A Comparative Study between Ceftobiprole and Vancomycin in treating Skin and Soft Tissue Infections (SSTIs) due to Methicillin Resistant *staphylococcus aureus*in Mice

[a]Orooba M.S. Ibrahim, Ahmed Abdullah Najim
[a]Department of Physiology and Pharmacology College of Veterinary Medicine, Baghdad University, Baghdad, Iraq**.**

Abstract

The present study was carried out to compare between the antibacterial activity of Ceftobiprole and vancomycin*in-vitro* and *in-vivo* after inducing complicated skin and soft tissue infection in mice by injecting methicillin resistance *staphylococcus aureus*(MRSA)subcutaneously.In-vitro antibacterial study showed that methicillin resistance *staphylococcus aureus* was more sensitive to Ceftobiprole in comparison with vancomycin, and showed that ceftobiprole have a low MIC against MRSA (1µg/ml) when compared with vancomycin (2µg/ml). In-vivo study indicated that doses of 25mg/kg/day of ceftobiprole succeeded to reduce the lesion volume in infected mice and reduce the acute inflammation symptoms after four days, while vancomycin with same dose treated mice after seven days. Gross examination of mice skin treated with ceftobiprole and vancomycinshowed a reduction in lesion volume from (4.94 ± 0.59 cm^3)to(2.65 ± 0.63 cm^3) and (4.41 ± 0.8 cm^3) to(3.31 ± 0.46 cm^3) with recovery percentage (48%) and (24%) after 7 days of treatment respectively.

KEYWORDS:: Ceftobiprole, *S.aureus*, Vancomycin, Skin and Soft tissue Infections, methicillin.

Introduction

Skin and soft tissue infections (SSTIs) are common in outpatient clinic and emergency department visits and include a wide variety of infections of the epidermis, dermis, subcutaneous tissue, fascia and muscle(Edelsberg,2009 and Dryden,2009). The SSTIs usually result from traumatic, surgical or healthcare-related skin break down with secondary inflammatory microbial invasions(Edelsberg,2009).The severity of SSTIs ranges from mild superficial to deeper or potentially fatal necrotizing infections requiring hospitalization or intensive care(George *et al*.,2008).

The SSTI could be caused by a multitude of bacterial agents. The most important pathogenic agents are gram-positive bacteria, primarily *Staphylococcus aureus*and group A *streptococci*. In recent studies more than 50% of allSSTI were caused by *S. aureus.*(Wilson,2009).Methicillin resistant *Staphylococcus aureus*(MRSA), has complicated management of cSSTIs. These pathogens, previously confined to healthcare settings, are increasing commonly in the community(Fridkin*et al*.,2005and Chua *et al*.,2011).

Ceftobiprole is active in vitro against Gram-positive strains, including methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant coagulase-negative staphylococci (MRCoNS), penicillin-resistant *Streptococcus pneumoniae* (PRSP) and vancomycin-resistant *enterococci* (VRE), and Gramnegative bacteria, including *Escherichia coli* and *Pseudomonas aeruginosa*(Pillar *et al*.,2008 and Silva *et al*.,2010).Ceftobiprole has been shown to be effective in several animal models,

including infections caused by MRSA.(Silva *et al*.,2010) Currently, ceftobiprole has completed Phase III trials for complicated skin and skin structure infections (cSSSIs), and has been selected for the treatment of community-associated and hospital-acquired pneumonia**.**Present study was design to found out new antimicrobial effectiveness in treating of complicated skin infection caused by MRSA,and avoiding resistance toantimicrobial drugs. These aims should be accomplish by studying the *in-vitro* antibacterial activity of ceftobiprole, vancomycin and *in-vivo* throw treatment of experimentally cSSSIs with MRSA.

