Bacteriological and Immunological Study of Diabetic Foot

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ABSTRACT

Diabetes mellitus (DM) is a serious health problem and remains an important cause of morbidity and mortality worldwide. Patients with uncontrolled diabetes develop complications, some of the most clinically important are foot ulcers, retinopathy, neuropathy and macrovascular complications. Foot complications such as foot ulcers constitute a major public health problem and impose a heavy burden in health service. The aim of the work was to isolate, identify the most common bacterial causes of diabetic foot lesions, and to assess the susceptibility pattern of the isolated organisms to the commonly used antibiotics. Phagocytic index of neutrophils of diabetic foot patients was also evaluated and its change over a short treatment course. The study was carried out on 35 patients with diabetic foot wound admitted to the General Surgery Department in Benha University Hospital. Phagocytic index of neutrophils was determined for each case by the phagocytic test at the beginning of the study and 2 weeks later. Pus aspirates were collected from the foot wound and cultured to identify the causative bacteria and its antibiotic susceptibility pattern. The results of the bacteriologic study revealed that, pure culture was found in 12 patients (34.29%) and mixed infection was found in 23 patients (65.71%). Gram negative isolates considered a high ratio (58.33%) than gram positive isolates (41.67%). Most isolates were aerobes (90%), however anaerobes were (10%). Staph aureus and Pseudomonas aeruginosa were the most commonly isolated bacterial species from diabetic foot wounds. The results of the immunologic study (phagocytic test) concluded that there is a statistically significant correlation between phagocytic index and the mean value of blood glucose.

INTRODUCTION

Diabetic patients are more prone to infection. Prior to the advent of modern antimicrobial therapy, infection account for much of morbidity and mortality in diabetes[1].

Uncontrolled diabetes rapidly promotes infection, as sugar is a good medium for rapid and abundant growth of organisms. The occurrence of infection in a diabetic patient initiates a vicious cycle in which infection results in uncontrolled hyperglycemia which in turn causes further aggravation of infection[2].

Many infections are common in patient with diabetes; as pyelonephritis, and soft tissue infection (Diabetic foot, necrotizing fasciitis, mucocutaneous candida infection)[3].

Foot infection is the most common soft tissue infection associated with diabetes, related peripheral neuropathy and peripheral vascular disease play a major role in this complication of diabetes mellitus (D.M.). More serious complications are osteomyelitis, amputation and death. Infection begins after minor trauma and may progress to cellulitis, soft tissue necrosis and may extend into bone[4].

A number of studies have found that staphylococcus aureus is the main causative pathogen in diabetic foot infection but other studies reported a predominance of gram-negative aerobes, while role of anaerobes is unclear[5].

Increased susceptibility to infection in diabetic is multifactorial. Several immunological factors are related to increase risk of infection, first, neutrophil function is depressed affecting adherence to endothelium, chemotaxis, phagocytosis and bactericidal activity. Also cell mediated immunity (CMI), serum level of Igs (Immunoglobulins), complement function all are reduced in diabetic patient, these impairment are exaggerated by hyperglycemia, academia, but reversed by normalization of pH (the measure of the acidity of a solution) and blood glucose level[6].

Furthermore, susceptability of target tissue to hyperglycemia, vascular disease, and nerve damage cause proness to infection, and reduction of antibiotic absorption due to microangiopathy lead to persistence of infection[7].

MATERIAL & METHODS

The study was carried out on 35 inpatients with diabetic foot wound admitted to the
General Surgery Department in Benha University Hospital. They were 19 males and 16 females and their ages ranged from 50 to 70 years during the period from July 2009 to February 2010. The studied patients were under standard therapy of: intensive insulin therapy, antibiotics and surgical debridement. The control group consisted of 10 healthy persons. Blood samples were collected aseptically from studied patient group and from healthy persons of control group in a screw-caped tubes containing heparin. Pus specimens were collected using a sterile ordinary swabs or the pus aspirated by a sterile syringe and transferred to a sterile screw-caped container and swabs for anaerobic culture were transferred in Cooked meat broth.

