In vitro and *in vivo* Activity of some Antibiotics against Staphylococcal Biofilm and Planktonic Cells Isolated from Diabetic Foot Infections.

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Abstract: The diabetic foot syndrome is clearly one of the most important complications of diabetes and is the most common cause of hospitalization among diabetic patients. *Staphylococcus aureus* is found to be the commonest pathogen present in diabetic foot infections. The aim of the present study is to determine activities of three kinds of antibiotics against Staphylococcal biofilm and planktonic cultures *in vitro*, and to indicate the difference in wound healing between staphylococcal planktonic and biofilm stage of colonization *in vivo* by using diabetic rat models. Biofilm forming staphylococci were identified by using the modified microtiter plate method. And the effect of different concentrations of several antibiotics (including ciprofloxacin, gentamycin and amoxicillin-clavulanic acid) on eight isolates was determined. The result showed that out of 86 Staphylococcal isolates, eight strains were found to be strong biofilm forming. It was found that the preformed biofilm was very difficult to remove with most isolates even with multiples of the MIC and that the biofilm MBC reached 46 times the planktonic MBC in some isolates. This was also noticed in case of the diabetic foot infection of the rat model, as the treatment was more efficient when it started immediately after infection, before the formation of the biofilm, as the bacterial infection was eliminated within 3-4 days, while it could not be completely eliminated when treatment started after the biofilm formation. This was also observed from the rate of healing and confirmed by histological examination.

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1. Introduction:

The diabetic foot syndrome is clearly one of the most important complications of diabetes and is the most common cause of hospitalization among diabetic patients (Wrobel *et al.*, 2001), people with diabetes are 25 times more likely to have a leg amputated than those without the condition, according to the International Diabetes Federation (Lau *et al*., 2008). The combination of peripheral neuropathy, peripheral arterial disease and infections would result in unhealing ulcers, gangrene and amputation (Aulivola *et al*., 2004). Kandemir *et al*. (2007) found that the most frequent etiological agent for diabetic foot infections was staphylococci. In tissues removed from patients with recurrent staphylococcal infections, the cells are frequently organized in confluent colonies with a biofilm-like appearance. The pathogenesis of staphylococcal infection begins with primary bacterial adhesion and colonization of the host tissues (Morikawa *et al*., 2005). Once a biofilm has been established, it is a major concern for clinicians in the treatment of infectious disease because of the resistance to a wide range of antibiotics (Amorena *et al*., 1999). Therefore, a better understanding of bacterial biofilms is needed, and this may ultimately result in development of novel therapeutics for the prevention and treatment of wound infections (Davis *et al*., 2006).

Biofilm formation occurs upon initial rapid attachment of staphylococci to the surface, followed by multilayered cellular proliferation and intercellular adhesion in an extracellular polysaccharide matrix excreted by the bacteria (Gotz, 2002). Cell adhesion between staphylococci is mediated by polysaccharide intercellular adhesin (PIA), a linear homopolymer of ß-1,6-linked *N*-acetylglucosamine residues (Arciola *et al*., 2001).

Biofilm infections are difficult to treat due to their inherent antibiotic resistance. Once staphylococcal biofilm has formed on damaged tissue, it is difficult to disrupt. Most antimicrobial therapies for biofilms have largely proven unsuccessful. The mechanism of biofilm-associated

antibiotic resistance is uncertain and likely multifactorial. A number of factors have been postulated, including binding of antibiotic to the slime, poor penetration of antibiotic into the biofilm, slow growth rate of organisms in the biofilm, high bacterial density, and changes in gene expression in biofilm bacteria. Bacteria released from biofilms retain susceptibility to antibiotics characteristic of free-growing bacteria rather than biofilms, implying that the mechanism of resistance is not genetic change (Saginur *et al*., 2006).

The objective of this study was to determine the effect of different antibiotics on biofilm removal, also to compare the planktonic and biofilm MBC of staphylococcal isolates retrieved from diabetic ulcers to these antibiotics, and then to study the effect of planktonic and biofilm stage of colonization on an *in vivo* diabetic rat model.

