Passive immunization against *Pseudomonas aeruginosa* Infected in burn wound in mice

M. J. Alwan*, R. S. Al-Kamisi* and S. H. AlKutbi**
***College of Veterinary Medicine\ University of Baghdad**
****College of Pharmacy\ University of Baghdad**

Abstract

The present study aimed to know the efficacy of a ntisera of *Pseudomonas aeruginosa and Salmonella typhimurium* in protect burn mice against *Pseudomonas aeruginosa* infection.

Twenty mice have been used (6-8 weeks age) and exposed to burn wound by hot water ($1.5x1.5cm^2$, 90^0C). After 24hrs, the animals were divided randomly into four groups equally. The 1st, 2nd and 3rd groups were inoculated with 0.3ml of bacterial suspension contain $1x10^9$ cfu/ml, under the burn area. Immediately, the 1st and 2nd groups were inoculated 1/V with 0.1ml of antisera of *S.typhimurium and P.aeruginosa* respectively. The 4th group was inoculated with 0.3ml of sterile normal saline under the burn area as hegative control group.

The results showed that all infected-non-passive immunizaed animals died during 24-96hrs post-burn wound infection with heavy bacterial isolation from examined internal organs and sever pathological lesions characterized by neutrophils infiltration in the stroma of the tissues. Congestion of blood vessels with neutrophils in their lumen was seen, in addition to degenerative and necrotic changes as well as thrombus in the blood vessels in some organs while infected-passive immunized animals were survived with good healthy as well as no bacterial isolation and no or mild pathological lesions in their internal organs.

التمنيع المنفعل ضد خمج الحروق بجراثيم الزوائف الزنجارية في الفئران

محمد جويد علوان* ورائد سليم* وسهير حسن علي**
***كلية الطب البيطري/ جامعة بغداد**
****كلية الصيدلة/جامعة بغداد**

الخلاصة

هدفت هذه الدراسة معرفة كفاءة أضداد الزوائف الزنجارية والسالمونيلا على حماية الفئران المحروقة من الإصابة بالزوائف الزنجارية استخدم (20) من الفئران السويسرية البيضاء تراوحت أعمارها بين 6 إلى 8 أسبوع. تم حرق 1.5 × 1.5 سم^2 من سطح جسم الحيوان باستخدام الماء المغلي بدرجة حرارة 90° مئوية لمدة 10 ثواني بعد ذلك قسمت الحيوانات عشوائياً إلى أربعة مجاميع بالتساوي وحقنت حيوانات المجاميع الأولى والثانية والثالثة بجرعة 0.3 مل من العالق الجرثومي الحاوي على $1x10^9$ cfu/ml من الزوائف الزنجارية تحت منطقة الحرق وعولجت حيوانات المجموعتين الأولى والثانية بأمصال ضد السالمونيلا والزوائف الزنجارية على التوالي عن طريق وريد الذيل بجرعة 0.1 مل، أما المجموعة الرابعة فلقد حقنت بجرعة 0.3 مل من المحلول الملحي المتعادل المعقم تحت منطقة الحرق واعدت مجموعة سيطرة سالبة.

أظهرت النتائج ان جميع الحيوانات المصابة غير المعالجة بأضداد المصول هلكت خلال 24 إلى 96 ساعة بعد حقن منطقة الحرق وحدوث الخمج الحقن مع عزل جرثومي كثيف من الأعضاء الداخلية وحدوث آفات مرضيه شديدة تميزت بارتشاح العدلات إضافة إلى وجود تغيرات تنكسيه وتنخرات في خلايا بعض الأعضاء الداخلية مع وجود الخثرة الدموية بينما لم يسجل أي هلاك في الحيوانات المصابة المحقونة بأضداد الأمصال سواء ضد السالمونيلا أو الزوائف الزنجارية مع عدم وجود عزل جرثومي من الأعضاء الداخلية وقلة أو عدم وجود تغيرات مرضية في هذه الأعضاء.

Introduction

Burns are one of the most common and devastating forms of trauma, in united states, about 5000 patients die each year from burn-related complications.

In patients with sever burns over more than 40% of the total body surface area,75% of all deaths are currently related to sepsis from burn wound infection or other infection.

Significant thermal injuries induce a state of immunosuppression that predisposes burn patients to infectious complication (2) bacteria rapidly colonize open skin wound after burn injury. M. O colonizing burn wound originate from the patients endogenous skin and gastrointestinal and respiratory flora(3) as well as M.O may be transferred to patients skin surface via contact with contaminated external environmental surface(4).

