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Measure the levels of TGF-β in Induced Wound Infection with *Staphylococcus aureus*

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Abstract

Aim: *Staphylococcus aureus* wound infections are the leading cause of mortality and morbidity in patients around the world, Animal models is standard tools for studying a wide range of traumatic wound infections. The present paper is aim to study the effect of *Staphylococcus aureus* (SA) in wound and how the innate immune system interacts with the infection , by Measure TGF-β cytokine , and there's roles on recovery of wound. **Methodology :** 51 female albino Rats were used at the age of (6-9) week , the animals were divided in to three group (17 animal in the group) the healthy control (Group I), wound control group(Group II), and infection contamination group with SA (Group III), , To mimic clinically occurring infections caused by SA infection , excision wound were made on dorsal side of the animals , and SA 2×10^8 (CFU)/mL were inoculated on the wound site in infection contamination group , The blood collected after 24hours post infection for three days (24, 48, 72 hours) , histopathological examination with ELISA tecinque was made for Measure TGF-β. **Result :**Immunological assay results of this study showed that the serum level for TGF-beta were statistical significant between different values at different time when ($P \leq 0.02$).

Conclusion: TGF-β is a potent immunomodulatory that initiates and terminates tissue repair. It is released in wound areas after tissue damage by the inflammatory cell .

Keyword: Animal model , Bacterial Inoculum ,*Staphylococcus aureus* , Wound Infection , TGF-β ,

Introduction

The skin is part of the integumentary system, and it's the largest organ of the body based on the surface area makes about (6-7) % of body weight, despite that the muscular and skeleton systems have more mass **(Treuting, et al., 2018).** Wound is breakdown or loss of the protective function of the skin, leading to compromise immune system, any microorganism that colonizes the skin or is introduced to a wound can cause an infection **(Shankar, et al., 2014) ,** thus Skin wounds infection with *Staphylococcus aureus* (SA) are a major public health problem **(Tsige, et al., 2020).** Because of its ability to express many virulence factors and it ability to escape the immune system , During skin infection MAMPs of SA are recognized by PRRs of the host , leading to host defense system being activated **(Mohammad, et al., 2021),** to do so, platelets secrete several chemokines and growth factors implicated in early wound repair stage such as IL-1, TNF, TGF-α, TGF-β, platelet factor IV (PF4) to form **(Nguyen, 2015).** plug in a short period, , neutrophils invade the wound in large numbers, within a few minutes, and it secret IL-1, TNF, TGF-β locally**(Kim, et al.,**

2008), activating local macrophages or endothelial cells, leading to the release of Proinflammatory cytokines and growth factors, including TGF- β, TGF-α, and VEGF, **(Nguyen, 2015)** , Macrophages are the cells responsible for ending the local inflammation steps These related two types of macrophages, so called the M1 and M2 macrophages **(MacDonald, et al., 2010),** and the last one lead to secrete TGF-β , that is an anti-inflammatory cytokines , involved both negative regulators of the immune response, The main function of (TGF-β is to inhibit the growth and activation of T cells as well as in the process of wound repair by enhancing the synthesis of collagen by stimulation of wound healing **(Levinson, et al., 2014)**. also TGF-β induces fibrosis while inducing fibroblast differentiation into collagen-producing myofibroblasts and increasing fibroblast proliferation **(Zaiss, et al., 2019).** TGF-β induces alpha-smooth muscle actin expression in fibroblasts, providing the motive force for contraction of the wound margins **(Levinson, et al., 2014);** therefore the objective of present study is to highlights the role of TGF-β in wound infections with ***S. aureus***.

Materials and Methods

Experimental study from March 2021 to April 2021:

Rat animals Model used, 6-9 week old female (51) Albino Rats, were placed in an environment -controlled animal house. In the University of Kufa / Faculty of Veterinary Medicine, fllowing three weeks adaptation period , and Rat were divide in to three group 17 each. healthy control (Group I), control wound (Group II) without contamination, and Contamination with staphylococcus aureus (Group III).