2. Materials and methods

Bacterial strains: In this study 140 clinical swab form foot ulcers of diabetic inpatients in the university hospital (Alexandria, Egypt) were collected. Out of 197 isolate 86 isolates were identified as Gram positive bacteria (Staphylococi). Identification of the staphylococcal strains was done by Gram staining, catalase, coagulase tests, and cultivation on mannitol salt agar, further confirmation was carried out by using the API Staph system. Out of the 86 staphylococci isolated 62 (70.4%) were *Staphylococcus aureus* and 25 (29.6%) were Coagulase negative staphylococci (CONS).

Quantification of biofilm formation: This was done by using the modified microtiter plate method (Stepanovic *et al*., 2000), where the strains were grown in TSB supplemented with 2.5% glucose (Christensen *et al*., 1985). All strains were categorized as non, weakly, moderately, or strongly adherent, based upon the ODs of bacterial films as described by Stepanovic *et al*. (2000).

Antimicrobial agents used: Gentamycin (CN) (Alexandria Co., Egypt), Ciprofloxacin (CIP) (Amriya, Egypt), and Amoxicillin-Clavulanic acid (AMC) (GlaxoWellcome) were used.

MIC and MBC determination: Susceptibility testing to each drug was performed on planktonic cultures using the two-fold dilution method according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2003). MICs were performed in 96-well microplates (Greiner, Wemmel, Belgium) and results were recorded after incubation at 35 °C for 18 h. For MBC determination, 10µl aliquots were removed from the wells after incubation and spread onto Mueller–Hinton agar in Petri dishes and incubated overnight at 35 °C (Tre-Hardy *et al*., 2008).

Effect of antimicrobial drugs on pre-formed biofilms:

Biofilms were allowed to form as described above. The content of the wells was then aspirated and the wells were washed and fresh TSB containing two fold serial dilution of the antimicrobial agent was added to the wells as described above. The plate was then incubated at 35^{o}C for 24h. The content of the wells was aspirated then washed and stained by using crystal violet as described above. The optical densities were determined after elution as before. Biofilm persistence in the presence of antimicrobial agents was calculated using the following formula (Tre-Hardy *et al.*, 2008).

Percentage of biofilm persistence = $[(A_{590x} - A_{590\text{negative control}})/ (A_{590\text{ positive control}} - A_{590\text{negative control}})] \times 100$, where x corresponds to the antimicrobial agent used.

Animal model

Alloxan-induced diabetes model:

Diabetes was induced by a single intraperitoneal injection of 130 mg/kg monohydrated alloxan (Sigma, St. Louis, MO, USA) dissolved in sterile 0.9% saline. Rats were made to fast prior to alloxan administration. After 12 h, a 10% glucose solution was offered to the animals to prevent hypoglycemia (Diniz *et al*., 2008). After 72 h, blood samples were collected from the eye of the animals for evaluation of plasma glucose levels by the glucose-oxidase enzymatic colorimetric method (Biomedical Systems, Spain). Animals presenting glucose levels above 300 mg/dL were included in the diabetic group.

Diabetic foot ulcer study:

The adult diabetic rats (>300 mg/dl) were used for wound induction. On the day of wound induction (defined as day 0) each rat was anesthetized with an intraperitoneal injection of 50 mg/kg thiopental sodium. A rectangular pattern was marked on the dorsal surface of the foot using a flexible transparent plastic template, and then a layer of skin in full thickness with standard area of 2 mm × 5 mm was removed as shown in Figure 1. The initial wound size was measured on day 1 (Lau *et al*., 2009).

Treatment groups:

A total of 16 rats were used in this study, they were divided into control and treatment groups, each group was composed of three rats and one rat was sacrificed on day 2 for the histological studies to confirm the formation of the bacterial biofilm within the wound bed. The treatment groups included two groups, the first received ciprofloxacin and the second received amoxicillin clavulanic acid. Half the

number of rats in each group started the treatment 15 minutes after inoculation (representing the planktonic stage), the other half started the treatment after 48 hours (representing the colonized bacteria forming the biofilm). Antimicrobial agents were administered orally by gavages. This experiment was made once with the *S. aureus* isolate (25S), and another time with the *S. epidermides* (78S).