Gram-negative bacteria, such as *Pseudomonas aeruginosa*, from patients gastrointestinal tract flora rapidly colonize the burn wound surface in the first few days after injury(5). The antibiotic resistance showed by this M.O and morbidity in patients which has encouraged into the development of vaccines affective (active and or passive).the development of vaccines for *P. aeruginosa* infections has become hindered due to complexity of the organisms pathogenesis elaboration of wide array of virulence factors such as'(cell-associated adhesions), alginate, pili, flagella and lipopolysaccharide as well as extracellular factors (6).

The 0-side chain portion of LPS/ principal target of immune response including many subgroups of this microorganism,has become identified and based on different of saccharide composition and structure of the O-Ags, the complexity is the bases for problem associated with developing LPS vaccine(7). AI-kmisi,(8) reported cross protection between *P.aeruginosa* and *S. typhimurium* in mice, therefore the present study attempt to use anti-sera *against S.typhimurium* and *P.aeruginosa* in passive immunized against burn wound infected with *P.aeruginosa*.

Materials and Methods

- **Bacterial isolates:** *Salmonella typhimurium and Pseudomonas aeruginosa* isolates were obtained from the unit of zoonotic disease of collage of Vet. Medicine,the biochemical tests were done to these isolates to confirm their diagnosis and identification (9).
- **Culture Media:**Blood agar base, brain heart infusion broth and agar, and Pseudomonas agar base, these medias were prepared according to the production manuals. Whole *S,.typhimurium* soniccated antigens(WSSAgs) have been prepared according to Mitove, et al.,(10)**.** Extracellular secretion antigens *of P.aeruginosa* was prepared according to Al-kmisi,(8).
- **Determination of the challenge dose:** The preparation of bacterial suspension of the counting was made using Miles, etal.(11) method.
- **Preparation of anti-sera:** 20 white mice both sex were randomly divided into two groups equally, the 1st group was twice immunized with WSSAgs, S/ C (2mg/ml concentration of protein) with 2 weeks intervals, the 2nd group immunized as the 1st

group but using extracellular secretion of *P.aeruginosa.at* 30 days postimmunization, blood was collected via intracardial puncture from each anesthetized immunized animals, the samples were allowed to stand at room temperature for 4 hrs and then were incubated overnight at 4^0C, then kept in standing position ,then centrifuged at 3000 rpm for 3 minutes, stored frozen until used.

- **Determination of antibodies titer:** Titer of antisera was determined according Hudson and Hay(12)**.**
- **Experimental design:** Twenty white mice, both sex, 6-8 weeks age were exposed to burn via boiling water (90^0C for 10 second, after preparation 1.5X1.5cm^2 skin area in right flank region, about 15% of the total surface area of the mice), at 24hrs post-burn, the animals were randomly divided equally into four groups and treated as following:

1. The 1st group was inoculated S/C with 0.3 ml of bacterial suspension contain $1x10^9$cfu of *P. aeruginosa* under the burn and immediately passive transfer of sterilized undiluted *antisera (S. typhimurium* Abs) by Millipore filter 0.45 mm of pooled antisera 0.1 ml/iv, via dorsal tail vein(titer of Abs 51.2), antihistamine (0.01, 1/M with 1ml of normal saline.
2. The 2nd group was treated as the 1st group but antisera of *P.aeruginosa* was used.
3. The 3rd group was inoculated with same route and dose of *P.aeruginosa* only as 1st group and served as control positive group.
4. The 4th group inoculated with 0.5ml of sterile normal saline S/C under the burn and served as negative control group. At 20 days post-burn infection, all animals were sacrified, postmortem examination done and recording any gross lesions,pieces from internal (liver, lung, spleen, kidney and heart) were taken for bacterial isolation and other pieces(1x1x1cm) fixed in 10% normal buffer formalin for 72 hrs then used the routen procedure for histopathological section preparation according to Luna.

