EVALUATION OF THE ROLE OF CHITOSAN IN THE IMMUNOPATHOLOGY OF OSTEOMYELITIS IN DIABETIC RABBITS

Zainab Abdulkareem Maktoof* , Jihad Abdulameer Ahmed**

College of dentistry, University of Basrah, University of Basrah, Basrah,Iraq

Department of Pathology ,College of Veterinary Medicine, University of Basrah,Basrah,Iraq

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Corresponding Author: dr.jihad.vet@gma2il.com

ABSTRACT

This study was conducted to investigate the beneficial effects of chitosan in the immunopathology of osteomyelitis in diabetic rabbits; therefore, the experimental design was carried out on 40 rabbits. They were divided into 5 groups each of 8 animals, diabetes mellitus was induced in rabbits , then infected by *Staphylococcus aureus* and treated as following: First group (G1) was induced diabetes mellitus then immunized by whole sonicated *S. aureus* antigens (WSSAG) and induced experimentally osteomyelitis. The second group (G2) was induced diabetes mellitus, then immunized by (WSSAG) and induced experimentally osteomyelitis and fed on diet containing chitosan. Third group (G3) was induced diabetes mellitus, and induced experimentally osteomyelitis only. Fourth group (G4) was induction of diabetes mellitus, and induced experimentally osteomyelitis and fed on diet containing chitosan. Fifth group (G5) was induced experimentally osteomyelitis only without diabetes mellitus induction. Then at day 28th - 30th post immunization, skin test was performed to each of the immunized groups, and at day 30th the antibodies titer was measured by passive hemagglutination assay and phagocytic activity, then the animals were sacrificed and the treated bone taken for histopathological examination. In the present study , a significant increase was noted in the value of skin thickness of G2 at 48 and 72 hrs PI. A significant increase was also noted in the value of antibodies titers of G2. We also showed a marked decrease in the $t_{1/2}$ of carbon clearance of G2.

The histopathological results of G2 showed normal periosteal surface and compact bone with active osteoblasts lining the trabecular bones 30 days PI. However, other groups showed many histopathological lesions like infiltration of inflammatory cells and proliferation of fibrous connective tissue in G1, G3, and G4.The results also showed necrotic bone, hemorrhage, inflammatory cells infiltration and fibrosis in G5. Taken together, these findings indicate that the chitosan had a beneficial effect in bone healing of diabetic animals after infection in *S. aureus*.

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INTRODUCTION

Osteomyelitis is an inflammatory process accompanied by bone destruction and caused by an infecting microorganism. It can be limited to a single portion of the bone or involved several regions such as marrow, cortex, periosteum and the surrounding soft tissue (1). The commonest infecting organism isolated from osteomyelitis was *Staphylococcus* (54%) followed by *enterobacteriaceae* (23%) that includes *proteus spp* (12.5%), *E.coli* (8%), *Klebseilla* (2.5%), *P. aeruginosa* (18%), anaerobes (2.5%) and miscellaneous (2.5%) (2). *Staphylococcus aureus* osteomyelitis is a significant complication for orthopedic cases undergoing surgery, particularly with fracture fixation and arthroplasty, given the difficulty in studying *S. aureus* infections in human subjects and other domestic animals. The animal models serve an integral role in exploring the pathogenesis of staphylococcal osteomyelitis, and aid in determining the efficacy of prophylactic and therapeutic treatments (3). Chitosan was the deacetylated form of chitin, composed of glucosamine. It was produced commercially by deacetylation of chitin that naturally occurring polysaccharides structural element in the exoskeleton of crustaceans like crabs, shrimp. It exhibits various potential biological activities such as antitumor, immunostimulatory, antibacterial, and antifungal properties (4). On other hand, diabetes mellitus is a disease with many manifestations secondary to hyperglycemia which include peripheral arterial disease, peripheral neuropathy, and immune dysfunction. Osteomyelitis is often found in diabetic patients and can be difficult to treat in this complex and growing patient population (5).This work aimed to investigate the effectiveness of chitosan in improvement of immunity against diabetic rabbits infected by Staphylococcal osteomyelitis.

MATERIALS AND METHODS

Experimental Design

The study was carried out on 40 rabbits of both sexes (900-1100g), aged between 4 and 6 months. They are fed on special feed pellets and given ad libitum water. Rabbits were kept in standard environmental conditions (temperature (25±2°C) for adaptation at animal's house in the College of Veterinary Medicine-University of Basrah. They were divided into 5 groups randomly each of 8 animals. Animals of Group 1 were induced diabetes mellitus then immunized by whole sonicated *S. aureus* antigens (WSSAG) and induced experimentally osteomyelitis. Group 2 animals were induced diabetes mellitus, then immunized by whole sonicated *S.aureus* antigens (WSSAG) , induced experimentally osteomyelitis and fed on diet containing chitosan. Animals of Group 3 were induced diabetes mellitus, and induced experimentally osteomyelitis only. Group 4 animals were inducted of diabetes mellitus, and induced experimentally osteomyelitis and fed on diet containing chitosan. Animals of Group 5 were induced experimentally osteomyelitis only without diabetes mellitus inducted.

At day 28th to 30th post immunization, skin test was performed each of the immunized groups. At day 30th ,the antibodies titer was measured using passive hemagglutination assay. Then the animals were sacrificed and the treated bone taken for histopathological examination.