Materials and methods

Test organisms: A methicillin resistant *Staphylococcus aureus*isolate was obtained from the College of Veterinary Medicine/ Department of Zoonotic diseases/Baghdad University.These isolates spp. were identified bystudying morphological and some biochemicalcharacteristics. The organisms were maintained on agarslants stocks and were subsequently sub cultured intonewly prepared chromogenic and staph 110 agars and mannitol salt agar.

Determination of MIC: Minimum Inhibitory Concentration (MIC) of methicillin ,ceftobiprole and vancomycinwas determined by using standard two-fold microdilution broth methodology (NCCLS, USA, 2000). A stock solution of each antibiotic was serially diluted in 96-wells microtiter plate with Mueller Hinton broth to obtain a concentration of 1024, 512, 256, 128, 64, 32,16, 8, 4 , 2 µg/ml for methicillin and 64, 32,16,8,4, 2, ,0.5,0.25, 0.125 µg/ml for ceftobiprole and vancomycin. A standardized inoculum for each test organism was prepared so as to give an inoculum size of approximately 5 1.5 $\times 10^8$ cfu/ml in each well. Microtiter plates were then incubated at 37°C for 24hrs. Following incubation, the MIC was calculated as the lowest concentration of antibacterial inhibiting the visible growth of test organism using reflective viewer.

Antibacterial Susceptibility Assay:The agar well diffusion method was adopted according to (Perez*et al*.,1990), for assessing the antibacterial activity, standardized bacterial stock suspensions (1.5 $\times 10^8$ cfu/ml) of methicillin resistant *Staphylococcus aureus* was thoroughly mixed to sterile Mueller Hinton agar and distributed into sterile Petri dishes of each. The agar was left for 10 minutes to allow solidifying , and making 4 wells for each of these plates , 6 mm in diameter were cut using a sterile Pasteur pipette and the agar discs were removed by a sterile forceps, after that wells were filled with 0.1ml of each concentration of antibiotics (2 , 4 , 6 , 8 , 10) µg/ml. The plates were then incubated in the upright position at 37 C^o for 24 hrs. Three replicates were carried out for each concentration of the antibiotics and the activity of these antibiotics was determined by measuring the diameter of inhibition zone around each well by millimeter against the tested organism. The results and standard errors means values were tabulated.

Experimental Animals

Forty healthy male Swiss mice (White BALB/c) , aged 8-12 weeks, and weights 25-30 gm. Mice were housed in plastic cages and placed in a special housing room / Department of Physiology and Pharmacology / College of Veterinary Medicine for two weeks for adaptation period.

Preparing the miceand inducing infection

After the adaptation period, each mouse was anesthetized by isoflurane inhalation. preparing the left flank region by clipping the hair with electrical clipper after that removing the hair by sterile gauze. Bacterial inoculums used to induce infection (skin infection) was (2.7 x 10^6cfu/ml) of MRSA suspension, 0.25 ml was s/c administered into the left flank of each mice and watched for symptom of inflammation. (Bitton, 2005; Quinn *et al* ., 2004).

Experimental design: Forty mice were divided equally into four groups(ten mice / each, and treatment began after 24 hrs. after inducing complicated skin and soft tissue infections). Group (A): negative control, Group (B): positive control, Group (C): infected with MRSA and treated with ceftobiprole 25 mg/kg B.W.S/C for 7days and Group (D): infected with MRSA and treated with Vancomycin 25 mg/kg B.W. S/C for 7 days.

Estimation of skin lesion volume

To evaluate the antibacterial effect of vancomycin and ceftobiprole on skin and soft tissue infection, skin lesion volume was measured by digital caliper 48 h after infection,at 2^{nd} day of treatment and at the end of treatment in day 7^{th}.Evaluation of treatment on lesion size was not blinded. A lesion volume score was calculated from the following equation;$\mathbf{LV=(\pi/6)(L\times W^2)}$

where LV = lesion volume.

L=length of the lesion in mm.

W=width of the lesion in mm.