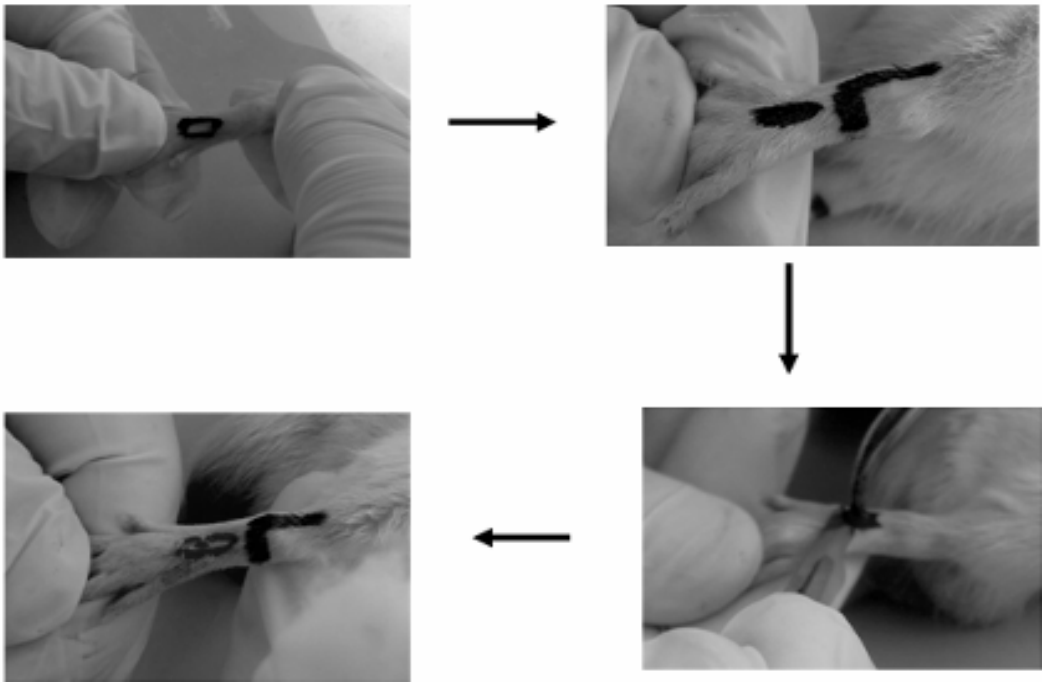

Figure 1: Wound formation in the dorsal surface of the rat hind paw.

Preparation of bacterial inoculums:

Overnight cultures of the staphylococcal isolate were used to inoculate the wounds. The wounds representing the planktonic stage of bacterial colonization started the antimicrobial treatment 15min. after inoculation, while the wounds representing the biofilm-associated cells were inoculated with the initial inoculum and left for 48 hours to allow wounds to be colonized (Davis *et al.,* 2008a)

Sampling and Wound area measurement:

In this study we selected the one-point sampling method because of the relatively small size of wounds that can be induced in rats and to better avoid contamination with peri-wound flora. In this method the swab cultures were taken from the center of each surgical wound by rotating the swab three times clockwise. Ulcer digital photographs and swabs were taken on days 1, 3, 5 and 9. Ulcer area measurement were made by using the digital photographs of the wounds perpendicular to a metric reference scale (Sullivan *et al.,* 2004), which allowed us to enlarge the photograph in order to increase the accuracy of wound area measurement and the photographs were processed by using Microsoft Word where the wound area was traced and measured on the scale present in the same photograph as shown in Figure 2.