S. aureus strains were kindly provided by the department of Biology , College of Science- University of Basrah. Confirmative biochemical, and microbiological assays were performed as described by (6). Vitik assay was also applied to confirm the serotype of bacteria. The bacterial suspension was prepared as described by (7) and the challenge dose was 7.9×10^{10} CFU/ml.

The whole sonicated *Staphylococcus aureus* antigens (WSSAgs) were prepared as described by (8). The total protein concentration of the antigen was measured according to Biuret procedure in which 15mg/ml was diluted by phosphate buffer saline to 0.5mg/ml, then stored at -20°C till used, in which inject the 0.2ml in the animals footpad.

To determine the cellular mediated immunity, the skin test was performed as described by (9). To determine the humoral immunity, the test was applied as described by (10). The phagocytic activity was determined as described by by (11) in

which the phagocytic activity was calculated as a rate of carbon elimination of reticulo-endothelial and other inflammatory cells using carbon ink suspension.

Induction of diabetes mellitus via injecting 150 mg / kg bw alloxan monohydrate into the rabbits was applied as described by (12). The experimental induction of osteomyelitis in rabbit model was performed as described by(13). The bone specimens fixed, treated, and then stained as described by (14). The statistical analysis was performed using SPSS (15).

RESULTS

I. Immunological study:

1. Delayed type hypersensitivity (Skin test): Analysis of the Skin data revealed no-significant difference ($P \geq 0.05$) in the value of skin thickness between G1 and G2 after 24 hours, . However, a marked increase ($P \leq 0.05$) was noted in the value of skin thickness of G2 after 48 and 72 hours (table1).

Table (1): The mean values of skin test after 24-72 hrs of test.

Groups	After 24 hrs Mean ± SE	After 48 hrs Mean ± SE	After 72 hrs Mean ± SE
G1 Immunized + infection	0.64 ± 0.02 a	0.83 ± 0.02 b	0.71 ± 0.5 b
G2 Immunized + infection + chitosan	0.7 ± 0.03 a	1.28 ± 0.03 a	1.0 ± 0.4 a

- Results are expressed as Mean ± Standard Error of the mean of the eight replicates
- Different letters vertically refers to presence significant differences between groups.
- Skin test measured by mm.

2. Passive hemagglutination test:

Analysis of the passive hemagglutination test data in the day 30 post immunization revealed that the serum antibodies titer was significantly increased ($P \leq 0.05$) in the G2 (12.0 ± 1.78) compared to G1 (4.0 ± 0.89) (table 2).

Table (2): Antibodies titers post 30 days of immunization.

Groups	Abs titers (Mean ± SE)
G1 Immunized + infection	**4.0 ± 0.89 b**
G2 Immunized + infection + chitosan	**12.0 ± 1.78 a**

- Results are expressed as Mean ± Standard Error of the mean of the eight replicates
- Different letters vertically refers to presence significant differences between groups.

3. Phagocytic activity:

The statistical results of phagocytic activity revealed that G2 was significantly decreased ($P \leq 0.05$) in the half-life time t1/2 (2.58 ± 0.30) to clear the carbon by reticulo-endothlial system and inflammatory cells compared to G1 (5.6 ± 0.76) (table 3).

Table (3): Phagocytic activity of immunized groups after 30 days

Groups	$t_{1/2}$ of carbon clearance / minute (Mean ± SE)
G1 Immunized + infection	**5.6 ± 0.76** a
G2 Immunized + infection + chitosan	**2.58 ± 0.30** b

- Results are expressed as Mean ± Standard Error of the mean of the eight replicates
- **Different letters vertically refers to presence significant differences between groups.**

II. Pathological results:

1. First group (G1) (Immunized with infected with *S.aureus*):

Microscopic analysis of femoral bone at day 15 revealed congestion of blood vessels and infiltration of mononuclear cells. A few neutrophils were also noted in haversian canals and infiltrate in the periosteum (Figure 1). At day 30 post infection (PI), the trabecular bone lined by active osteoblasts and proliferation of fibrous connective tissue were noted (Figure 2).

2. Second group (G2) (Immunized and fed on chitosan with infected in *S.aureus*):

Histopathological analysis showed infiltration of active macrophages and lymphocytes. No clear lesions in bones of these group at 15 days PI was noted(Figure 3). At 30 days PI normal periosteal surface and compact bone with active osteoblasts lining the trabecular bones was also noted (Figure 4).

3. Third group (G3) (diabetic and infected in *S.aureus*):

Histopathological analysis of femural bone at day 15 revealed severe congestion with severe infiltration of neutrophils and mononuclear cells particularly macrophages in the bone marrow (Figure 5). In addition, 30 days PI showed abscess formation in the bone marrow surrounded by fibrous capsule. Fragments of necrotic bone (sequestra) surrounded by neutrophils and mononuclear cells and haversian canals filled with inflammatory cells, narrowing of trabeculae and no active osteoblasts was also noted (Figure 6).

4. Fourth group (G4) (diabetic with chitosan fed and infected in *S.aureus*):

Histopathological analysis of femural bone at day 15 PI and chitosan fed revealed infiltration of inflammatory cells with early granulation tissues (Figure 7), 30 days PI presence of active osteoblasts lining the trabeculae, also the bone marrow showed fibrus tissue formation in bone marrow. Formation of granulomatous reaction surrounding fragments (sequestra) of bone necrosis was also noted(Figure 8).