Results

- **Clinical finding and bacterial isolation:** The results showed that all animals of control positive group were died during 24-96 hrs post-burn/infection with heavy bacterial isolation from examined internal organs, while all animals which were infected burn wound and passive immunized with antisera of *S.typhimurium or P.aeruginosa* remain survive during the cource of the experiment with healing of the burn wound. No bacterial isolation was recorded from examined internal organs at 20 days post-burn wound infection.
- **Pathological changes:** Congestion of most examined organs was the main gross lesions in the infected non-immunized animals while these lesion was not recorded in the examined organs of animals in both infected -passive immunized groups.
- **Histopathological examination.**
- **Infected-nonimmunized animals:**

1. **Liver:** The liver showed congested central veins and sinusoids with neutrophils in their lumen as well as multiple area of coagulative necrosis characterized by pyknotic or disappeared neuclei of hepatocytes (Fig. l) together with thrombus in the blood vessels.
2. **Kidney:** Microscopic section revealed congested of blood vesels between renal tubules with neutrophils in their lumen (Fig.2), together with acute cellular degeneration characterized by vacuolation of the cytoplasm of epithelial cells lining of renal tubules and narrowing or occluded lumen of the renal tubules, in addition to neutrophils infiltration in the interstitial tissue(Fig.3).

3. **Lung:** The histopathological examination of the lung showed large amount of neutrophils aggregation in the interstitial tissue in the animal died at 4 days post-infection as well as increased thickness of interalveolar septa due to congestion of capillary blood vessels and neutrophils infiltration (Fig.4). In addition to thrombus was seen in the blood vessels.
4. **Spleen:** The spleen showed neutrophils infiltration in the congested red pulp with modereate depletion of white pulp.
5. **Heart:** There is congestion blood vessels between muscle fiber with inflammatory cells in their lumen (Fig.5).
6. **Infected-passive immunized animals:** Animals were infected their burn skin wound and treated with antisera of *P. oeruginosa* or *S. typhimurium* showed no clear histopathological lesion in their examined organs such as kidney (Fig.6), heart and lung, however, liver showed proliferation of kupffer cells with few mononuclear cells aggregation around central veins (Fig.7) in addition, hyperplasia of white pulp of spleen was also recorded (Fig.8).

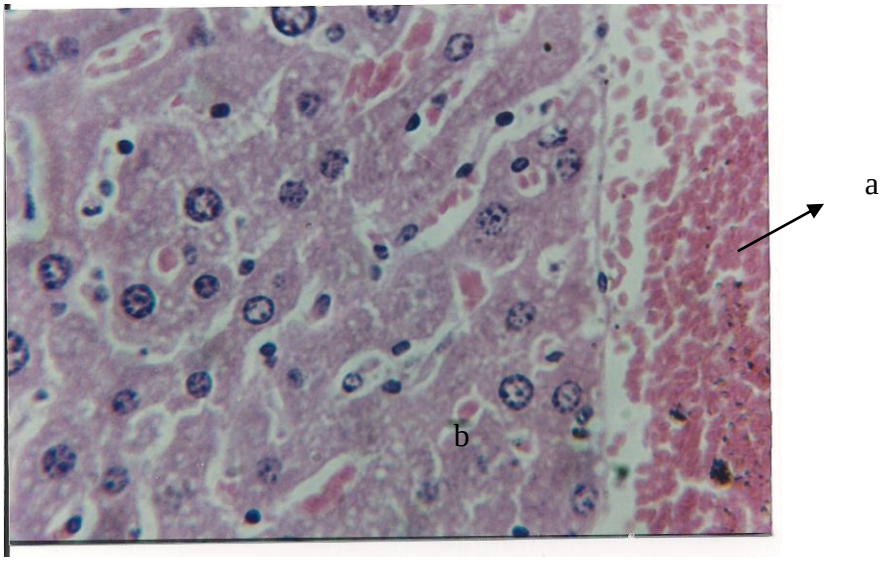

Fig. (l) Histological section in liver of one animals at 24hr post-burn infection showed congested central vein and sinusoids with neutrophils in their lumen (a) in addition to coagulative necrotic of hepatocyte (b) (H&E40X).