- **Phagocytic index of neutrophils:**
  - **A)** Phagocytosis against *Candida albicans* was done according to Wilkinson,1997). Neutrophils were incubated with heat-killed *Candida albicans* at 37°C for 15 minutes. The number of organisms ingested by the phagocytic cell was counted. The procedure was done as follow:
  1. **Preparation of blood leukocytes:**
     - 5 ml of heparinized venous blood was collected from studied patients and healthy persons of control group in a sterile screw-caped tubes containing heparin and shaked gently.
     - Then transferred to a sterile centrifuge tube containing 5 ml Hank's balanced salt solution and mixed well using pastuer pipette.
     - In another sterile centrifuge tube containing 5ml Ficol-Hypaque ,the content of the first tube was poured on the inner side of the second tube without allowing the solutions to become mixed by keeping the pipette against the tube wall.
     - The tube was centrifuged for 20 minutes at 2000 rpm .The leukocytes were localized as a whitish layer (buffy coat) on the upper meniscus of the solution .
     - A fine Pasteur pipette was used to take up the zone containing the leukocytes (buffy coat) which were then washed twice with Hank’s solution and centrifuged for 10 minutes at 2000 rpm after each wash.
     - After each wash with Hank’s , the bottom of the tube was knocked to release the deposite.
     - The supernatant was discarded and the pellet cells were suspended in a Hank’s solution .

  2. **Preparation of suspension of candida:**
     - Pathogenic strain of *Candida albicans* was isolated. After overnight broth culture, The *Candida* was inactivated by heating at 60°C for 1/2 hour in a water bath. The concentration of candida suspension was adjusted to 2 x 10^6 cell/ml using turbidity tube.

  3. **Phagocytosis:**
     - 0.1 ml of heat killed *Candida albicans* and 0.1 ml of AB serum were added to leukocyte suspension at 37 °C for 15 minutes followed by centrifugation. From the sediment, thin smears were made fixed with methanol and stained by Leishman stain .

  4. **Calculation of phagocytic index:**
     - The slide was examined under light microscope using oil immersion lens. The ingestion of *Candida albicans* by neutrophils was demonstrated. The total number of *Candida albicans* within 100 neutrophils were counted divided by 100 gives the **phagocytic index**.

- **B)** Phagocytosis against *Staph aureus* was done as follow:

  1. **Leukocyte suspension** was made as previously described for *Candida albicans* and the concentration was adjusted to 1-2 x 10^6 cell/ml .

  2. **Preparation of bacterial suspension:**
     - Pathogenic strain of staphylococcus aureus was isolated from pus specimen and its pathogenicity was checked according to standard biochemical tests. It was kept on a slope of nutrient agar medium. This strain was used throughout the study. Overnight broth culture of Staphylocossici was done. Staph were collected by centrifugation for 10 minutes at 2000 rpm. After washing twice with 0.85% saline, staphylococci were suspended in Hank’s solution to give a concentration of 1-2x10^7 cells/ml (10 organisms for each cell) using Macferland turbidity tube. The viable count of staphylococci was calculated by utilizing a standard pour plate technique with nutrient agar.

  3. **Phagocytosis:**
     - A mixture of 0.4 ml of staphylococcal suspension at a concentration of (1-2 x 10^7 cell/ml), 0.2ml of AB serum and 0.4 ml of leukocyte suspension at a concentration of (1-2 x 10^6 cell/ml) was put in plastic tube. The mixture was then incubated in shaking water-bath at rate of 80 stroke per minute at 37° C for an hour