5. Fifth group (G5) (infected with *S.aureus* only):

histopathological analysis of femural bone at day 15 PI showed neutrophils infiltration, edema, and hemorrhage. Haversian canals filled with neutrophils and infiltration of neutrophils in periosteum was also noted (Figure 9). At day 30, there was an erosion of the cortical surface of the bone, subperiosteal fibrosis, and neutrophils infiltration present of fragments of necrotic bone (sequestra) surrounded by active osteoblasts and pool of exudate consists of neutrophils, macrophages and fibrous connective tissues (Figure 10) .

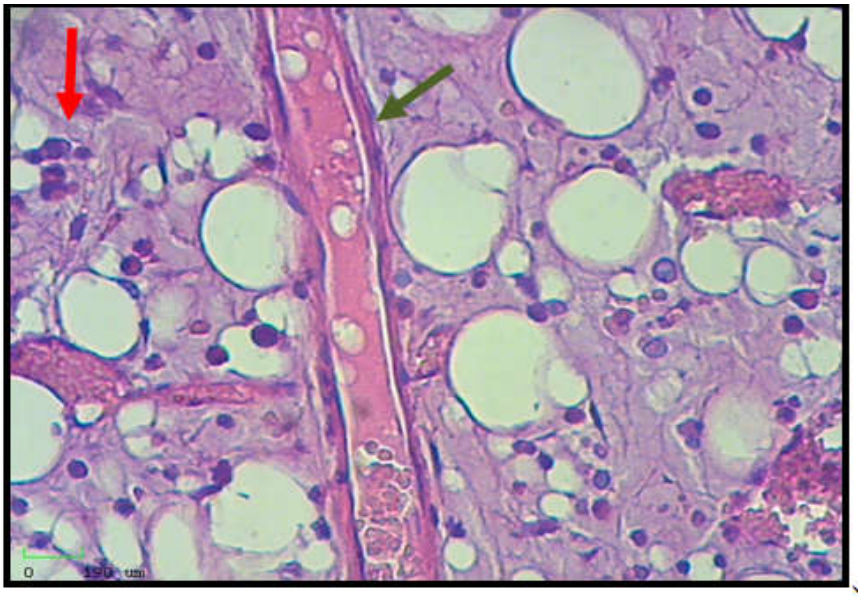


Figure (1): Histological section of femural bone of G1 15 days PI. Femural bone of G1 15 days PI shows congested blood vessel (green arrow), infiltration of mononuclear cells particularly active macrophages and lymphocytes (red arrow). (H&E stain. 40X).

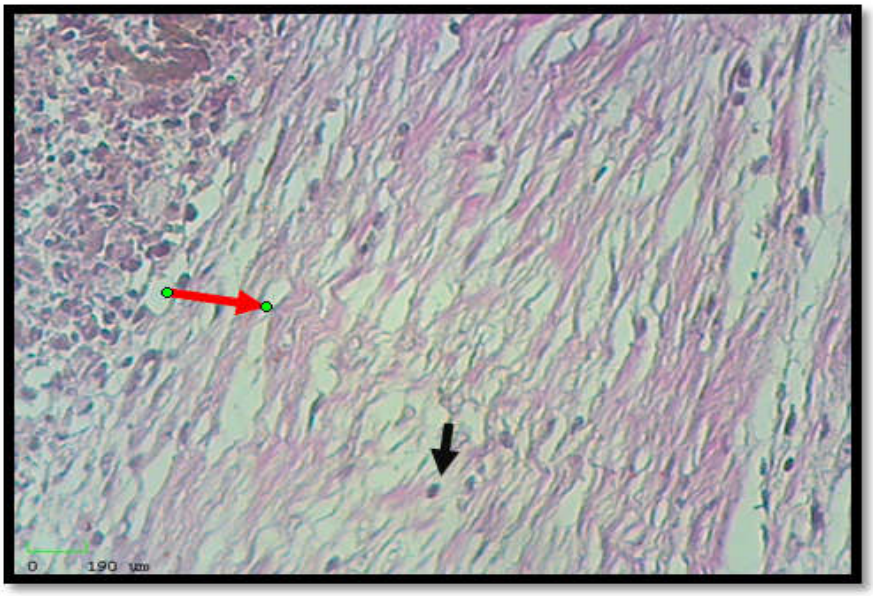


Figure (2): Histological section of femoral bone of G1 30 days PI. Femural bone of G1 30 days PI shows neutrophils and mononuclear cells infiltration (black arrow). Proliferation of fibrous connective tissue was also noted (red arrow) .(H&E stain, 40X).

