Fig:12,13).

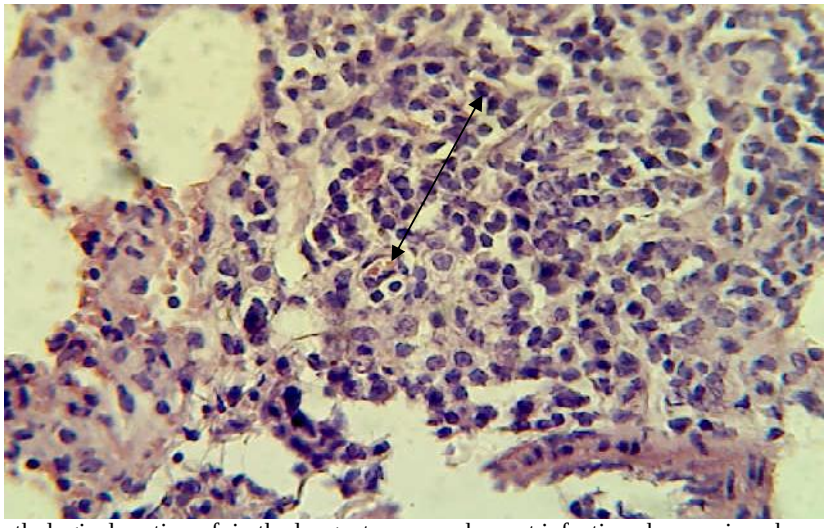

FIGURE 1: Histopathological section of in the lung at one weeks post infection shows microabscess in the interstitiak tissue (H and E stain 40X)

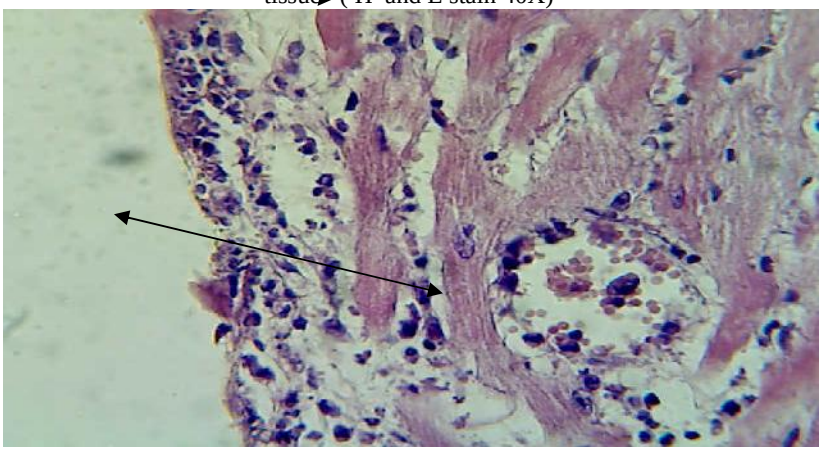

FIGURE 2: Histopathological section of the heart animal at 2 weeks post infection shows neutrophils infiltration in the pericardium and in and around congested blood vessels between cardiac muscle fiber (H and E stain 40X)

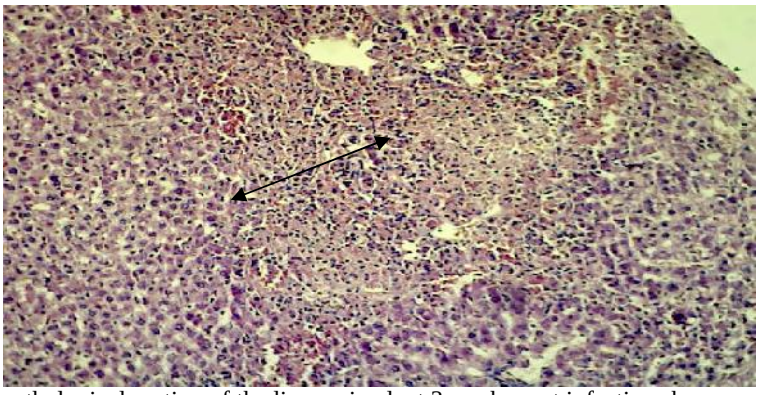

FIGURE 3: Histopathological section of the liver animal at 3 weeks post infection shows necrotic area around granulomatous lesions granulomatous lesions consisting from