Animal Preparation

Animals were anesthetized prior to wound creation by using combination xylazine (20mg/ml) and ketamine (50mg/ml) in a 2:1 ratio , sequentially in a dose from 0.10 to 0.15 ml/100g at body Wight" **(Silva, et al., 2013)** . A full-thickness excision wound was made using a surgical blade, forceps, and pointed scissors on the dorsal side of the rats **(Nayak, et al., 2006)**. The animal's fur was shaved, two kinds of antiseptic were apply (alcohol 70%, povidone-iodine) on the area where the wound was made, sterilizing wound area for the first time with alcohol 70% and that repeat for three times and then using povidone-iodine **(Grada, et al., 2018)** .

Bacterial isolates

Bacterial used was taken from milk sample Karbala its mecA positive and considered as MDR isolates due to the resistance pattern of 6 antibiotics of 9 classes.

Bacterial Inoculum and Bacterial inoculation

Methicillin-resistant Staphylococcus aureus (MRSA) Bacterial isolates was taken from milk sample , its mecA positive and considered as MDR isolates due to the resistance pattern of 6 antibiotics of 9 classes , were Inoculate into sterilized Tryptic soy broth (TSB) medium (from a frozen glycerol stock), Reagent Bottle with Screw Cap, Grow the bacteria for (16–18) hours at 37°C. when the bacteria reach the late stationary phase of growth, 15 ml of the broth were drowned by micro pepite from the bottle, 5 ml in each one of the three tubes, to be centrifugation at (6,000 × g, for 3 min) repeated three time for Collection the bacteria sedimentation by proses that involve through 3ml of the broth and leaving the bacterial sedimentation and adding

2ml of PBS this process was repeated for three-times except it the 3rd time centrifugation at (6,000 × g, for 4 min) and no Wash process is needed, the 2nd steep of Preparation of Bacterial Inoculum was to measure the optical density by using a spectrophotometer, The culture optical density at 600 nm (OD 600) exceeds 2.3 , 2×108 colony-forming units (CFU)/mL is the concentration. Then, Transfer the bacterial suspension to septum vials and maintain them on ice at ~4 °C until the use. **(Malachowa, et al., 2013) ,** The wounds were inoculated with 0.1 mL bacterial suspension after 24 hours from wounding with bacterial suspension that was equal to 2×10^8 CFU/mL , into the wound site in, The wound site were cover with Adhesive Wound Dressing , with changed daily **(Asada, et al., 2012).**

Blood samples

A total of (51) blood samples were collected , from wounded rats at specific time line (24, 48, 72) hours after wound infection with *Staphylococcus aureus*,each serum sample was divided into five small (200 µl) aliquots and kept at deep freeze (-20°C) until used in ELISA.

Enzyme Linked Immune Sorbent Assay (ELISA)

TGF-β from (Bt lab)

Tissue preparation for Histopathological analysis

from the wounded rat in exaction model were collected after (24, 48 , 72 , 96 hour) for both the control wound group and the contamination group with ***S.aureus.***

Ethics approval

the experimental processes protocols was approved, by Research Centre's Ethic Committee's suggestion, of the of University Kufa, Council on Guidelines to ensure that experimental animals were properly cared for and used were. (authorisation from the Animal Experimentation Ethic Agency; Ethic no. 12- 7-2021).

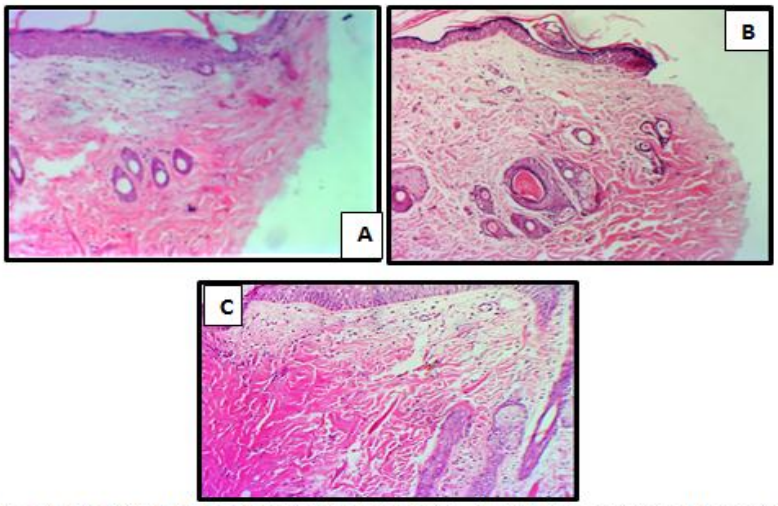
Statistical Analysis:

Data of the present study were translated into codes by using a computerized data program Statistic Package for the Social Sciences (SPSS version 23) in order to be statistically analyzed in to graphs.. In statistical programme we depend on P value level ,when $p \leq 0.05$, were considered statistically significant.

Results

Table (1-1) Macroscopic Evaluation , Microscopic Evaluation

Macroscopic Evaluation	
Infected group (Group III)	**Control wound group (Group II)**
Abscess, swelling, redness, hemorrhage, and pain at the wound inoculation site	No signs of infection, such as swelling or pus formation, discoloration, and hemorrhage. In the control wound, the local inflammation lasted for 24h after post wound
Microscopic Evaluation	
Infected group (Group III)	**Control wound group (Group II)**
The wound area at 24 hr post- wound Infection showed a normal features of wound healing process, where no signs of inflammation in dermis layer were observed (**fig**1-2(A and B)), in the 48hr post- wound Infection Necrosis of fibrous tissue was observed in dermis layer of wound area, where the necrosis of fibrocytes formed a spaces filled with exudate in affected areas. Also, infiltration of inflammatory cells was observed in affected areas (**figure** 1-3(A ,B and C)) , in the 72hr post- wound Infection (figure 1-4(A ,B ,C)) Necrosis of fibrous tissue was observed in lower dermis layer of wound area, where the necrosis of fibrocytes of dermis layer formed a spaces filled with infiltrated inflammatory cells , (**figure** 1-4 D) Infiltration of inflammatory cells that occupied the necrosis affected areas .	wound area showed a normal features of wound healing process The Epidermis layer shows damaging in the epithelial cells , and without any sign of inflammation in dermis layer at 24hr post wound **(Figure 1-1(A)** , while in 48hr post wound The wound area showed a normal features of wound healing process without any sign of inflammation in dermis **(Figure 1-1(B)** layer, and in 72hr post wound The wound area showed a normal features of wound healing process without any sign of inflammation in dermis layer **(Figure 1-1(C).**



(Figure 1-1 A,B,C) A-24 hours post-wound induction, B- 48 hours post-wound induction , C- 72 hours post-wound induction. H&E. x100

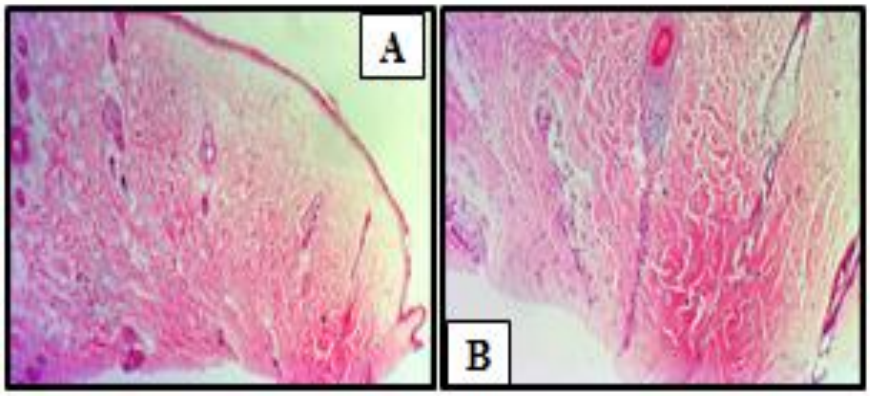


Figure (1-2 A,B): Staph infected cutaneous wound 24 hours post- wound Infection H&E. A: x40 and B: x100.