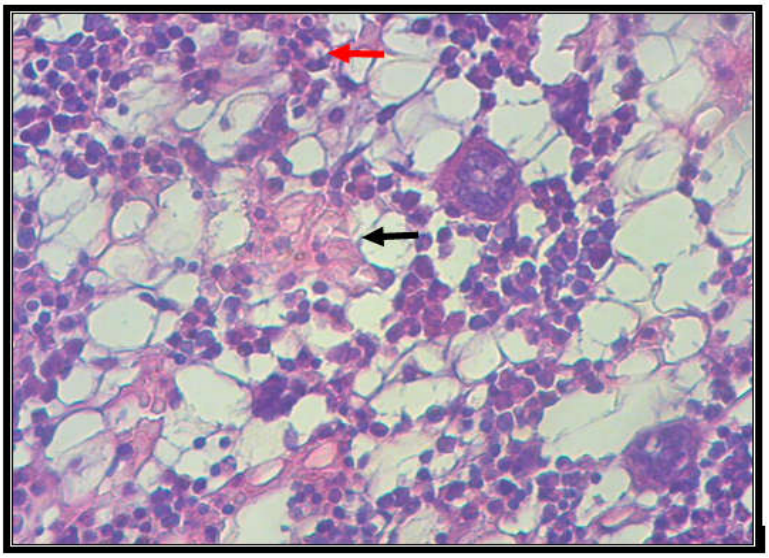


Figure (3): Histological section of femoral bone of G2 15 days PI. femoral bone of G2 15 days PI shows no clear lesion in bone marrow tissue, presence of active macrophages and lymphocytes (red arrow), mild congestion (black arrow).(H&E; 40X).

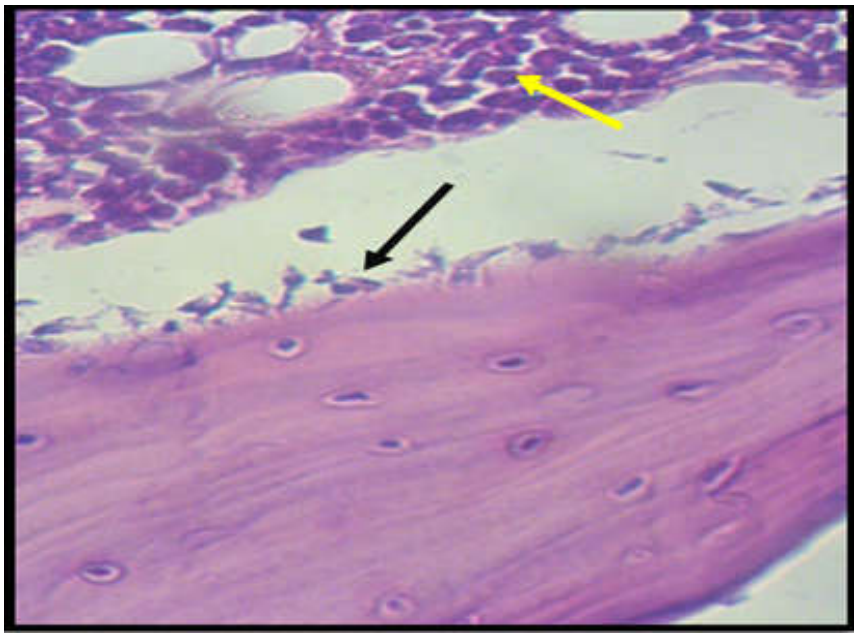
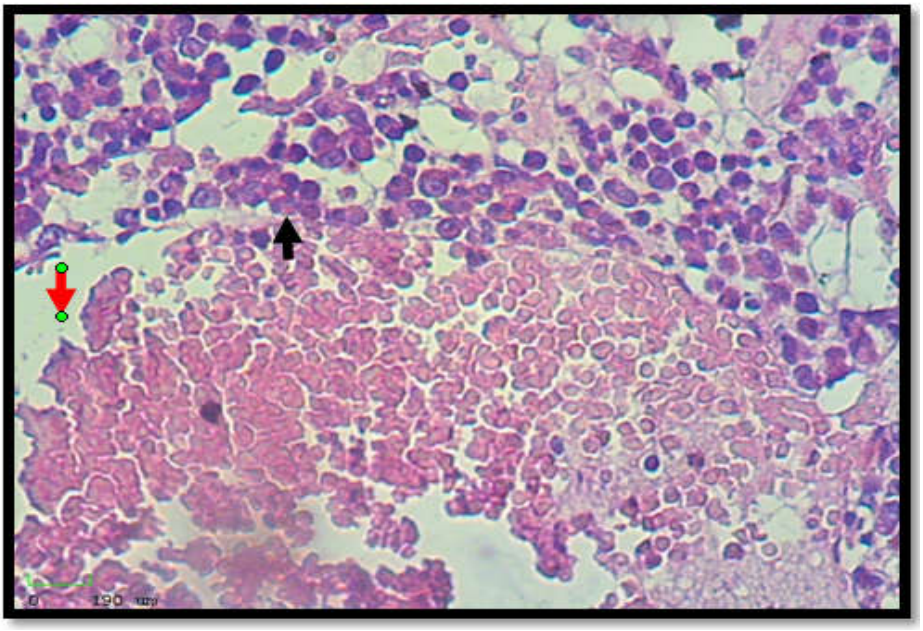


Figure (4): Histological section of femoral bone of G2 30 days PI femoral bone of G2 PI shows reactive osteoblasts (black arrow) in the surface of trabeculae. Infiltration of active macrophages and lymphocytes in the bone marrow was also noted (yellow arrow). (H&E. 40X).



Figure(5): Histological section of femoral bone marrow in G3 15 days PI. Femural bone marrow in G3 15 days shows severe infiltration of neutrophils and mononuclear cells (black arrow), severe congestion (red arrow). (H&E stain. 40X).