FIGURE 4: Histopathological section of the cerebelum at 1 weeks post infection shows (central cheomatolysis of purkinje cells characterized by homogenous round cell body without neuclei as well as oligodentrocytes attached with degenerative cell (H and E stain 40X)

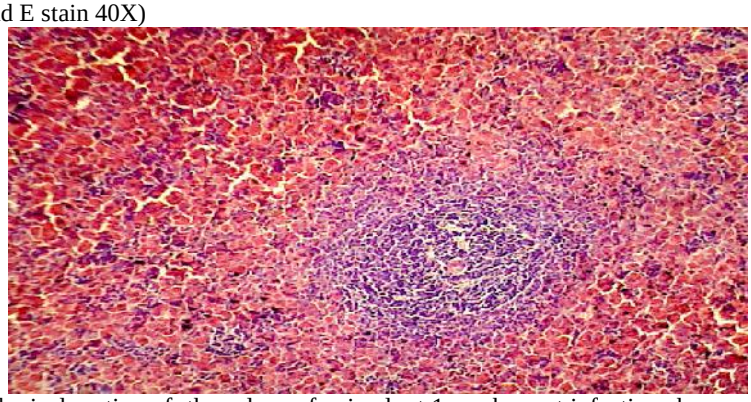

FIGURE 5: Histopathological section of the spleen of animal at 1 weeks post infection shows severe hemorrhage of red pulp with atrophy of white pulp (H and E stain 100X)

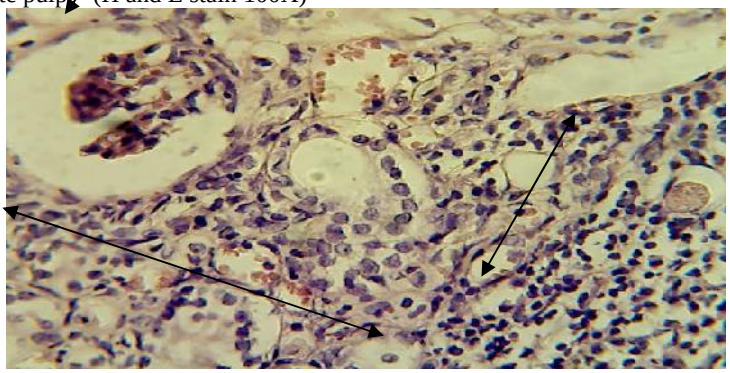

FIGURE 6: Histopathological section of the kidney of animal at 3 weeks post infection shows neutrophils and mononuclear cells infiltration in proliferation fibrous connective tissue the interstitial tissue, hyaline cast in the lumen of renal tubules and atrophy of glomerular tuft with dilated of Boman space (H and E stain 40X)

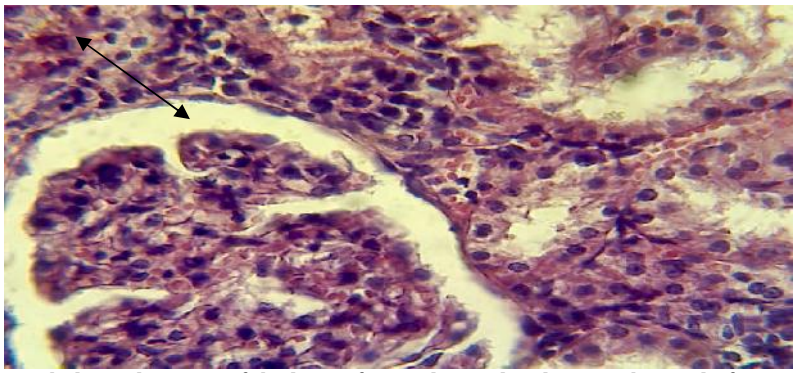

FIGURE 7: Histopathological section of the liver of animal inoculated S/C with 0.2ml of AgNPs shows shows mononuclear cells aggregation around glomerula (H and E stain 40X)