Antimicrobial therapy:

Antimicrobial doses were chosen to approximate, in the rat, the serum or tissue AUCs achieved in humans. The doses were 350/87.5 mg/kg and 200 mg/kg for amoxicillin/clavulanic acid and ciprofloxacin respectively (Berry *et al*., 2000). Rats receive the antimicrobial treatment twice daily for 7 days (Cagni *et al*., 1995).

Histological examination:

Biopsy specimens were obtained 48 hours after inoculation and colonization and at the end of the treatment for evaluation with light microscope. Specimens were placed in 10% formaldehyde and stained with hematoxylin and eosin and with Gram crystal violet (Kugelberg *etal.,* 2005)

3. Results

Quantification of biofilm formation:

The experiment performed was carried out to measure the degree of adherence and subsequent biofilm formation of all staphylococcal isolates. Out of the 86 staphylococcal isolates 4 (4.65%) were non-adherent, 35 (40.7%) were weakly adherent, 39 (45.35%) were moderately adherent and 8(9.3%) were strongly adherent. The eight strong biofilm forming strains (16S, 17S, 18S, 25S, 45S, 78S, 90S and 108S) were selected for further investigations. Three of which (16S, 25S and 45S) were *S. aureus*, while 17S, 18S, 78S, 90S and 108S were CONS.

MIC and MBC determination: MICs and MBCs recorded for the tested antimicrobial agents against the eight staphylococcal isolates are summarised in Table1.The biofilm MBCs were found to be much higher than the planktonic MBCs. These results are shown in Figure 3.

In case of ciprofloxacin, the biofilm MBC was 2 to 512 times higher than the planktonic MBC. Also, the biofilm MBC for gentamycin was 2 to 256 times higher than that of the planktonic culture. Whereas, the biofilm MBC in case of amoxicillin-clavulanic acid was 4 to 64 times higher than that of the planktonic culture.

Variable behavior in biofilm formation for the 8 staphylococcal isolates was observed in biofilm and planktonic cultures; no fixed increase was noticed for all strains with the different antibiotics used.

Detachment of established staphylococcal biofilms after antibiotics exposure:

The effect of MIC and its multiples on the pre-formed biofilm was tested in order to determine the biofilm removing capacity of the studied antibiotics. It was found that ciprofloxacin showed relatively the best results in biofilm detachment as it removed from 40-80% of an already formed biofilm in five (62.5%) of tested isolates. However, amoxicillin-clavulanic acid showed the lowest ability for biofilm detachment. On the other hand, two strains (25S, 45S) showed increase in the biofilm formation by the addition of antibiotics (Figure 4).