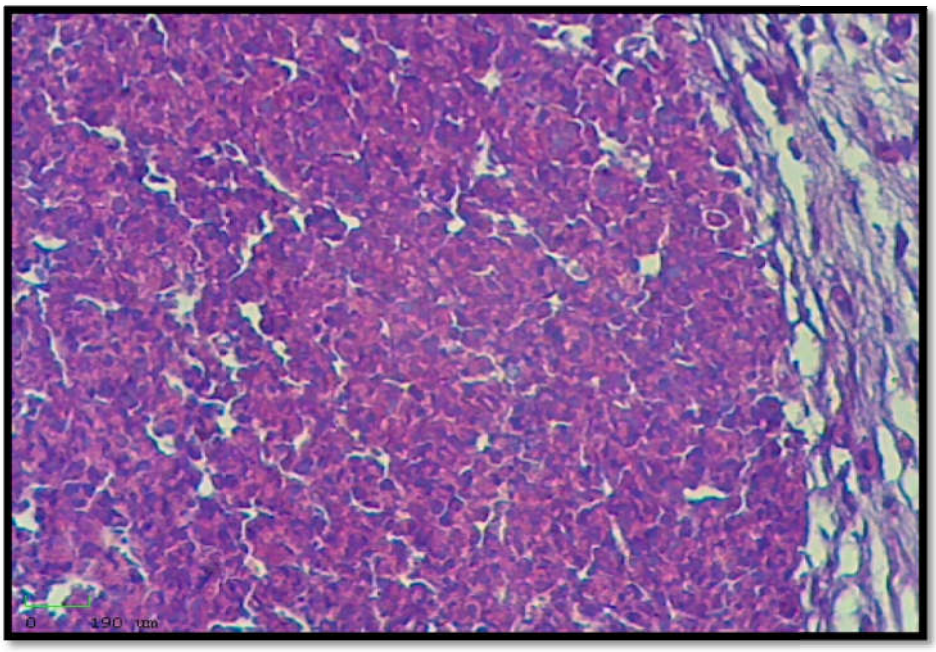


Figure (6): Histological section of femoral bone in G3 30 days PI. Femoral bone in G3 30 days PI shows abscess in bone marrow tissue (H&E stain. 40X).

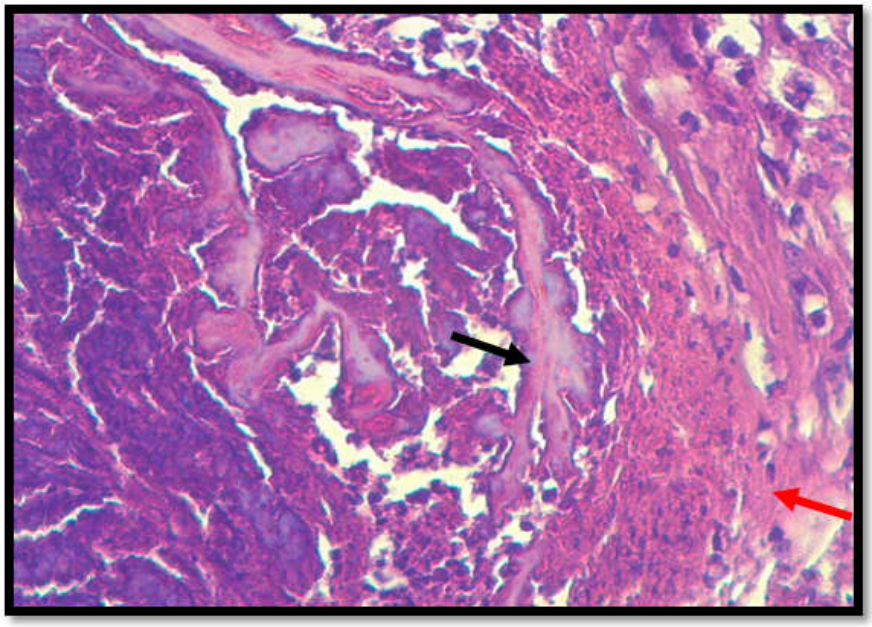


Figure (7): Histological section of femoral bone of G4 15 days PI. Femoral bone of G4 15 days PI shows early granulomatous reaction (red arrow) surrounding fragments of necrotic bone (black arrow). (H&E stain. 40X).