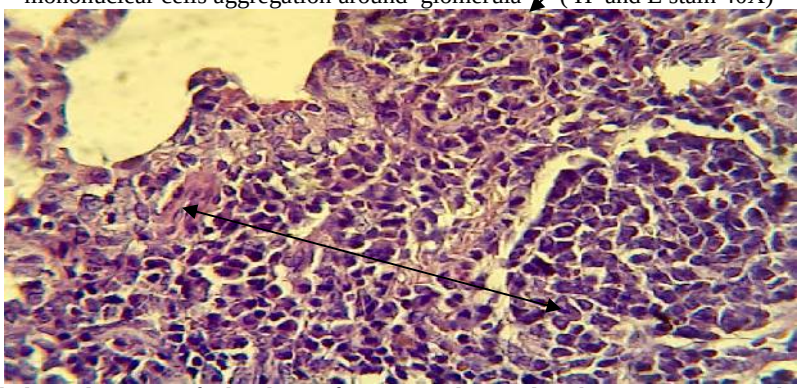

FIGURE 8: Histopathological section of the lung of immunized animal with CFAgs at 3 weeks post infection shows granulomatous lesion consisting from aggtregation f macrophages with severe mononuclear cells aggregation in the interstial tissue (H and E stain 40X)

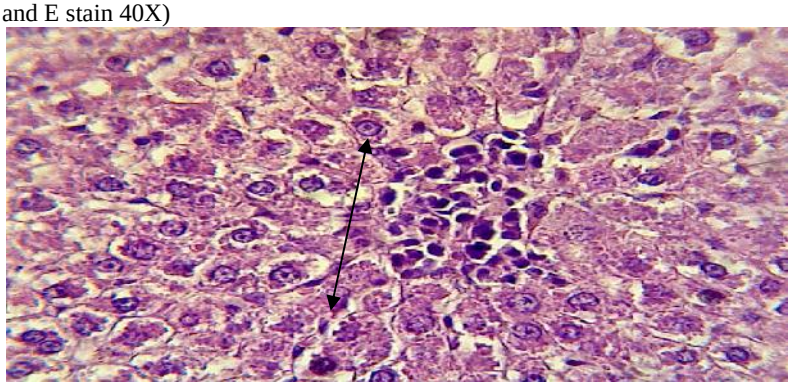

FIGURE 9: .Histopathological section of the liver of immunized animal with CFAgs at 3 weeks post infection shows granulomatous lesion consisting from aggregated macrophages and lymphocytes in liver parenchyma (H and E stain 40X)

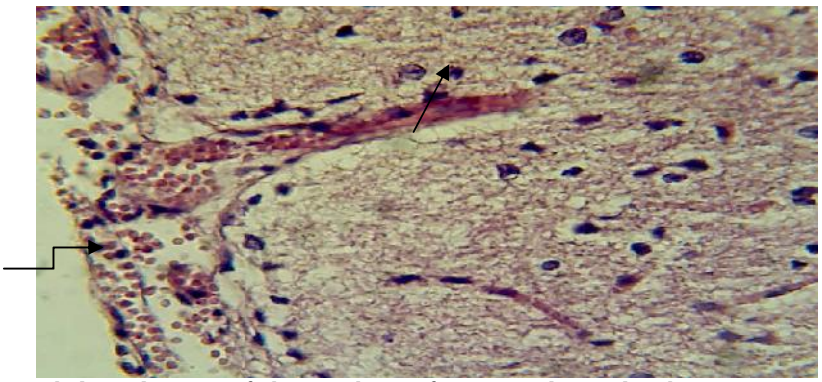

FIGURE10: Histopathological section of the cerebrum of immunized animal with CFAgs at 3weeks post infection shows congested blood vessels in the pia matter (H and E stain 40X)