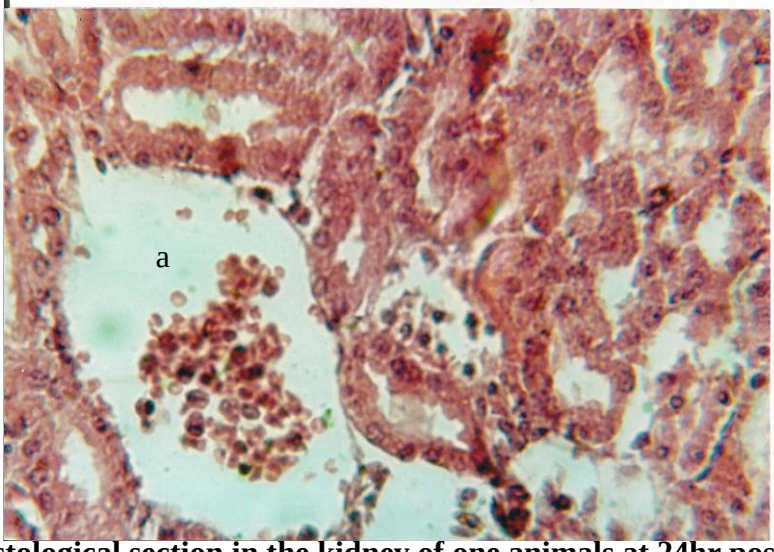

Fig. (2) Histological section in the kidney of one animals at 24hr post burn infection showed congested of blood vessels between renal tubules with neutrophils in their lumen (a) (H&E40X).

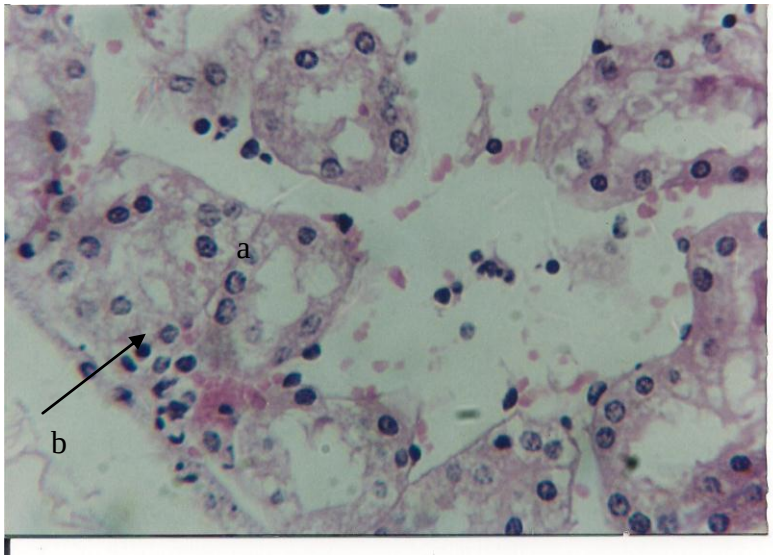

Fig. (3) Histological section in kidney of one animal at 48hr post infection showed acute cellular degeneration of epithelial lining cell of renal tubules(a) with neutrophils infiltration in the interstitial tissue and in luman(b)of B.Vs. (H&E40X)

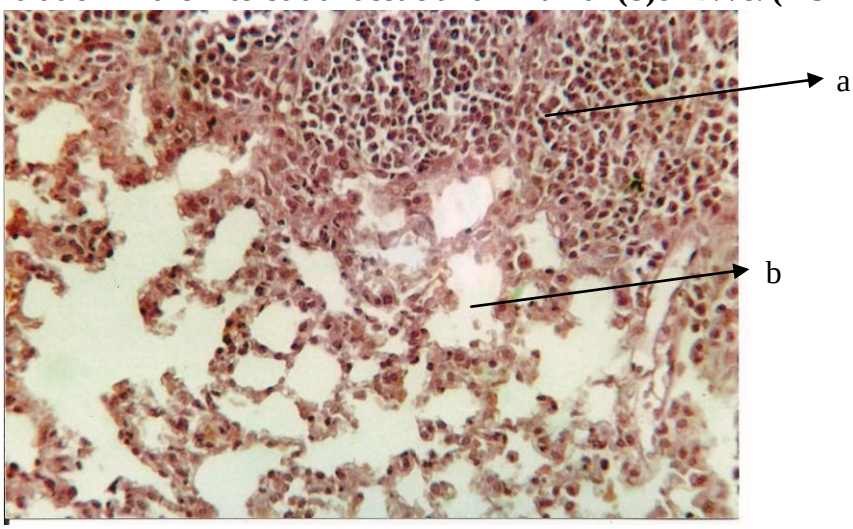

Fig. (4) Histological section in lung of one animal at 96hrs post infection explained aggregation of neutrophils in interstial tissue(a) with increase thickness of interalveolar septa (b).(H&E40X).