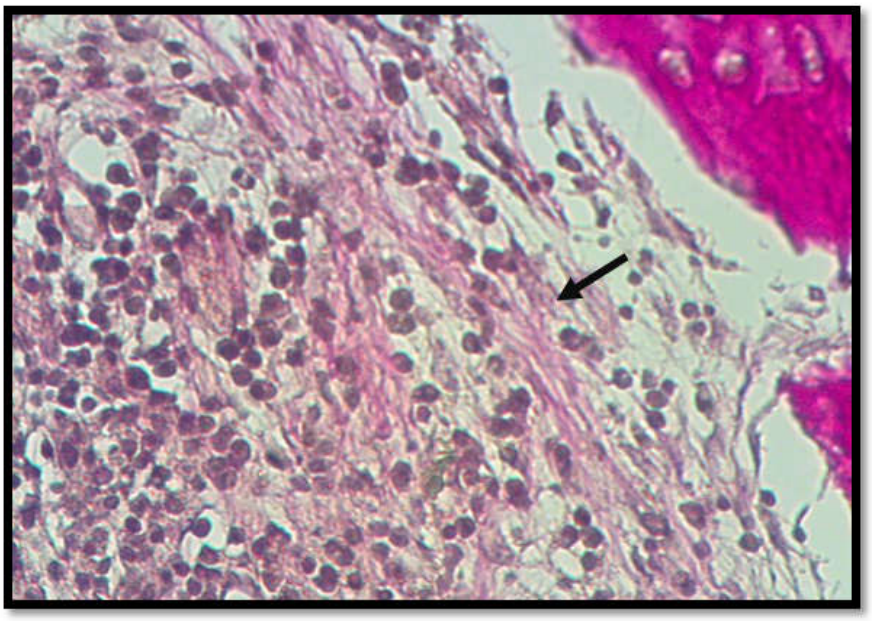
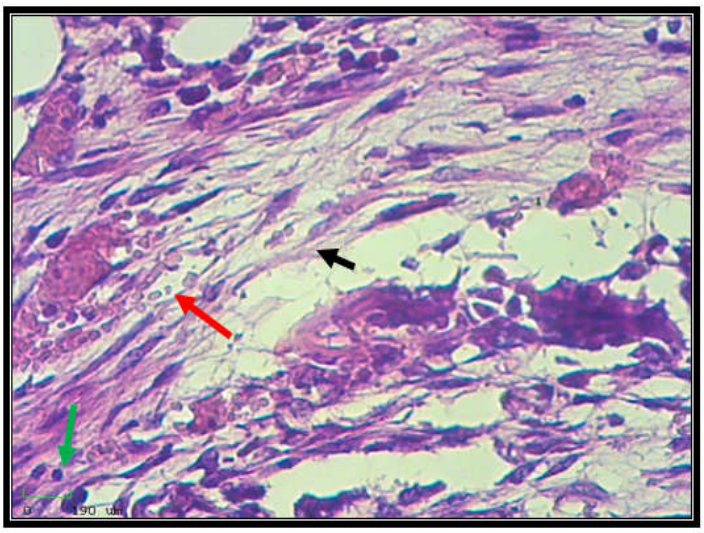


Figure (8): Histological section of femoral bone of G4 30 days PI. femoral bone of G4 30 days PI shows fibrosis in bone marrow tissue with mononuclear infiltration (black arrow). (H&E stain. 40X).



Figure(9): histological section of femoral bone in G5 at 15 days of infection showed neutrophils infiltration(green arrow) , hemorrhage(red arrow)and connective tissue proliferation(black arrow)(H&E stain; 40X).

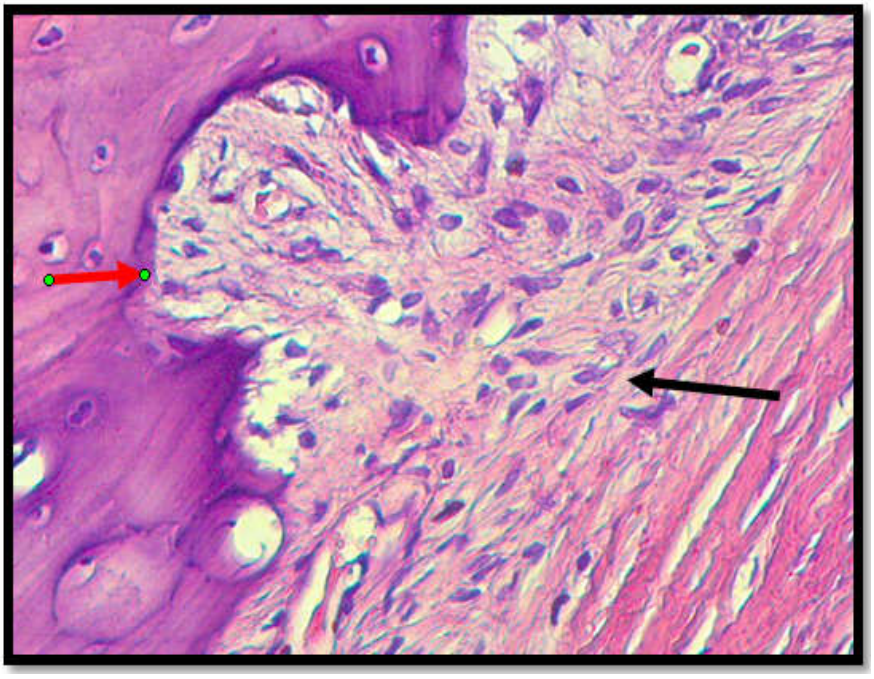


Figure (10): Histological section of bone in G5 30 days PI. Section of bone in G5 30 days PI shows erosion of cortical bone surface (red arrow) and subperiosteal fibrosis (black arrow).(H&E; 40X).

DISCUSSION

I. Immunological study:

Skin thickness test showed that DTH induced by soluble sonicated *S. aureus* antigen in immunized groups. This reaction **started** after **24 hours** and **continued** for 72 h. The DTH reaction involves cellular activation of T-helper cells (CD4+) and/or cytotoxic Tcells (CD8+ CTLs) leads to activation of macrophages which appeared as erythema and oedema at the injection site. This finding is in agreement with previous study (16) in which the DTH is characterized by its observed time course in which the reactions take approximately 12–24 h to develop, the reactivity persists for 2–3 days, and is dependent on the direct action of T- lymphocytes which recognize processed and presented antigen, unlike the other hypersensitivities, the type IV hypersensitivity is not antibody-mediated but rather defined as a T- lymphocyte-mediated response that can occur in response to a variety of deferent stimuli.