  4. **Assessment of phagocytosis:**
     - 0.5 ml of mixture of bacteria , leukocytes and serum was taken after one hour of incubation added to 1.5 ml of Hank’s balanced salt solution, centrifuged for 4 minutes and washed twice with Hank’s solution.
• The cells were suspended in 0.5 ml of Hank's balanced salt solution.
• 2-3 drops of suspension were put on the slide, allowed to dry, then cells were fixed with methanol and stained with Giemsa stain, and the percentage of cells that have ingested bacteria was determined.
• To determine the number of ingested bacteria by phagocytic cells the following was done:
  o Suspension containing phagocytic cells and bacteria was cultured on nutrient agar after doing ten fold serial dilutions to determine the remaining viable bacteria.
  o The remaining viable bacteria were substracted from the total number of bacteria added at the start of the experiment.
• The phagocytic index was calculated by dividing the number of ingested bacteria by the number of leukocytes performed phagocytosis.

\[
\text{phagocytic index} = \frac{\text{The number of ingested bacteria}}{\text{The number of leukocytes performed phagocytosis}}
\]

• Bacteriological examination of pus specimens collected from infected diabetic foot:
• Antibiotic susceptibility pattern was performed using standard disc diffusion method.

**RESULTS**

The results of the bacteriologic study revealed that, pure culture was found in 12 patients (34.29%) and mixed infection was found in 23 patients (65.71%).

Gram negative isolates considered a high ratio (58.33%) than gram positive isolates (41.67%). Most isolates were aerobes (90%), however anaerobes were (10%). Staph aureus and Pseudomonas aeruginosa were the bacterial species most commonly isolated from diabetic foot wounds. Bacterial isolates from diabetic foot in the study group are shown in table(1).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Frequency</th>
<th>Number of isolates</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>15</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>11</td>
<td>18.33</td>
<td></td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>9</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Enterococci</td>
<td>6</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Gram negative Anaerobes</td>
<td>6</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>4</td>
<td>6.67</td>
<td></td>
</tr>
<tr>
<td>Klebsiella</td>
<td>3</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Citrobacter</td>
<td>2</td>
<td>3.33</td>
<td></td>
</tr>
<tr>
<td>Coagulase negative staph</td>
<td>2</td>
<td>3.33</td>
<td></td>
</tr>
<tr>
<td>Sterptococcus pyogens</td>
<td>2</td>
<td>3.33</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>60</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

The results from susceptibility testing revealed that, the isolated bacteria showed a high resistance ratio (42.3%) to used antibiotics. Vancomycin was the most effective antimicrobial agent against the gram positive isolated species. On the other hand Ciprofloxacin was the most effective antimicrobial agent against the gram negative isolated species. Antimicrobial resistance pattern of gram positive isolates and gram negative isolates are shown in table(2), and table(3) respectively.
Table (2): Antimicrobial resistance pattern of gram positive organisms in the study group:

<table>
<thead>
<tr>
<th>Organism</th>
<th>Staph. aureus (n=15)</th>
<th>Sterpt. pyogens (n=2)</th>
<th>Enterococci (n=6)</th>
<th>Coagulase -ve Staph (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibiotic</td>
<td>No %</td>
<td>No %</td>
<td>No %</td>
<td>No %</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>--/--</td>
<td>0/0</td>
<td>1/16.67</td>
<td>--/--</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>2/100</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>7/46.67</td>
<td>0/0</td>
<td>1/16.67</td>
<td>2/100</td>
</tr>
<tr>
<td>Methicillin</td>
<td>7/46.67</td>
<td>--/--</td>
<td>--/--</td>
<td>--/--</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>2/13.33</td>
<td>--/--</td>
<td>--/--</td>
<td>--/--</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>1/6.67</td>
<td>--/--</td>
<td>--/--</td>
<td>0/0</td>
</tr>
</tbody>
</table>

Table (3): Antimicrobial resistance pattern of gram negative organisms in the study group:

<table>
<thead>
<tr>
<th>Organism</th>
<th>Pseudomonas aeruginosa (n=11)</th>
<th>Proteus mirabilis (n=9)</th>
<th>E. coli (n=4)</th>
<th>Klebsiella (n=3)</th>
<th>Citrobacter (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibiotic</td>
<td>No %</td>
<td>No %</td>
<td>No %</td>
<td>No %</td>
<td>No %</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>0/0</td>
<td>6/66.67</td>
<td>2/50</td>
<td>3/100</td>
<td>0/0</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>1/9.09</td>
<td>1/11.11</td>
<td>0/0</td>
<td>3/100</td>
<td>0/0</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>9/81.81</td>
<td>0/0</td>
<td>0/0</td>
<td>3/100</td>
<td>0/0</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>1/9.09</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
</tbody>
</table>

The results of immunologic study revealed that, improvement occurred in phagocytic index over the treatment course, a significant difference was observed in phagocytic index before and after the 2 weeks therapy as shown in table (4).

Table (4): Comparison of phagocytic index (PI) values of study group before and after therapy:

<table>
<thead>
<tr>
<th>Phagocytic index (PI)</th>
<th>Number of patients</th>
<th>Mean ± SD</th>
<th>t</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>before therapy</td>
<td>35</td>
<td>0.697 ± 0.3417</td>
<td>16.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>after therapy</td>
<td>35</td>
<td>1.694 ± 0.2531</td>
<td></td>
<td>(HS)*</td>
</tr>
</tbody>
</table>

*P = <0.001 is highly significant(HS)

The results of immunologic study revealed a statistically significant negative correlation between phagocytic index and the mean value of blood glucose as shown in figure (1).

Figure (1): Correlation between phagocytic index (PI) & mean blood sugar values of study group
The organisms that were isolated from the diabetic foot infections in this study, were 60 bacterial isolates. As regard gram staining of the bacterial isolates, they were 25 (41.67%) gram positive organisms and 35 (58.33%) gram negative organisms. These results are in agreement with the study carried out by Shanker et al.\(^\text{[8]}\).

As regard the type of respiration of the bacterial isolates, they were 54 (90%) aerobes and 6 (10%) anaerobes. These results coincide with Tahawy\(^\text{[9]}\) who reported the same aerobes - anaerobes ratio in his study in King Abdulaziz University Hospital, Jeddah, Kingdom of Saudi Arabia. He reported a ratio of (89%) for aerobes and (11%) for anaerobes.

As regard frequency of Bacterial isolates from diabetic foot, the most frequent isolates in the present study were Staph aureus represents (25%) of the isolated bacteria, followed by Pseudomonas aeruginosa (18.33%), Proteus mirabilis (15%), then Enterococci and Gram negative Anaerobes each represents (10%) of the isolates. The predominance of Staph aureus was in agreement with the majority of studies performed on diabetic foot lesions.

Tahawy\(^\text{[9]}\); Unachukwu et al.\(^\text{[10]}\) and Alvai et al.\(^\text{[11]}\) all had reported that the Staph aureus were the most frequent isolates. On the other hand, Anandi et al.\(^\text{[12]}\) had recorded the predominance of Ecoli and proteus mirabilis over staph aureus in the isolates.

The present study showed that the most frequent gram negative isolates were Pseudomonas aeruginosa (18.33%), similar to that reported by Fernandes et al.\(^\text{[13]}\) who carried out a study on diabetic foot patients admitted to The Emergency Room of The Main University Hospital in the state of Goiás, Brazil. In the present study Pseudomonas were followed by Proteus mirabilis (15%), and then gram negative Anaerobes (10%).

Based on the results from susceptibility testing, the isolated bacteria showed (42.3%) resistance to used antibiotics. This was a higher resistance compared to similar work of Jakelic et al.\(^\text{[18]}\) who performed a similar study in Turkey.

The present study reported a statistically significant correlation between phagocytic index and the mean value of blood glucose which coincides with the study performed by Jakelic et al.\(^\text{[18]}\).