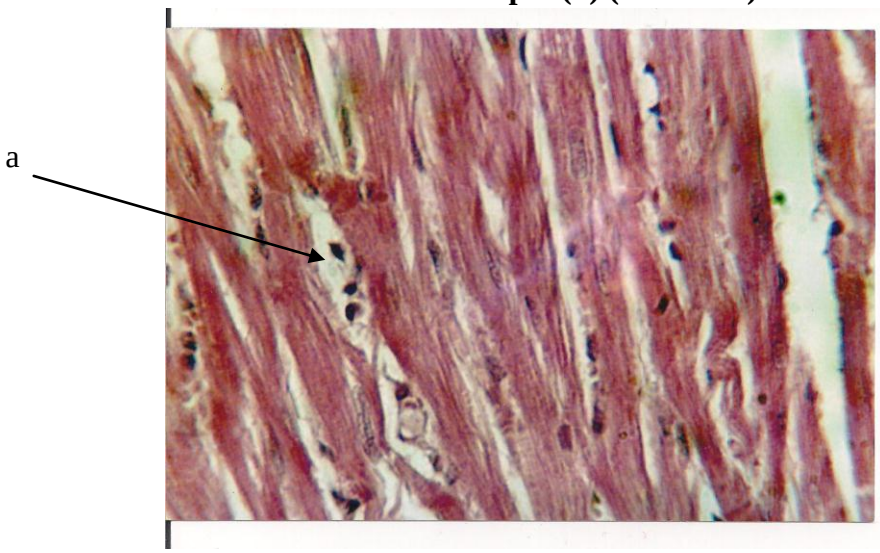

Fig. (5)Histological section in heart of one animal at 24hr post-infection showed congested blood vessels between muscle fiber with few neutrophils in their lumen(a) (H&E40X)

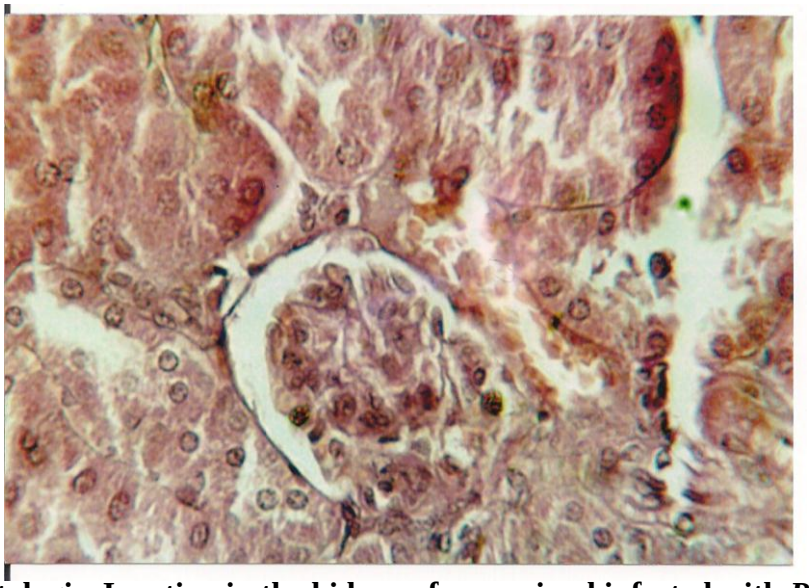

Fig. (6) HistologicaI section in the kidney of one animal infected with *P. aeruginosa* and treated with antisera of *S. typhimurium* showed no clear pathological lesions (H&E40X).