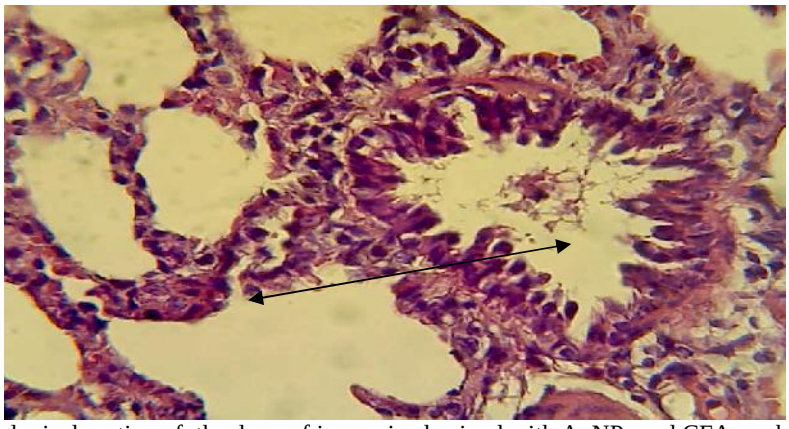

Figure 11:Histopathological section of the lung of immunized animal with AgNPs and CFAgs shows at 3weeks post infection shows no clear lesions (H and E stain 40X)

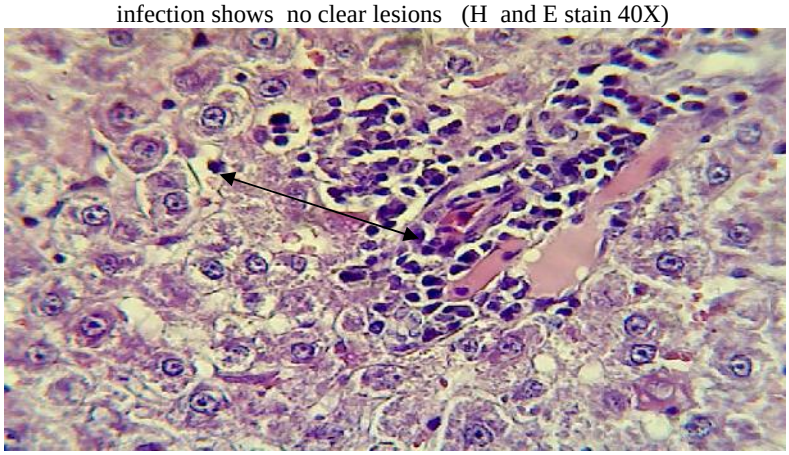

FIGURE12: Histopathological section of the liver of immunized animal with AgNPs and CFAgs shows at32 weeks post infection shows mononuclear cells aggregation in the portal area and small granule in liver parenchyma ↙ H and E stain 40X)

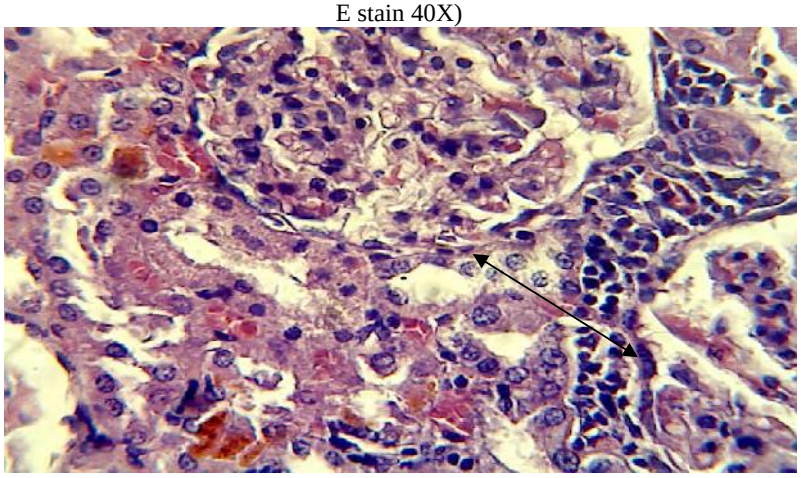

FIGURE 13: Histopathological section of the kidney of immunized animal with AgNPs and CFAgs shows at3 weeks post infection shows mononuclear cells aggregation around glomerula ↙H and E stain 40X)