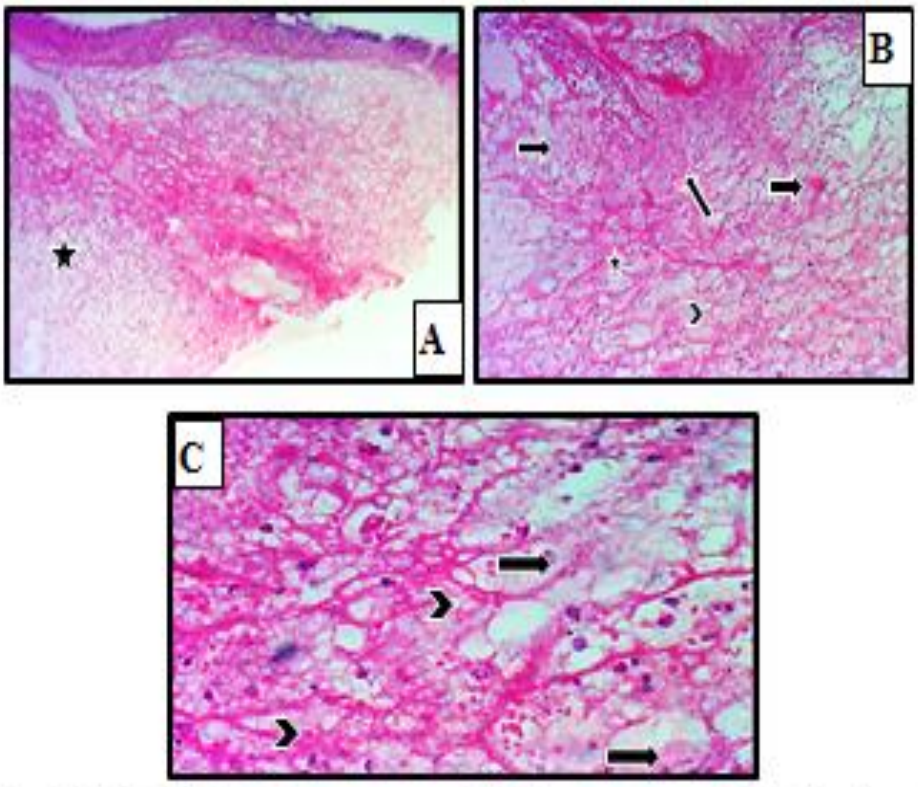


Figure (1-3 A,B and C): Staph infected cutaneous wound 48 hours post- wound Infection Necrosis IN dermis layer (stars), necrosis of fibrocytes (arrowheads), and inflammatory cells (arrows) H&E. H&E. A: x40, B: x100 C: x400.