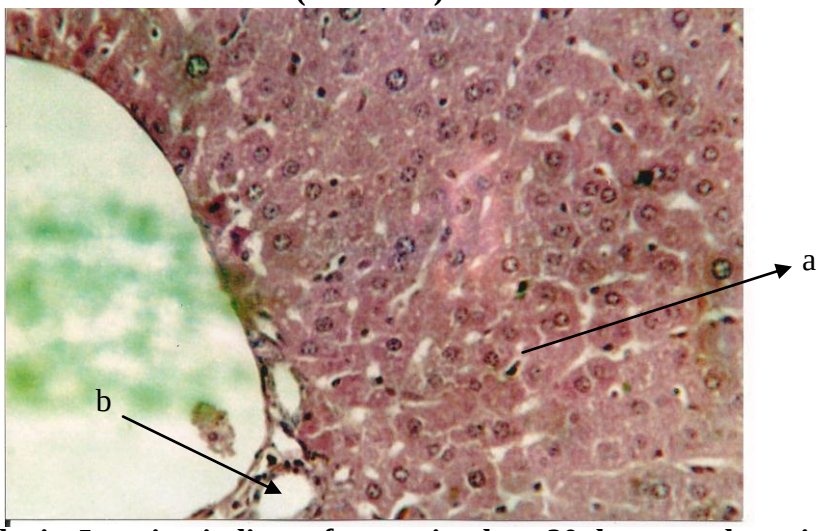

Fig.(7) HistologicaI section in liver of one animals at 20 days post-burn infection and treated with antisera of *P. aeruginosa* revealed kupffer cells proliferation(a) with few mononuclear cells aggregation around cenral veins (b) (H&E50X)

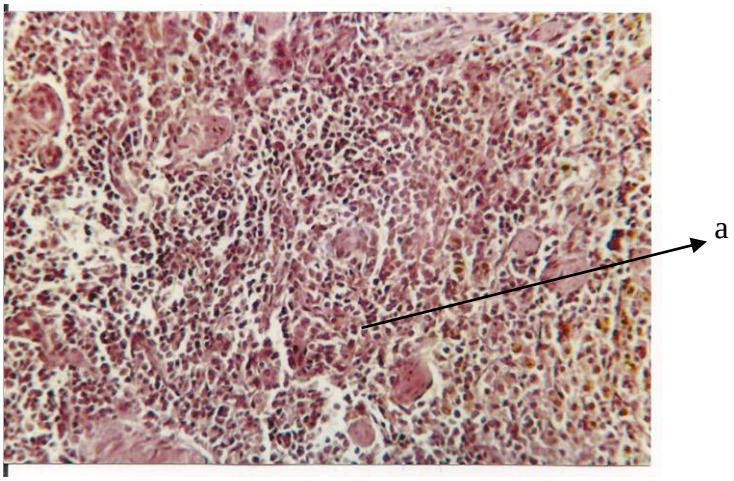

Fig. (8) Histological section in spleen of one animal at 96hrs post burn infection and treated with antisera of *P.aeruginosa* showed hyperplasia of white pulp (a) (H&E40X)

Discussion

The death of infected non immunized animal during 24-96hrs post-burn/infection with *P.aeruginosa* indicate that the animals were infected with virulent pathogens that proliferation in the burn area and overcome innate immunity and disseminated to internal organs that cause bacteremia, septicemia and septic shock and death of infected mice ,this results supported the investigation that mentioned by Allan et al.,(14) who explained that the *P.aeruginosa* is extremely efficient at colonizing burn wounds, spreading systemically, and causing sepsis, which often results in a systemic inflammatory response, multiple-organ failure and death.

The heavy bacterial isolation from examined organs may be due to large number of these bacteria ,that heavily proliferated in burn area, reach to internal organs.

As a result of thermal destruction of the skin barrier and concomitant depression of local and systemic immune responses as well as the burn wound surface is a protein-rich media to bacterial growth, this idea was agreement with Gamelli etal.,(15) and Nathan et al., (16) who investigated that the avascularity of the eschar results in impaired migration of host immune cells and restricts delivery of systemically adminsistered antimicrobial agents to the area, while toxic substances released by eschar tissue impair local host immune responses.

Our results was consistent with (3) who showed that by 24 hr post burn/infection, *P.aeruginosa* CFU in the eschar increase from $lX10^2$CFU/g tissue (infecting dose) to approximately $lX10^9$CFU/g tissue and can also be isolated from the liver and by 48 hr post-burn/infection, the majority of mice succumb to the infection.

The present study revealed that the main histopathological lesions in the examined organs were thrombus,congestion of blood vessels and neutrophils infiltration/this results may be due to the virulence factors of *P.aeruginosa* which induced cytokine storm and these observation agreed with Steinhauser et al.,(20) who explained that the inflammatory response of *P. aeruginosa* infection is characterized by vigorous neutrophils response,elevates neutrophil elastase activity, LTB4, IL-l, IL-6, IL-8/TNFa. Essner et al.,(21) revealed that the combination of thermal injury and *P.aeruginosa* infection results in an acute and exaggerated inflammatory response that is characterized by elevated mRNA levels of several cytokine and chemokine genes and this cytokine storm can be detected locally in the skin and systemically within the livers of burned -infected mice by 24h postburn/infection.