DISCUSSION

Immune response

High mean skin thickness and high mean serum antibodies titers in immunized animals may indicated that these bacterial secretion antigen stimulated both humeral and cell mediated immune response ,particularly DTH reaction was considered a better indicator of stimulated cell mediated immune response that occur by Th1 producing INF-Y these cytokines can produced by activated CD4 and CD8 T cells [22] as a result of protein nature of CFSAgs which considered a good stimulator of cell mediated immune response.The current results were similar to result that recorded by[23] in rabbits immunized with whole sonicated *Staphylococcus aureus* antigens. It was found in the current study that usage AgNPs mixed with CFSAgs in immunized animals stimulated good cell mediated and humeral immune response as comparing with those result in immunized animals with CFSAgs alone, these result may indicated that AgNPs formulated can augument both arms of acquired immune response,

these idea was agreement with [24] who found that Ag NPs adjuvant better stimulated humeral and cellular immune response due to activation of CD4 and CD8 T cells that actively produced INF Y which play role in induce Ig class switching to Ig2a, also the present result was agreement with [25] who recorded that activated immune response by NPs attributed to activated transmission of antigen into regional lymph node efficient activation and maturation of dentric cells and activated rapidly memory T cells differentiation in draining LNs. High levels of serum antibody titers (768 hem agglutination units of Ab) in immunized animals with CFSAgs AgNPs was in constant of observation of[16] who showed that intraperitoneal and subcutaneous inoculated AgNPs as adjuvant with two type of antigen ,ovalbumin and bovine serum albumin in mice lead to significantly increase levels of serum IgG of immunized animals as compared with control animals particularly IgG1/IgG2a ratio also they found increased in levels of TNF-α and IFN-Y in abdominal lavage of immunized animals .AgNPs can easly phagocytosis by peritoneal macrophages without damage of antigens also recruitment and activation of local leukocytes particularly neutrophils and macrophage. Dead of the animals in the current finding may indicated the isolates strain of *S. aureus* is pathogen do not normal flora contaminated bovine meat samples and these pathogen overcome host immune system ,proliferation and toxins that cause damage of multi organ lead to animals died. These idea was agreement with [26] who found that *S. aureus* can infected any organs of the body due to possess wide range of virulence factors including proteins ,enzymes and toxins, *S. aureus* produced 23 serologically difference toxins such as Staphylococcal enterotoxins and Staphylococcal enterotoxin like proteins [27], addition these pathogen cause toxic shock syndrome by toxic shock syndrome toxin I (TSST 1) [28] .

Absent mortality with mild or no bacterial isolated in immunized animals particularly those immunized by (CFSAg+AgNPs) may indicated these Ags can stimulated a good protective immune response against bacterial infection, activated CD4 and CD8 Tcells by CFSAgs lead to produce INF y that activated innate immune cells particularly macrophages and these observation was coincident with result of DTH sensitivity in immunized animals which associated with activated of immune cells ,these idea was agreement with[29] who investigated that activated of innate immunity play role in eradicated of bacterial infection. also absent bacterial isolated from examined organs of animals immunized by CFAg AgNPs indicated these Ag was significantly active production of INF y that activated peritoneal macrophages which rapidly and engulfed and killed all inoculated bacteria at the site of inoculation,, these idea was in consistent with [25] when used nanoparticles as adjuvant including polysaccharide PS with cubosomes (Cub-PS) nanoparticles as compared with these ratio when used Cub and PS group alone and these nanoparticles can improve immune reaction against pathogen

Histopathological examination

The main lesions in the examined organs of non-immunized infected animals were supportive reaction, these lesions may indicated that these pathogen associated with production of pro inflammatory cytokines and chemokines that attracted the neutrophils to site of infection, these idea was agreement with[30] who demonstrated that the main lesions associated with *S. aureus* infection were suppurative reaction with tissue destruction abscess formation in the examined organs particularly may indicated that these pathogen has virulence factors associated with abscess formation these evidence was agreement with[31] who demonstrated that *S. aureus* can, disseminate to different body tissue and seed abscess formation in which the bacteria survival and multiple in the center of the abscess that separated the bacteria from immune cells by pseudo capsule [32].