DISCUSSION

From this study we can conclude that, there is a statistically significant correlation between phagocytic index and the mean value of blood glucose. The derangement of carbohydrate metabolism may underlie the impairment of phagocytic function of neutrophils of poorly controlled diabetic patients. The data revealed that phagocytic index of neutrophils of diabetic patients with foot infections improves during the short treatment course and might enable the monitoring of efficacy of treatment modalities in these patients.

REFERENCES


دراسة بكتيريولوجية ومناعية للقدم السكري

أحمد عمر شقنق، رشان عرفة، محمد جودة عوض الله، جمال عامر، ماسية مصطفى

الميكروبولوجيا والمناعة: كلية طب بنها، جامعة بنها.

بحد ذات القدرا من أخطر المشاكل الصحية ومن أهم أسباب الأمراض والوفيات في العالم. وتحدث مضاعفات مرض السكري الخطرة المتصلة، من أهمها: فرخ البذور، واعتلال الأوعية، وارتفاع الكوليسترول، وارتفاع الكبد، وارتفاع السكر في الدم وغيرها من مضاعفات القدم. تظهر مضاعفات القدم كفرح القدم في حالة ضعف الدم، و ההתورم او البكتيريا الستيرول، و نقص التغذية او أو التغذية العلوية، و دهون الدم، و الكوليسترول، و السكر في الدم. و هذه الالفتاح هو عزل و التعرف على البكتيريا المسببة للقدم السكري و تقديم علاجات للمضادات الحيوية المانيية الاستخدام، و كذلك تقييم معالجات القدم لعلاج القدم السكري ومدى تأثره بالعلاج.

تم إجراء البحث على 35 مريضاً بتجميع السكري تنقسم الجراحة العامة بمستوى جهاز، و 10 أيضًا أخذت كما مجموعة ملزمة. تم سحب عينات مريضية من الدم، و تحديد معالجة الالتهابات في ثلاث مراحل، لكل حالة عن طريق اختبار الالتهاب. و أخذت عينات من جروج المرضى من كل مريض ثم تم عزل و التعرف على الالتهابات السببية لنوع الجسم، و يمكن الالتهابات السببية للمرضى، و يمكن الالتهابات السببية للمرضى، و يمكن الالتهابات السببية للمرضى.

- يتم عزل ميكروب واحد في 12 مريض (32,4%) و يتم عزل أكثر من ميكروب في 23 مريض (57,6%). وكانت معظم الميكوريات الملعنة من البكتيريا البيانية (95%) بينما البكتيريا اله-East كنا با (5%).
- البكتيريا الميكروبات المعولفة من البكتيريا اله-East كنا با (95%) حيث هي أكثر الميكروبات التي تم عزلها من جروج المرضى، و كانت نسبة البكتيريا سلبية الجراحة (73,3%) أعلى من البكتيريا موجبة الجراحة (17,7%).
- قد أظهرت الدراسات الحساسية أن نسبة معاوضة البكتيريا الملعنة لل مضادات الحيوية المختلفة هي (74%). و كان الفاينوسين هو أكثر مضادات الالتهابات التي تحدثت عن فعالية ضد الالتهابات الموجبة الجراحة، كذلك كان أداقة فعالية مضادات البكتيريا تصل إلى 60%.

و قد نتائج هذه الدراسة أن استخدام الالتهابات الموضعية الفعالة فعالة ضد البكتيريا موجبة الجراحة. و قد نتج عنها أن استخدام المعولنة أسلوبات التحكم في الالتهابات الموضعية الموضعية قد يؤدي إلى تعافى الطبية، و يمكن استخدامها للالتهابات الموضعية. و يمكن استخدامها في العلاج.

و بنانتي فيمكن استخدامها لل치료ات في العلاج الناجح بعد مدة قصيرة من العلاج، و بالتالي يمكن استخدامها للعلاج الناجح بعد مدة قصيرة من العلاج.