Syndecanl (Sdc-l) is a major heparin sulfate proteoglycan present on many host cells and this factor can be cleaved in a process termed ectodomain sheding and results in the release of intact, soluble proteoglycan ectodomains that have diverse role in innate immunity, thermal injury results in shedding of cyndecan1 from host tissue(14). Also LasA produced by *P.aeruginosa* activates syndecan 1 sheding(22). Shed Sdc-1 is a decisive host factor contributing to *P.aeruginosa* pathogenesis in burn wound infections and play a role in the blood vessel invasion and subsequent systemic spread of *P.aeruginosa(23). Shed* Sdc-1 is known to regulate the expression and function of many cytokines(24). It stimulates the release of TNFa, IL-lb AND 1L-6 from dendritic cells and IL-1,IL-12 and PGE2 from macrophages(24). These above observations were supported the bacterial isolation and pathological changes in the present study.

The results of bacterial isolation and pathological examination of the internal organs of burn/infection animals with *P.aeruginosa* and treated with antisera of *S.typhimurium* or *P.aeruginosa* indicated that the passive immunization are highly effective in destroying all M.O in the blood circulation and this results supported the observation of Al-kmisi,(8) who revealed that passive immunized mice with antisera of *P.aeruginosa or S.typhimurium* protected the animal against infection by both of these

organism) and he suggested that a present specific cross -protection among these microorganisms.

Kaniga et al.,(25) recorded that the amino terminal domain of salmonella invasion protein-P(SptP) showed a sequence similar to that of the ribosyle-transferase exotoxin S from *P.aeruginosa,* according to these idea we suggested these protein may be elicited Antibodies share between these two microorganisms.

Passively administered Abs, exerts their protective action in a number of ways, it is recruitment of the complement pathway to destruction or removal of the microorganisms/Abs bound to bacterial surface and promotes opsonophagocytosis ,also Abs neutralized the bacterial toxin and facilated its removal by phagocytic cells,in addition, Abs initiated Abs dependent cell mediated cytotoxicity(26).

In early infection, *P. aeruginosa* is considered extracellular pathogens, there fore we suspected that the administration of antisera of *P.aeruginosa* or *S.typhimurium* play role in destruction of these pathogen ,these idea consistent with previous studies which explained that prophylactic intravenous administration of monoclonal antibodies directed against OMP F of *P.aeruginosa* protective against corneal damage(27).

Our results supported the investigation of Neely et al.,(28) who showed in a burn mouse model, that Abs elicited from immunization with PcrV enhanced survival of mice challenged with different serogroups of *P.aeruginosa*.

References

1. AmericanBurn Association. (2000). Burn incidence and treatment in the US:2000.
2. Wertheim, G. Uber die Vesandesungen vesbrennungen, Weiner. Medical Press, Vienna/Austria. P.309.
3. Barret, J. P. & Herndon, A. B. (2003). Effects of burn wound exicion on bacterial infection. Plast. Reconstri., 744-750.
4. Wysocki, A.B. (2005). Evaluating and managing open skin wounds: colonization versus infection. AACN Clin. Issues.,1:382-397.
5. Mnson, W. L.; Coenen, J. M.; Klasen, H. J. & Horwitz, E. H. (1996). Intestinal bacterial translocation in experimentally burned mice with wound colonized by Pseudomonas aeruginosa. J Trauma., 33:654-658.
6. Tredget, E. E.; Shankowsky, H. A.; Rennie, R. & Logsetty, S. (2004). Pseudomonas infections in the thermally injured patient. Burn., 30:3-10.
7. Pier, G. B. (2003). Promises and pitfalls of *Pseudomonas aeruginosa* lipopolysaccharide as a vaccine antigen. Carbohydr. Res., 338: 2549-2556.
8. Al-Kmisi, R. S. S. (2009). ImmunopathologicaJ study of cross protection between *Salmonella typhimunum and Pseudomonas aeruginosa a thesis* -College of Vet Med. Baghdad University, Iraqi.
9. Holt, J. G.; Kreig, N. R.; Sneath, P. H.; Staley, J. T. & Williams, S. T. (1994). Bergeys, manual of determinative bacteriology 9th ed. William, S and Wilkins. USA, PP:93,94.
10. Mitove, L.; Dennchen,V. & Linde, K. (1992). Humeral and cell mediated immunity in mice after immunization with live oral vaccines *of Salmonella typhimunum: auxotrophic* mutants with two attenuating markers vaccine., l0: 61-66.
11. Miles, A. A.; Misra, S. S. & Irwin, J. V. (1938). The estimation of the bacteriocidal power of blood. J. Hyg., 38: 732-749.
12. Hudson, L. & Hay, F. C. (1980). Practical immunology 3rd. Blackwell scientific publication, Oxford. London.