Histopathological Examination

The skin lesion was sectioned from the sacrificed animals before infection, at 2^{nd} day and at the end of treatment at day 7^{th} . These sections were fixed in 10% formalin saline after fixation, the tissue was trimmed and the specimens were washed with saline for (1-2hrs) and transferred to the following steps: Dehydration, Clearing, Impregnation with Paraffin wax, Blocking, Sectioning andStaining with Haematoxylin and Eosin (Luna,1968).

Statistical Analysis

Data were analyzed statistically using the Microsoft Program (SPSS). Statistical analysis of data was performed on the basis of Two-Way Analysis of Variance (ANOVA) using a significant level of ($P<0.05$). Specific group differences were determined using least significant differences (LSD) as described by **(**Snedecor and Cochran, 1973).

Results and Discussion

Identification of the test microorganism

The microscopic appearance of methicillin-resistant *Staphylococcus aureus*spherical single cocci, diplococci, , but the predominant shape was grape-like clusters of blue color under light microscope, gram-positive. Goldenyellow to orange-pigmented colonies on Staph 110 agar, deep pink to magenta colonies on chromogenic media and on blood agar, colonies appeared as golden yellowproduces a zone of hemolysis surrounding the colonyand it was non-motile. All *staphylococci*produce theenzyme catalase when introduced to hydrogenperoxide. It is also producesthe enzyme

coagulase which allows the organism toproduce a clot in rabbit plasma. This is a key test todifferentiate *S. aureus*from other *staphylococci*. Thisdescription agrees with Subhankari*et al*. (2011) but*S. aureus*negative oxidase test.

Determination of MIC: The results of MIC estimated by tube dilution method showed that methicillin had high MIC (512µg/ml).This result is in agreement with Parves*et al*.,(2008) who referred that all the MRSA strains examined were resistant to methicillin MIC, 64 - 1024 µg/ml).Fuda*et al*., (2006).who found that the MRSA strains are oxacillin-resistant (MIC ≥ 128µg/ml). MIC of ceftobiprole relatively had low MIC (1.0µg/mL) while for vancomycin is (2.0 µg/mL). The highest sensitivity was noticed with ceftobiprole and the lowest with vancomycin. This result is in agreement with Entenza*et al*. (2011) who showed The MICs of ceftobiprole and vancomycinforMRSAwere1 and 2 µg/mL, respectively.