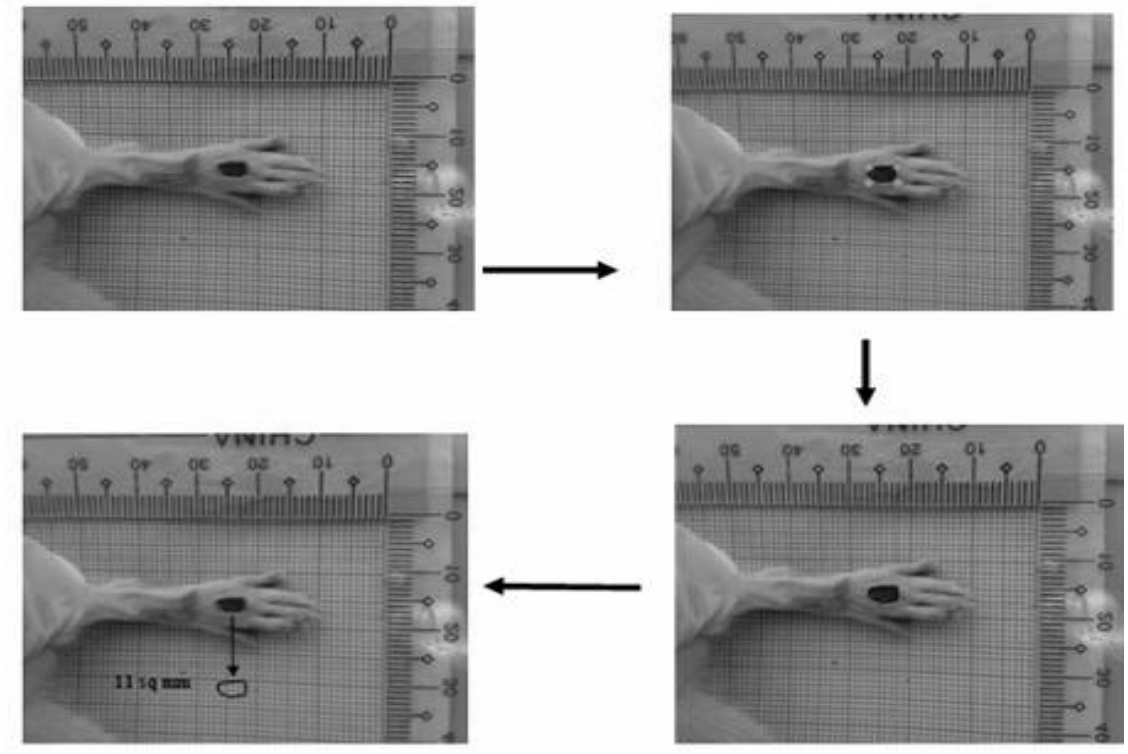

Figure 2: Wound area measurement in the diabetic rat model.

Table 1: MIC and MBC values for the tested antibiotics against the 8 staphylococcal isolates. The equivalent MIC breakpoints for *staphylococcus* spp. (CSLI 2005) were R: ≥4, S:≤ 1 for ciprofloxacin; R: ≥8, S:≤ 4 for gentamycin and R: ≥ 8/4, S: 4/2 for amoxicillin-clavulanic acid.

Strain	Ciprofloxacin		Gentamycin		Amoxicillin-clavulanic acid	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
16S	0.5	1	2	4	8	32
25S	0.5	1	4	8	8	32
45S	128	512	128	256	64	256
18S	32	128	128	256	32	32
17S	8	8	64	128	8	8
78S	0.5	0.5	0.25	0.5	0.25	0.25
108S	0.125	0.5	1	2	0.25	1
90S	8	16	128	128	8	32

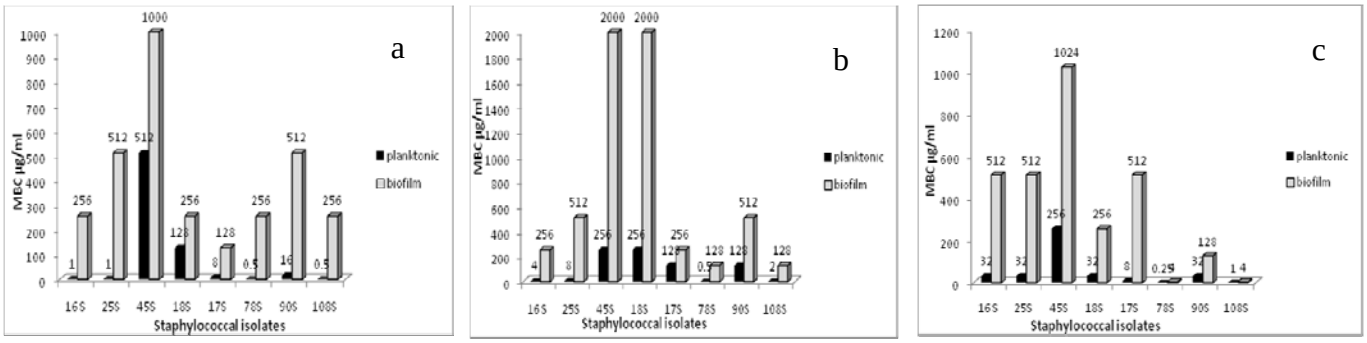

Figure 3: Comparison between planktonic and biofilm MBC of ciprofloxacin (a), gentamycin (b), and amoxicillin-clavulanic acid (c) for the 8 staphylococcal isolates.