The present study also showed that the *S.aureus* vaccine stimulated humoral immune response, indicated that the vaccine stimulated both cellular and humoral immune response via activated proliferation and differentiation of CD4+T cell into Th1 and Th2 subsets and this cells mediated proliferation and differentiation of B-cells into antibodies producing plasma cells. This finding is in agreement with previous study (17) in which the peptides generated by antigen processing are expressed in the membrane of the APCs bound to the molecules of the major histocompatibility complex (MHC) class II, and presented to LTCD4+ (helper). The interaction between the peptide complex/MHC class II with the TL receptor (TCR) initiates a chain of events that lead the auxiliary TL to clonal expansion and production of cytokines that stimulate the proliferation and differentiation of B to produce immunoglobulins.

The current study found that immunized animals fed on diet supplementation of chitosan expressed high values of DTH reaction and serum Abs titers as compared to immunized animals only. This finding agrees with (18) who found that the immune response induced by chitin and chitosan is determined by the existence of antigen. It is believed that chitosan enhances both humoral and cell-mediated immune responses when rabbits were vaccinated with the antigen.

Adaptive immune response was dependent on innate immune cells, particularly phagocytic cell, the phagocytic activity test showed lower $t_{1/2}$ for carbon clearance in the blood in animals fed with chitosan indicating that chitosan activated macrophages and DCs which were essential innate immune cells also they found that chitosan activated directly T and B lymphocytes. This finding agrees with (19).

II. Pathological study:

Infected immunized rabbits with WSSAgs (first group) showed moderate inflammatory reaction in the medullary cavity and haversian canals of the cortical bone with few mononuclear cells infiltration in the periosteum at day 15 PI, indicating that the WSSAgs stimulated immune response that destroyed most of the microorganisms at the site of inoculation. These finding is in agreement with the results of immune response examination suggesting that both cellular and humoral immune responses have synergistic action against *S.aureus* infection. This finding is in agreement with (20) who suggested that repeated *S. aureus* infection or vaccination with live bacteria conferred clinical protection as well, in skin and soft tissue infection, both TH17 cells and antibodies contributed to the observed reduction of lesion size in *S. aureus* experienced animals.

The presence of fibrosis in bone marrow tissue at day 30 PI ,indicating that some microorganisms evade immune responses and the activated macrophages limited or destroyed most of these organisms. The current study showed active osteoblasts lining the trabecular bone may be due to activation of osteoblasts by Th-1 cytokines. These finding is in agreement with (21) who reported that multiple cytokines, chemokines and growth factors of the immune cells such as T and B cells, fibroblasts, DCs and macrophages directly or indirectly regulate osteoblasts and osteoclasts activity by producing or influencing the production of the RANKL/RANK pathway.

The current study showed no clear lesion in bone tissue of immunized-chitosan treated group (second group) during the course of the study, indicating that chitosan activated the defense mechanism of the bone tissue against inoculated *S.aureus*. This finding is in agreement with (22) who found that chitosan particles could activate components of the non specific immune system such as macrophages and NK cells and could induce nonspecific immunity to bacteria, fungi and tumors. Chitosan have immune stimulating activity such as increasing accumulation and activation of macrophages and polymorphonuclear cells, including cytokines in mice(23).

According to DTH reaction and phagocytic activity in these group, the results suggested that chitosan elicited a better CMI response that destroyed most of inoculated bacteria, however, the high level of antibodies titer in the immunized-chitosan treated group might be due to that co-administration of chitosan derivative, N-trimethyl chitosan (TMC) with the antigen lead to increase antibody production and protection when compared to administration of antigen alone(24). Moreover, the conjugation of the antigen to TMC improves immunogenicity of the antigen. This finding may be attributed to the fact the ability of chitosan to strengthen the humoral response that destroyed most *S.aureus* during the extracellular stage of infection. this finding is in agreement with previous study (25), in which the chitosan administrated at the mucosal interface increases antibody responses.

Chitosan and its derivatives chitosan oligosaccharides accelerated the absorption of calcium and other minerals in vivo also it increases calcium level in bone (26). This finding may be attributed to the fact that the active osteoblasts and the new growth of trabecular bone which seen at day 30 post inoculation indicating that chitosan activated the immune cells to secret cytokines that activated osteoblasts and improved osteoid tissue calcification. In addition , the bactericidal activity of chitosan may be attributed to the fact that chitosan have higher antibacterial activities than chitosan oligomers and stronger bactericidal effects on gram positive and gram negative bacteria(27).

The pathological changes in bone of diabetic rabbits only (third group) showed chronic inflammatory reaction characterized by abscess formation surrounded by fibrosis and infiltration of inflammatory cells in periosteum. This finding is in agreement with previous study (28), in which the pathological changes in bone of uncontrolled diabetics include chronic inflammation, fibrosis and scattered inflammatory cells. It has been found that the histological sections of bone in the chitosan group showed fibrosis and granulomatous lesions in bone marrow surrounding the fragment of bone necrosis compared to untreated ones. This finding may be attributed to the fact that chitosan activate immune response against these infected necrotic fragments Chitin and chitosan activate immunocytes and inflammatory cells such as PMN, macrophage, fibroblast (29). It has been found that fibrous tissue proliferation in bone marrow tissue in diabetic group fed on chitosan may be due to the stimulation of fibroblasts by effecting the fibroblasts growth factor lead to subsequent collagen fibers production (30).