Thrombus formation in the lung and other organs of infected animals in the current study may indicated that these pathogen possess factor for blood clotting , these result was agreement with the result of biochemical test that demonstrated that certain *S. aureus* isolates in the present finding were coagulase positive which considered important test to differentiate pathogenic *S. aureus* from non-pathogenic commensal Staphylococci[33], these enzyme can converted prothrombin into thrombin and fibrinogen into fibrin that responsible for clot plasma or blood [34]. Pyogranulomatous lesion in the liver with neutrophils infiltration may indicated these pathogen cause acute inflammatory reaction followed by chronic reaction, these idea was agreement with[35,36] who found that *S. aureus* infection cause acute inflammatory cells infiltration in the tissue followed by micro abscess formation, due to toxic effect of hemolysis that cause damage neutrophils and monocytes [37, 38], IL 1B secreted by activated macrophages which responsible for pyogranulomatous lesions formation [39-41]. Lesions in the liver in the current study were similar to these described by Al-Ani in experimental infection mice with *S. aureus*.

Severe hemorrhage in the spleen with depletion of white pulp in the current study may due to these pathogen secreted toxins and enzymes that destroyed endothelial cells of blood vessels lead to hemorrhage .similar result was detected by [42] who demonstrated that *S. aureus* secreted toxin including alpha toxin lead to rapidly depletion of ATP and cellular necrosis the present lesions in the brain and other organs of infected animals may indicated that the strain of *S. aureus* isolated from bovine meat is highly virulence and infected all organs of infected animals these idea was in consistent with [43] who found that *S. aureus* possess virulent factors such as IsdA and IsdB which o help SA to uptake iron from animal tissue hemoproteins also [44,45] demonstrated that SA has AdsA and protein A that diminish the immune responses mild pathological lesions in the organs of immunized animals with absent of abscess formation may due to CFSAgs consisted from all protein secretion of *S. aureus* that containing secreted virulence factor and these Ag stimulated Abs that neutralized or block activity these virulence factors, these idea was agreement with [46] who found that antibodies against IsdA or Iadb production by *S. aureus* can prevent *S. aureus* replication in host tissue and abscess formation, also[47] recorded that antibodies against SpA can help the immunized host to clearance of the *S. aureus* as well as neutralized B cell superantigen

stimulation antibodies against coa and vWbp may prevent binding coagulase to prothrombin or fibrinogen prevent abscess formation and bacteremia [48].

However, the presence granulomatous lesions in the lung and liver of immunized animals post infection may indicated the Ags used in the immunized animals in the present study stimulated cell mediated immunity in which granulomatous formation in immunized animals was considered a good marker to these type of immunity due to both CD4 and CD8 T cells producing INF y play essential role in induced granulomatous reaction[49]. These result may indicated that AgNPs adjuvant efficiency activated local peritoneal macrophages and other immune elements that destroyed most *S. aureus* at site of infection and few of them reached the liver, these idea was agreement with [50] who reported that AgNPs enhance both humeral and cellular immune responses in immunized animals with Ags carried by AgNPs as adjuvant in addition these Ags can activated local leukocytes including macrophages, lymphocytes and increased production cytokines, Mononuclear cells infiltration in the tissue with lymphoid tissue hyperplasia in immunized animals may indicated effective stimulated immune response [51].

However, immunized animals by CFSAgs alone expressed moderate to mild lesions as compared with animals immunized with Ag-AgNPs adjuvant, these result may indicated that the immune response induced by CFSAgs alone less efficiency than those elicited by usage AgNPs adjuvant.

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