13. Luna, L. G. (1959). Manualof Histological staining methods of the Armed forces Institute of pathology, 3rd ed. McGrow- Hillbook Company, NY.
14. Allan, H.; Frank, R.; Jeffery, O. & Rumbaugh, P. (2005). Syndecan1 shedding contributes to *Pseudomonas aeruginosa* sepsis. lnfect. lmmun., 73:7914-7921.
15. Gamelli, R. L.; He, L. K. & Liu, H. (1995). Marrow granulocyte-macrophage progenitor cell response to burn injiry as modified by endotoxin and indomethacin. J. Trauma., 37:339-346.
16. Nathan, P. E.; Law, J.; Murphy, D. F. & McMillan, B. G. A. (2003). laboratory method for selection of topical antimicrobial agents to treat infected burn wounds. Burns, 4:177-187.
17. Nasser, S.; Mabrouk, A. & Maher, A. (1999). Colonization of burn wounds in Ain. Burn., 29: 229-233.
18. Wessman, C. (1990). The metabolic response to stress: an overview andupdate. Anesthesiollogy., 73:308-327.
19. McLough lin, G. A.; Wu, A. V.; Saporosschetz, l.; Nimberg, R. & Mannich, J. A. (1980). Energy and a circulating immunosuppressive factor following major surgical trauma. aNN. Surg., l90:297-304.
20. Steinhauser, M. L.; Hogaboam, C. M.; Kunkel, S.; Lukacs, N. W. & Standiford, T. J. (1999). Il-10 is a major mediator of sepsis-induced impairment in lung antibacterial host defense. J. lmmunol., 162:392-399.
21. Essner, R. K.; Rhoades, W. H.; Morton, D. L. & Economou, J. S. (1990). IL-4 down-regulates IL-1 and TNF gene expression in human monocytes. J. lmmunol., 142:3857-3861.
22. Rumbaugh, K. P.; Colmer, J. A.; Griswold, J. A. & Hamood, A. N. (2003). The effects of infection of thermal injury by *Pseudomonas aeruginosa* PA01 on the murine cytokine response. Cytokine., l6:160-168.
23. Park, P. W.; Pier, G. B.; Goldberger, O. & Bernfield, M. (2008). Syndecan-1 shedding is enhanced by LasA, a secreted virulence factor of *Pseudomonas aeruginosa*. J. Biol. Chem., 275:3057-3064.
24. Park, P. W.; Foster, T. J.; Nishi, S. J. & Chen, Y. (2005). Activation of syndecan-1 ectodomain shedding by *Staphylococcus aureus* alpha-toxin and beta-toxin. J. Biol. Chem., 279:251-258.
25. Gotte, M. (2007). Syndecans in inflammation. FASEB. J., 17: 575-591.
26. Kaniga, K.; Uralil, J.; Blisska, J. B. & Galan, J. K. A. (1999). Secreted tyrosine phosphatase with modular effector domains encoded by the bacterial pathogen *Salmonella typhimurium.* Mol. Microboil., 21:633-641.
27. Kindt, T. J.; Goldsby, R. A. & Osborne, B. A. (2007). Immunology 6th Ed .Library of connnngress, Publisher, S. Tenney, WH. Freeman and Company, Priniting and Binding, RR. Donnelley, NY. USA.
28. Moon, M. M.; Hazlett, L. D.; Hancock, R. E.; Berk, R. S. & Barrett, R. (2006). Monoclonal antibodies provide protection against ocular *Pseudomonas aeruginosa* infection./lnvestig. Ophthalmol. Vis. SDI., 9:1277-1284.
29. Neely, A. N.; Holder, I. A.; Wiener-Kronish, J. P. & Sawa, T. (2009). Passive anti pcrV treatment protects burned mice against *Pseudomonas aeruginosa* challenge. Burn., 31:153-158