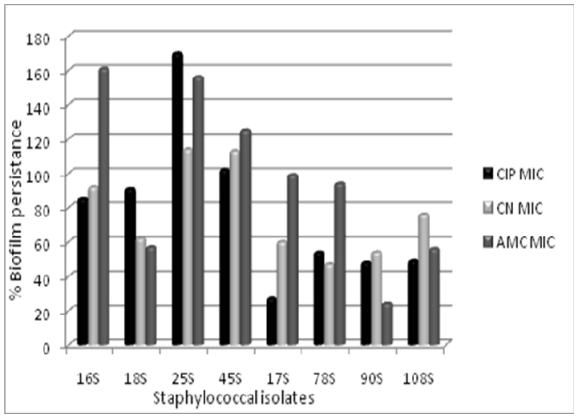

Figure 4: Effect of the MIC of each antibiotic on the removal of a pre-formed biofilm

Animal model:

Wound area measurement:

The wound healing effect of ciprofloxacin and amoxicillin clavulanic acid in case of planktonic stage of bacterial infection was compared with that after biofilm formation on days 1, 3, 5 and 9 in the diabetic rat foot infection model.

In case of the *S. aureus* isolate 25S, a trend of greater reduction of the ulcer area was observed in case of planktonic bacterial colonization treated with the antibiotic than that after the biofilm formation, where the average ulcer area in the control untreated groups decreased from 9mm^2 (100%) on day 1 to 3 mm^2 (33.33%) on day 9, whereas that treated with ciprofloxacin decreased from 13 mm^2 (100%) on day 1 to complete healing on day 9 (Figure 9), and that of the group treated with amoxicillin-clavulanic acid decreased from 14 mm^2 (100%) on day 1 to 1 mm^2 (7%) on day 9 .

Similar results were obtained in case of the wound inoculation with the isolate 78S, where the wound area started with 9, 18and 17 mm^2 (100%) on day 1 and was reduced to 3 mm^2 (33.33%), 1 mm^2 (5%) and 1 mm^2 (5.8%) in case of the control, ciprofloxacin and amoxicillin clavulanic acid treatment groups respectively.

It was noticed from the wound swab samples taken that complete eradication of the staphylococcal infection was achieved after four days in case of the isolate 25S when treated with either of the antibiotics, and three days for the isolate 78S in case of both

antibiotics. Whereas, after the formation of a biofilm it was difficult to completely eradicate the bacterial infection throughout the nine days of the experiment.

Histopathological studies:

Histopathological examination of rat skin sections of different treatment groups was made. Figures 6, 7 shows a wound made 48 hours before the examination, with the bacteria forming biofilm, this section was stained with H&E (Figure 6), and with Gram Stain (Figure 7) to visualize the Gram positive bacteria. Figure 8 showed complete healing and recovery from bacteria which was achieved with the ciprofloxacin treatment in case of the planktonic cells infection with the *S. aureus* isolate 25S. Whereas treatment after the biofilm formation with amoxicillin-clavulanic acid showed the persistence of bacterial infection with bacteria found around the hair follicles (Figure 9).

4. Discussion:

Biofilms, products of bacterial adherence, are structured communities of bacterial cells enclosed in a self-produced exopolysaccharide matrix and adherent to an inert or living surface. Establishment of a biofilm is the prelude to the development of various chronic, intractable infections, such as biomaterial-associated infections and pulmonary infection in patients with cystic fibrosis (Bonaventura *et al.*, 2004). Despite various efforts, treatment of an infection after biofilm is established is frequently futile because of the reduced susceptibility of biofilm to antibiotics. At least three mechanisms have been proposed to account for recalcitrance of biofilms to antimicrobial agents: (i) failure of the antimicrobial to penetrate the biofilm, (ii) slow growth and the stress response, and (iii) induction of a biofilm phenotype (Bonaventura *et al.*, 2004).