The lesion of fifth group (positive control) in the bone marrow at day 15 showed neutrophils infiltration and necrosis of hemopoietic component, This finding may be attributed to the fact that *S.aureus* used their virulence factors to evade the host defense mechanism by inducing necrosis of bone marrow elements. This finding is in agreement with (31) in which the anton-Valentine leukocidin (PVL) is a bi-component, poreforming cytotoxin produced by *S.aureus* and targets host defence cells such as human polymorphonuclear neutrophils, monocytes and macrophages.

The presence of neutrophils in Haversian canal indicating the spread of the acute infection from bone marrow to the cortical bone and periosteum. This finding is in agreement with previous study (32) in which acute the infections might be spread to adjacent bony areas through the haversian and volkmann canal systems and the infection can penetrate through the cortical bone and further spread along the bone under the periosteum, which is elevated by the inflammatory process.

The current study showed erosion, necrosis of bone tissues with bone sequestra formation.These finding may be attributed to the fact that the virulence factors of the pathogens, in addition to inflammatory response of the bone to the invaded pathogens.This finding is in agreement with (33) in which the oxidative arm of the neutrophil defense system is provided by NADPH oxidase enzyme complex that generates superoxide anion after the catalytic transfer of electrons from NADPH to molecular oxygen, superoxide in turn rapidly converted spontaneously and/or catalytically to several downstream reactive oxygen species including hydrogen peroxide and hypochlorous acid. In addition, the nonoxidative arm of neutrophil defense system is provided by a maltitude of proteases ,hydrolases, antimicrobial peptides and protein components present in neutrophils granules and these enzymes induce tissue damage.

تقييم دور الكايتوسان في المناعة المرضية لألتهاب العظم في الأرانب المصابة بداء السكري

زينب عبد الكريم مكطوف* ,جهاد عبد الأمير أحمد**

*كلية طب الأسنان, جامعة البصرة ,البصره، العراق.

**فرع الأمراض وأمراض الدواجن ، كلية الطب البيطري, جامعة البصرة ,البصره، العراق.

الخلاصة

هذه الدراسة تمت للبحث في التأثيرات المفيدة لمادة الكايتوسان على المناعة المرضية لألتهاب العظم التجريبي في الأرانب المصابة بداء السكري، لذلك اقيمت التجربة على ٤٠ أرنب .قسمت الأرانب الى خمس مجاميع ،٨ حيوانات لكل مجموعة.تم أستحداث السكري في الأرانب ثم اصيبت بعدوى المكورات الذهبية العنقودية وتم معاملتها كما يلي:

المجموعة الاولى:تم استحداث السكري ثم تمنيعها بواسطة المستضد الكلي المتكسر للمكورات الذهبية العنقودية وبعدها تم اصابتها بعدوى العظم التجريبية. المجموعة الثانية: تم استحداث السكري ثم تمنيعها بواسطة المستضد الكلي المتكسر للمكورات الذهبية العنقودية وبعدها تم اصابتها بعدوى العظم التجريبية وتمت معالجتها بمادة الكايتوسان.المحموعة الثالثة:تم استحداث السكري واصابتها بعدوى العظم التجريبية.المجموعة الرابعة: تم استحداث السكري واصابتها بعدوى العظم التجريبية وتم علاجها بمادة الكايتوسان. المجموعة الخامسة: تمت استحداث اصابة العظم التجريبية بدون اصابتها بداء السكري.

في اليوم ٢٨_٣٠ تم عمل اختبار سمك الجلد في المجاميع الممنعة وفي اليوم ٣٠ تم اختبار مستوى الاجسام المضادة في الدم بواسطة اختبار التراص الدموي السلبي والفعالية البلعمية.ثم تمت التضحية بالحيوانات و اخذت مقاطع من العظم المصاب للفحص النسيجي المرضي.

هذه الدراسة اظهرت زيادة معنوية في سمك الجلد في المجموعة الثانية بعد ٧٢ ساعة من الاصابة.كذلك زيادة معنوية في مستوى الاجسام المضادة في المجموعة الثانية. اظهرت النتائج ايضا ان المجموعة الثانية استغرقت وقت اقل للتخلص من الكاربون في الدم.

الدراسة النسيجية المرضية اظهرت ان في المجموعة الثانية لاتوجد تغييرات مرضية في سطح السمحاق او العظم اضافة الى وجود خلايا بانية للعظم على سطح العظم.اما في المجاميع الاخرى كان هناك تغييرات مثل وجود خلايا التهابية وانقسام النسيج الليفي في المجاميع الاولى ،الثالثة والرابعة.النتائج اظهرت ايضا تموت للعظم ،نزف ، تجمع الخلايا الالتهابية وتليف في المجموعة الخامسة.بالاخذ بهذه النتائج ،اظهر الكايتوسان تأثيرات مفيدة على شفاء العظم في الارانب المصابة بالسكري بعد اصابتها بعدوى المكورات الذهبية العنقودية.

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