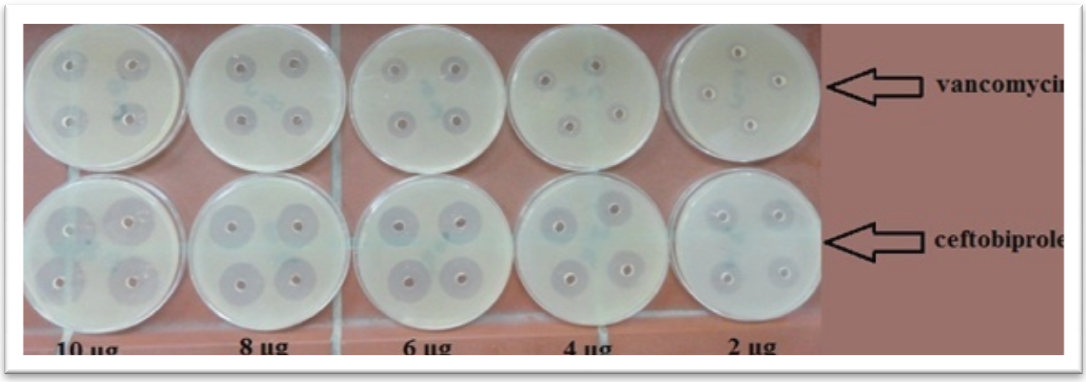
Antibacterial Activity

The size of inhibition zones were different according to concentration of ceftobiprole and vancomycin, the size of inhibition zone were proportionally increased with increasing of concentration, table (1) and figure (1). methicillin resistance *staphylococcus aureus*was sensitive significantly (P<0.05) to ceftobiprole more than vancomycin in a concentration dependent.Ceftobiprole exerts its antibacterial effect by binding to PBP, inhibiting transpeptidation and formation of the bacterial cell wall, leading to cell lysis and death. Ceftobiprole rapidly binds and forms a stable inhibitory acyl complex with PBP 2' (PBP 2a) and PBP 2x, which provide activity against β- lactam resistant *staphylococci* and *streptococci*, respectively. The stability of the enzyme complex, in combination with the long side chain that sits deep in the PBP 2'-binding pocket, enhances the stability of the bond and inhibition of the enzyme (Kontou*et al*.,2008; Kisgen and Whitney,2008; Bustos,2010).While vancomycin inhibits transpeptidation by binding to D-alanyl-D-alanine residues of the bacterial cell wall , historically has been the treatment of choice for MRSA infections, but adverse effects, the need for intravenous access, and growing resistance limit its use (Sharpe *et al*.,2005).

Table (1) Antibacterial activity of ceftobiprole and vancomycin in different concentrations (µg/ml) against MRSA.

Conc. µg/ml / Zone of Inhibition (mm)	2 µg/ml	4 µg/ml	6 µg/ml	8 µg/ml	10 µg/ml
Ceftobiprole	**20.25±0.45 Ae**	**22.125±0.12 Ad**	**24.37±0.37 Ac**	**25.75±0.32 Ab**	**28.5±0.35 Aa**
vancomycin	**0.00±0.00 Be**	**8.37±0.23 Bd**	**10.75±0.25 Bc**	**12.125±0.23 Bb**	**14.125±0.23 Ba**

Values represent mean ±S.E, Different capital letters mean significant (P<0.05) results between different groups .Different small letters mean significant (P< 0.05)results between concentrations within group.

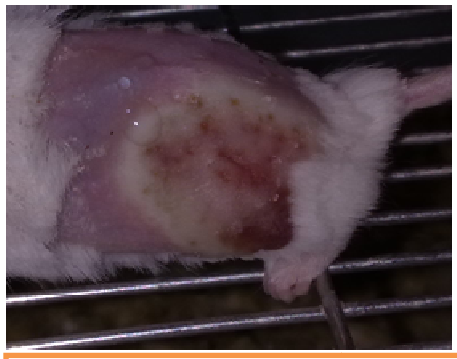


Figure(1):Sensitivity of MRSA to different concentrations ofceftobiprole andvancomycin

Pathological Gross Examination

The gross examination performed to all groups infected with MRSA show pus-filled lesions after 24 hours of infection with mean diameter (4.64 cm^3) in all infected group, after 7 days infected non-treated group lesions reached maximum diameter (6.50 cm^3), and when scarified animals, showed shin area with MRSA with severe ulcerative pyogenic lesion (figure 2,3).

Figure(2) Gross lesion of infected group after24hrs. of infectionshows pus-filledlesion.



Figure(3) Gross lesion of infected untreated group at day 7 post infection.

This resultsare in agreement with Godin *et al*., (2005) was observed animals infected with MRSA development of notable abscesses in the skin within 24–48 hrs. Treated group with ceftobiprole or vancomycin showed decrease in lesion volume but not significant difference when compared with untreated group after 48 hrs of treatment, after 7 day ceftobiprole significant reduction in lesion volume as compared with vancomycinand not treated groups, vancomycin showed less activity than ceftobiprole in reducing the lesion volume when compared with untreated group(fig. 4,5,6) and table (2). Reductions in lesion volume resulted in a decrease in staphylococcal bacterial density in the skin. The magnitude of the lesion volume score is generally in agreement with the magnitude of the decrease in colony forming unit. This result is in agreement with Fernandez *et al*.,(2009) who referred that treatment with ceftobiprole or vancomycin resulted in dose-proportional decreased in lesion volume for all doses ranging from 31% to 68% relative to untreated controls. Also, Hebeisen*et al* (2001) reported that ceftobiprole and vancomycin were highly effective, whereas linezolid was only slightly effective against an MRSA abscess infection in mice.