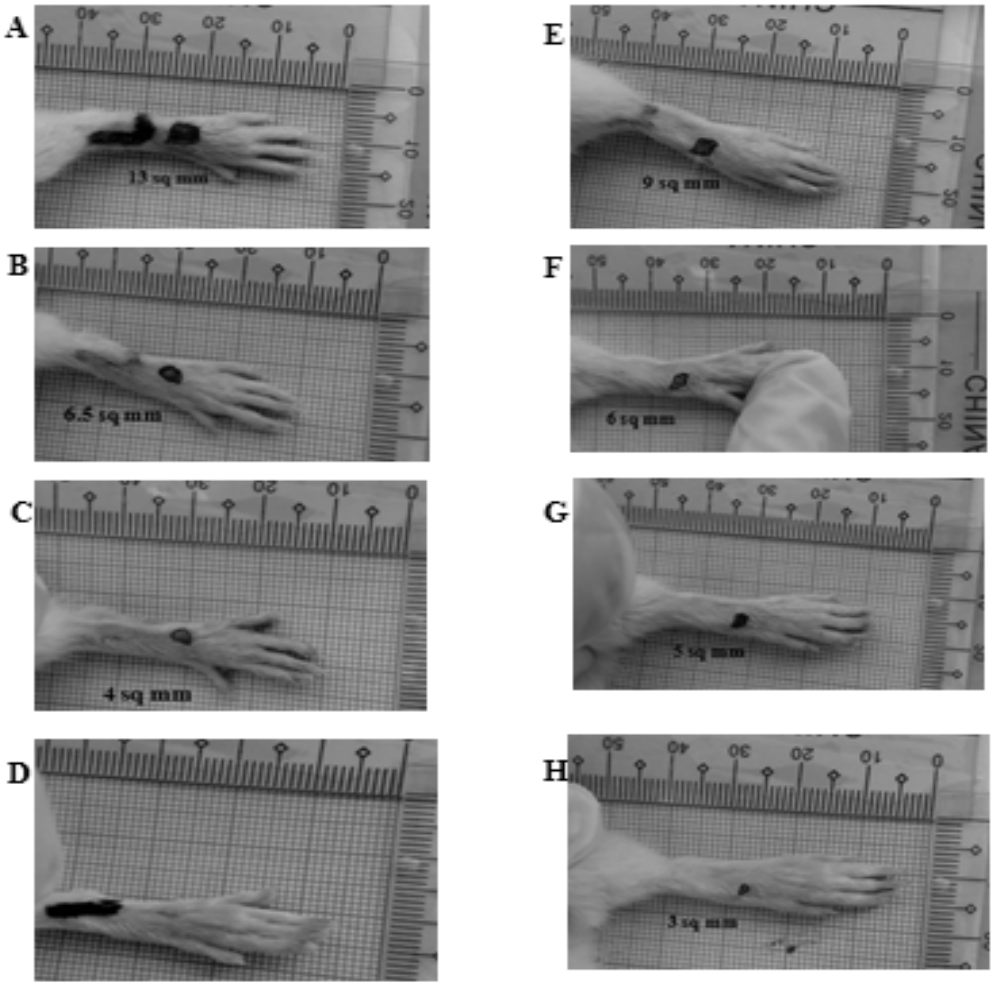

Figure 5: Effect of the treatment with ciprofloxacin on planktonic infection (on the left), compared to the control (untreated) (on the right) on wound healing of diabetic rat foot infection with *S. aureus* 25S.

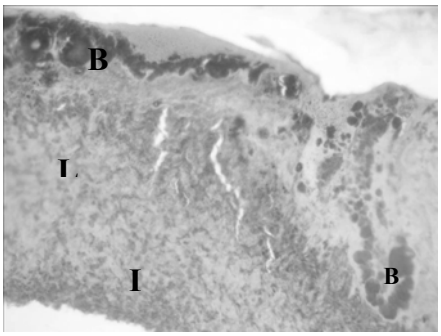

Figure 6: H & E stained section in diabetic rat skin 48 hours after wound formation and infection at X400 magnification using light microscopy. In the wound bed there are circular basophilic granular structures, which are multiple bacteria joined together living in biofilm-like structure (B) in the wound bed in the epidermis while the dermis shows lytic necrosis (L) and mononuclear cellular infiltrate (I). There is also hyalinizatrion and vaculation of the epithelial cells, and pathological changes in the epidermal cells was observed with mononuclear cellular infiltrate, hemorrhagic areas.