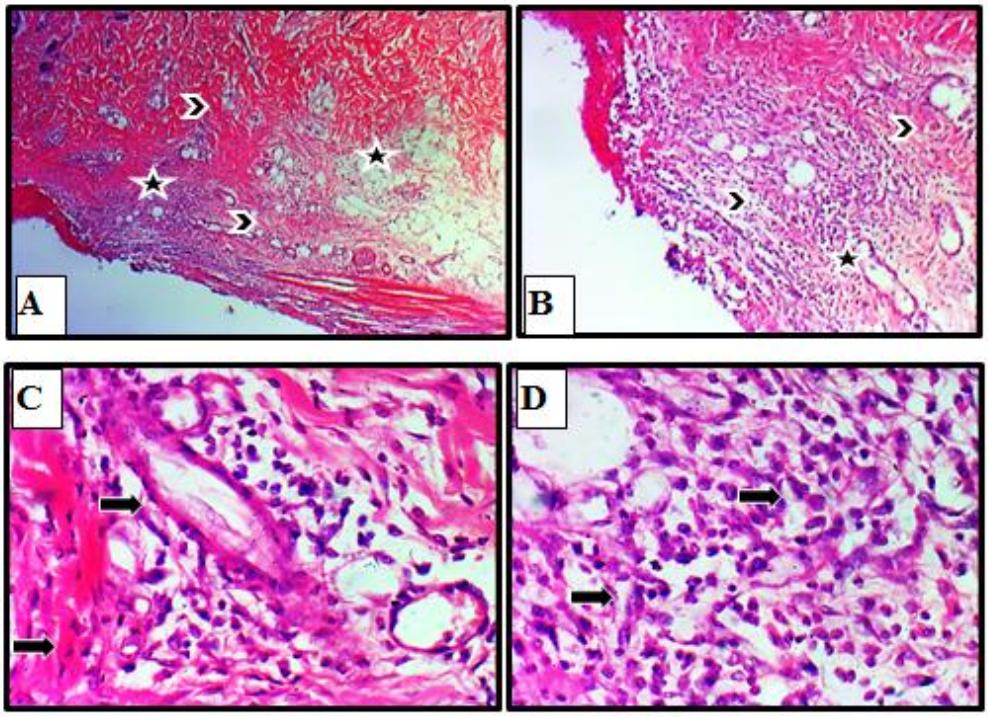


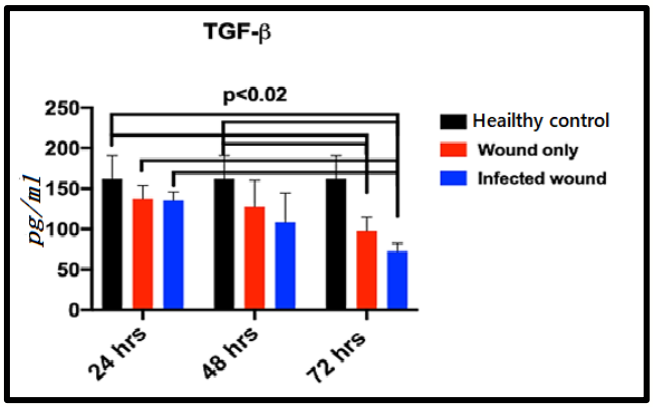
Figure (1-4 A, B, C and D): Staph infected in wound 72 hours post-infection. A, B&C/ Necrosis (star), necrosis of fibrocytes (arrowheads) , infiltrated inflammatory cells (arrows). D/ Infiltration (arrows). H&E. A: x40, B: x100 and C&D: x400.

Serological test :

The statistical analysis showed that there is a significant difference between different values at 24hr Group I vs. 72 hr. Group II when the average concentration is 63.89 pg/ml, 24hr Group I vs. 72 hr. Group II when average concentration is 89.55 pg/ml, at 24 hr Group I vs. 72 hr Group III when the average concentration is 65.42 pg/ml, at 24 hr Group III vs. 72 hr Group III when the average concentration is 63.25 pg/ml, at 48 hr Group I vs. 72 hr Group II when the average concentration is 63.89 pg/ml, and at 48 hr Group I vs. 72 hr Group III when the average concentration is 89.55 pg/ml P ≤0.02 (Figure 1-5).

Table (1-2) Serum concentration TGF-β protein in Group I, Group II, Group III

Post wounding	Group I (Mean ± SD)	Group II (Mean ± SD)	Group III (Mean ± SD)
24 hour	142.40± 45.03	137.83±13.91	135.66 ± 8.95
48 hour		128.33±27.78	108.76 ±30.83
72 hour		98.07±14.53	72.41±8.71



(Figure 1-5): The statistical analysis for TGF-β Serum concentration at (24, 48, 72) hr.

Discussion

Macroscopic Evaluation

The macroscopic evaluation of the wound for the Group III in the excision wound model were shown to form Abscess, swelling, redness, hemorrhage, and pain at the wound inoculation site with *Staphylococcus aureus*.