Figure(4): Gross lesion of infected group after 2 daystreated with ceftobiprole

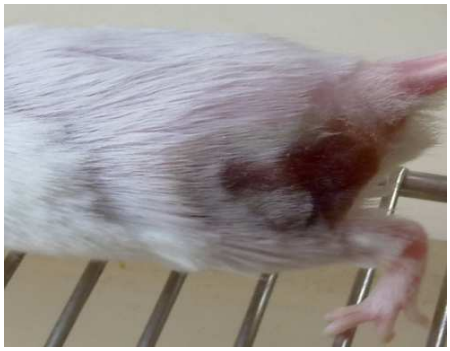
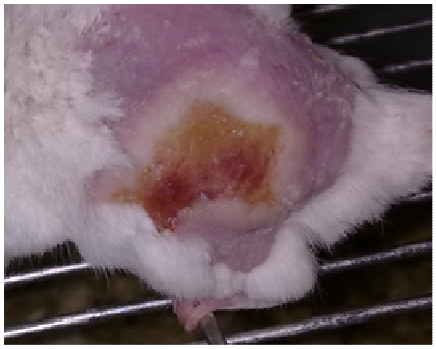


Figure (5): Gross lesion of infected group after 7 daystreated with ceftobiprole



Figure(6): Gross lesion of infected group after 7 daystreated with vancomycin

Table (2) The mean lesion volume (cm^3) of skin lesion in infected non treated group and in treated group with ceftobiprole and vancomycin .

Group	48 hour after infection	2nd day of treatment	7 day of treatment
+ ve control	4.58±0.43a	4.25±0.23a	5.22±0.35A
Ceftobiprole	4.94±0.59a	4.39±0.65a	2.65±0.63bC
Vancomycin	4.41±0.8a	4.07±0.46ab	3.31±0.46bB

Values represent mean ±S.E , Different capital letters mean significant (P<0.05) results between different groups.Different small letters mean significant (P< 0.05)results within group.

Histopathological examination

The main lesion characterized by severe neutrophil, macrophage and lymphocyte infiltration in the dermal layer and extended to adipose tissue and between muscle fibers which show severe zinker necrosis after 2 days of infected untreated group, In other sections, the lesion characterized by necrosis of desquamation epidermal layer with colonies of bacteria which were foud in necrotic area. Several inflammatory reactions extended to dermis and subcutaneous layer and characterized by neutrophils infiltration. Neutrophils were seen seen in the lumen of hair follicle. figure (7,8). After 7days of infection, there was severe inflammatory cell infiltration mainly neutrophils, macrophages and lymphocyte extended to subcutaneous tissue between sebaceous gland together with desquamation of the epidermis and inflammatory cell infiltration in the desquamation of sloughing of epidermis, Also marked inflammatory cell infiltration extended to adipose tissue , figure (9,10).

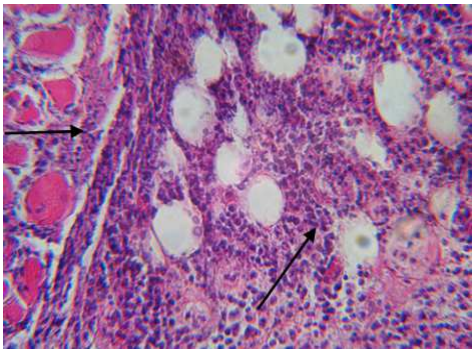


Figure (7): Shows several inflammatory cells infiltration in subcutaneous tissue & between muscle fibers in Infected non treated group.

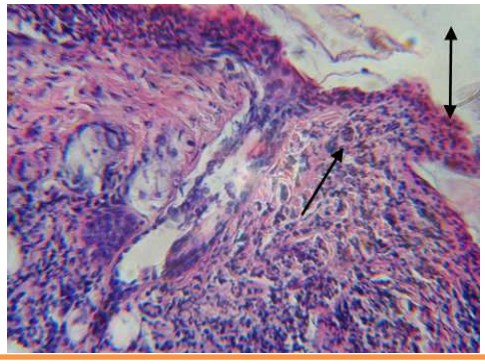


Figure (8): Histological section in the skin of animal at 7th day post infection with MRSA shows inflammatory cells infiltration in the dermis & subcutaneous tissue (→) and desquamated of the epidermis (↔) in Infected non treated group.

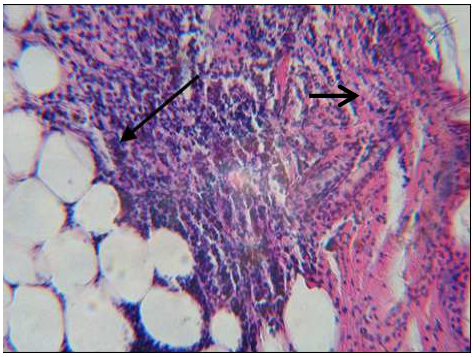


Figure (9): Histological section in the skin of animal at 7th day post infection with MRSA shows extending inflammatory cells infiltration into adipose tissue (→) in Infected non treated group.

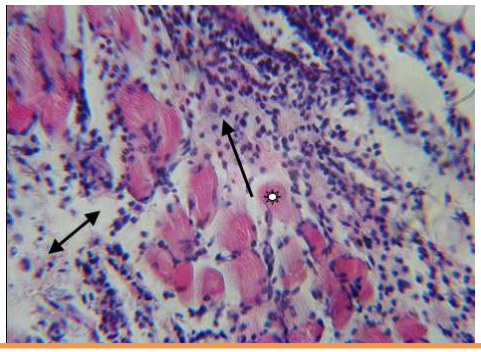


Figure (10): Histological section in the skin of animal at 7 day post infection with MRSA shows extending inflammatory cells infiltration ( ), edema ( ) & necrosis of muscle fiber ( ). in Infected non treated group.

Treated group with ceftobiprole lesion characterized by moderate mononuclear cell infiltration in the dermis layer and between hair follicle. In other sections granulation tissue formation consists from capillaries blood vessels with fibrous connective tissue proliferation replaces the suppurativeexudates in subcutaneous tissue and in the dermis figure(11,12). Animal treated with vancomycinafter 48 hour of treatment ,the lesion characterized by moderate erosion of the epidermal layer and moderate inflammatory cell infiltration particularly neutrophils, lymphocyte and macrophages in the dermis and subcutaneous tissue, after 7 days lesion characterized by granulation tissue consist from congested blood vessels and proliferation of fibrous connective tissue as well as mononuclear cell infiltration, and granulation tissue was extended to subcutaneous tissue and reached adipose tissue figure (13,14,15,16).The inflammatory response included most of the layers of the skin (Kugelberg*et al*.,2005). Four days after infection an acute subcutaneous phlegmonous inflammation with fibrin deposition and edema was observed with infiltration of a few neutrophils. The inflammation was the most pronounced in the subcutaneous and connective tissues , this result is in agreement with Kugelberg*et al*.,2005. The precipitation of fibrin from the coagulation cascade creates an environment that protects the *staphylococci* from the phagocytic mechanisms of the host. Coagulase also gives the particularly pyogenic character to staphylococcal infections. MRSAcould be also produce hemolysins and leukocidins. They, in turn, produce collagenase, hyaluronidase, and heparinase, which allow the extracellular matrix of the host tissues to be digested, with enhanced invasiveness of the pathogen being the consequence(Printzen,1996).