These results seem to agree with **(Kim, et al., 2014), (Pérez, et al., 2021).** They found that within 24h post-infection, an abscess lesion form with purulent exudate erythema, edema, and within 48h post-infection, purulent crusts developed, discoloration, Inflammation that triggered by *Staphylococcus aureus* and leading to cause swelling in both human and animal model leading to delay wound contraction to 96h post-infection.

In comparison with the control, wounds showed that there had been no signs of infection, such as significant swelling or pus formation, discoloration, and hemorrhage. In the Group II, the local inflammation lasted for 24h after post wound, and the clot formation was observed to be faster compared to the infected group.

These observations appear to be consistent agree with **(Yeng, et al., 2019) .** who found that the local inflammation reach to it peaked by 24hr and resolved within 48hr, with reducing swelling, contraction started by 72 h and Wound got smaller.

Histopathological change of the Group II

Microscopical exeamniation of the Group II revealed The wound area showed a normal features of wound healing process The Epiderm layer shows damaging in the epithelial cells , and without any sign of inflammation in dermis layer at 24hr , 48 and 72hr. post wound and that seems to agree with **(Khalaf, et al., 2019)** that shows a sections of skin tissue at 24hr. did not have any inflammatory reaction and exhibit only epidermal ulceration with mild dermal edema at the wound edge was seen.

Histopathological change of the Group III

Microscopical examination of the infected rat revealed the wound area at 24 hr post-wound Infection showed normal features of wound healing process, where no signs of inflammation in dermis layer were observed; these results seem to agree with **(d'Acampora, et al., 2006)** that shows Inflammation dependent on Both inflammatory mediators and inflammatory cells such polymorphonuclear leukocytes (PMN), macrophages, and lymphocytes are affected as well as . PMNs are responsible for bacterial phagocytosis and arrive at the site of tissue damage. They can stay for up to five days.

While at 48hr post- wound Infection Necrosis of fibrous tissue was observed in dermis layer of wound area, where the necrosis of fibrocytes formed a spaces filled with exudate in affected areas. Also, infiltration of inflammatory cells was observed in affected areas , these observation were similar to histopathological observation by **(Kugelberg, et al., 2005)** , who observed that inflammatory infiltrate of neutrophils and lymphocytes located in the upper dermis, bacteria coccoid are present in the superficial layers of the dermis and shown to have an inflammatory response that associated with marked fibrosis, oedema, and fibrin deposition, Mononuclear cells, including lymphocytes, and neutrophils, to a lesser extent, make up the inflammatory cell infiltration.

Also, mean of dermal necrosis results, organisms present in the superficial dermis these observation were similar to histopathological observation by **(Kennedy, et al., 2010)**.

also in the 72hr post- wound Infection Necrosis of fibrous tissue was observed in lower dermis layer of wound area, where the necrosis of fibrocytes of dermis layer formed a spaces filled with infiltrated inflammatory cells , Infiltration of inflammatory cells that occupied the necrosis affected areas these observation were similar to histopathological observation by **(Thurlow, et al., 2018)** where the observed tissues display dermal necrosis as well as necrosis of subdermal tissue. Due to increased bacterial burden at 72 hr. post infection that is eventually overlaid by a clot.

Serum concentration TGF-β protein produced from infected wound group, control wound, and healthy control group in the albino rat at different times

TGF-β is a potent immunomodulatory that initiates and terminates tissue repair and released in wound areas after tissue damage by the inflammatory cell **(Nguyen, 2015).** The present result of serum concentration of TGF-β at different time intervals of Group II shows that serum concentration of TGF-β reached the peak at 24hr and 135.66 pg/ml due to the TGF-β coincide with the peak of the inflammation. This result is the same as **(Wang, et al., 2017)**. Found an increase of TGF-β at 3 days post wounding in exaction full thickness wound. In addition, it gradually decrease to reach the low level at day 5.

In addition, the decrease of TGF-β in Group III was necessary step to prevent from adhesion to and invasion in wound area and that confirm with **(Zhao, et al., 2017)** , that shows adhesion and invasion of *S. aureus* into bovine mammary fibroblasts was significantly increased after treated with TGF-β.

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