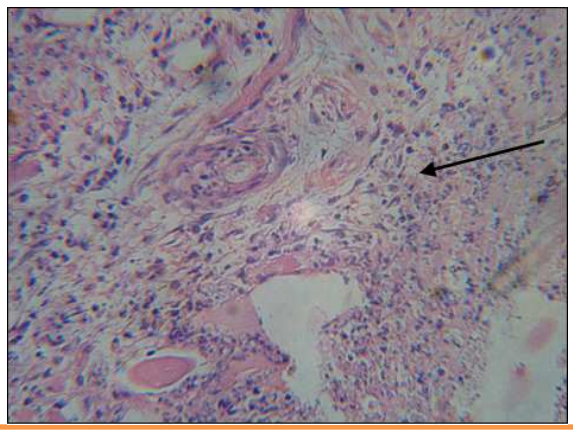


Figure (11); Histological section in the infected animal skin at 7th day post treatment with ceftobiprole shows granulation tissue & inflammatory cells infiltration in the dermis & between hair follicle (→) in Infected group treatedwith ceftobiprole.

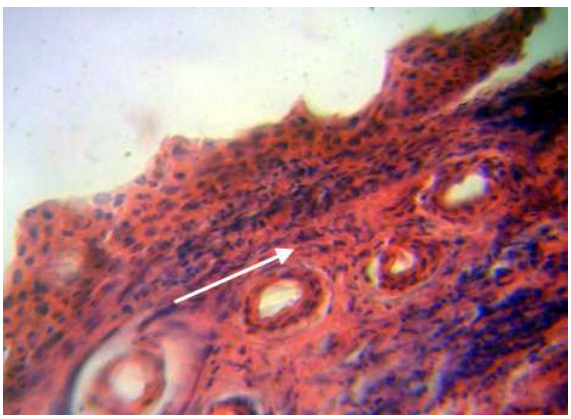


Figure (12): Histological section in the infected animal skin at 7th day post treatment with ceftobiprole shows granulation tissue replace the suppurated inflammation (→), in Infected group treated with ceftobiprole.

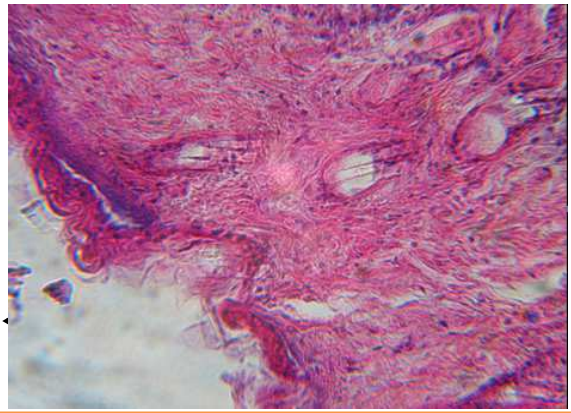


Figure (13) Histological section in the skin of infected animal at 48 hour post treatment with vancomycin shows moderate inflammatory cells with erosion of the epidermis, in Infected group treated with vancomycin.

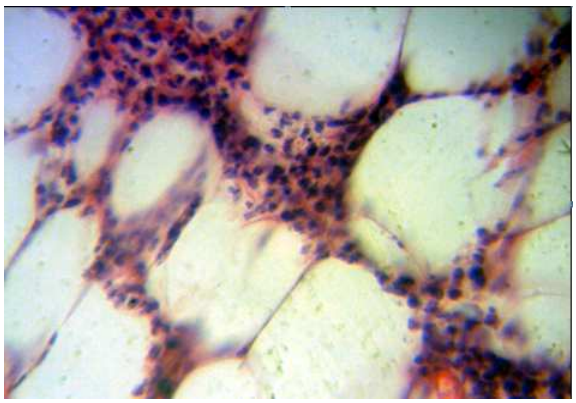


Figure (14) Histological section in the skin of infected animal at 48 hour post treatment with vancomycin shows moderate inflammatory cells between adipose tissue. in Infected group treated with vancomycin.

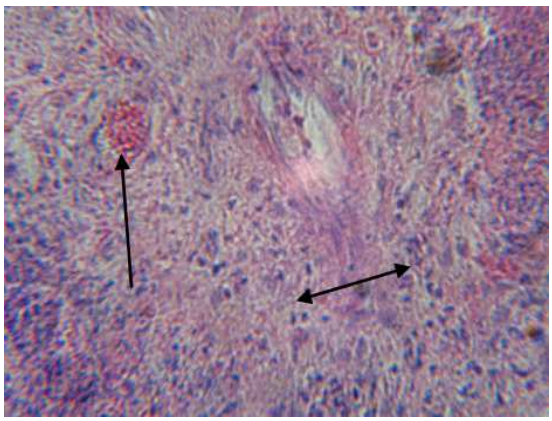


Figure (15) Histological section in the skin of infected animal at 7 day post treatment shows granulation tissue consist from congested blood vessels & collagen fiber (←→) & few mononuclear cells infiltration (←→) in the dermis layer & extended to subcutaneous tissue. in Infected group treated with vancomycin

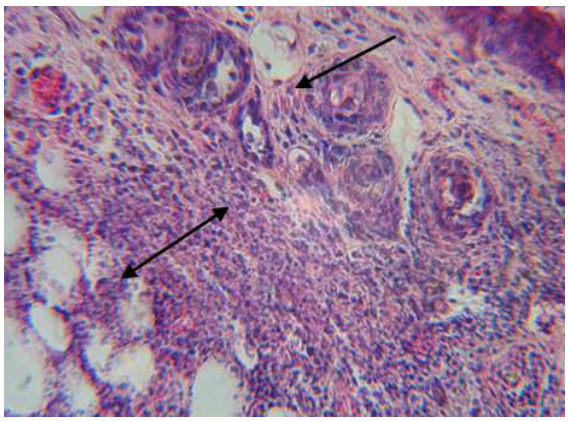


Figure (16) Histological section in the skin of infected animal at 7 day post treatment shows granulation tissue & inflammatory cells between hair follicle (⟶) & extended to adipose tissue ( ) . in Infected group treated with vancomycin.

The Inflammatory Response: Whether infection originates within a cutaneous wound or within a glandular structure, activation of the inflammatory response within the skin and soft tissues is the first line of the innate host response. Disrupted tissue, the release of tissue factor, exposed collagen, and released ADP activate the initiator events of human inflammation. The initiator events include the activation of the coagulation cascade, aggregation and degranulation of platelets, activation of the mast cells, activation of the bradykinin pathway, and activation of the complement cascade(Solomkin*et al*.,2003). These redundant and interactive responses result in an immediate vasoactive response and a secondary phago-cytic response. The efficiency and robustness of the response dictate whether contamination is eradicated before infection occurs, or whether the evolving infection can be contained and minimized.

The forces favoring infection are the inoculum of bacteria in the tissue, the virulence characteristics of the bacterial contaminant, and the local environment that enhances microbial aggressiveness(Fry,2003). The forces of pro-infection are then pitted against the intrinsic capacity of the host, which is then modulated by acquired clinical conditions that impair the efficiency of responsiveness . Clinical variable that are thought to impair the host include blood transfusion, hypoalbuminemia, corticosteroid therapy, hypoxemia, tissue ischemia, hypothermia, hyperglycemia, malnutrition, and coexistent diseases associated with immunosuppression. Whether the contaminant is eliminated before infection occurs, whether mild cellulitis or a small tissue abscess remains as a localized and relatively innocent event, or

whether an aggressive and life-threatening infection is the consequence is determined by this complex interaction. Understanding this relationship between the pathogen and the host sets the stage for a pathophysiologic approach to treat the patient with infections such as a soft tissue infection.

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