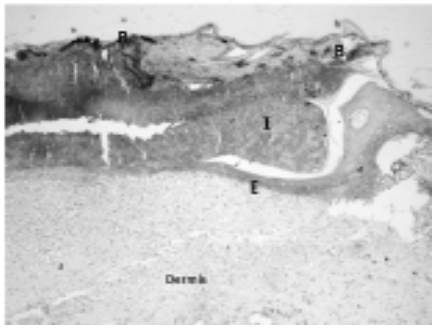

Figure 7: Section of 48 hours untreated wound stained with Gram stain showing Gram positive bacteria (B), cellular infiltrate (I), and reduced epidermis (E) (X100).

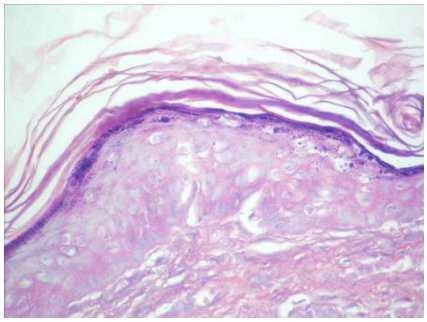

Figure 8: High power view of the epithelium showing normal skin, where complete healing was achieved in case of treatment of *S. aureus* 25S infection as planktonic cells with ciprofloxacin. (H&E stain X400)

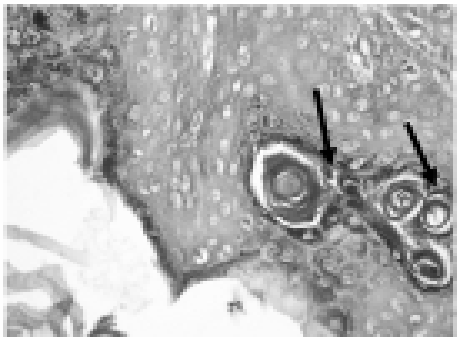

Figure 9: Another section in rat skin showing bacteria around transversely cut hair follicles with mononuclear inflammatory infiltrate. (H&E stain X400)

Microtiter plate systems for quantifying adherence and biofilm formation were used in this study, these techniques have been investigated with many different organisms and stains (Christensen *et al*., 1985) and they have been widely used because they are simple, reproducible, and quantitative methods. However, the staining measurements reflect the total amount of biofilm (sessile cells plus exopolysaccharide matrix) but do not give any information about its viability (Griffis *et al*., 2009). Thus, in this study, cell viability was also measured by determination of biofilm MBC, which was found to be very high (512 × planktonic MBC) in case of the isolates 25S, 78S and 108S when tested with ciprofloxacin. However, in other cases the difference between biofilm and planktonic MBC is much less (2 × planktonic MBC) in case of the isolate 18S when tested with ciprofloxacin also. These results was also noticed by other investigators who found that 4× MBC of the antibiotics used is not sufficient for killing *S. aureus* in biofilm (Amorena, *et al*., 1999). Also *Pseudomonas aeruginosa* in biofilm showed a 4-fold greater resistance against ciprofloxacin and gentamycin compared with free-living forms (Agarwal *et al*., 2005).

In the present study, the effects of several antibiotics on Staphylococcal adherence were tested. The studied antibiotics were chosen for several reasons. Amoxicillin-clavulanic acid was tested because it is frequently used in the therapy of Staphylococcal infections. Quinolone (ciprofloxacin) was chosen because of their interesting activity against gram-negative (Baskin *et al*., 2002) and gram-positive (Wilcox *et al*., 1991) bacterial biofilms. Currently there are no antibiotics on the market specifically indicated for the prevention of diabetic foot infections. Furthermore, diabetic ulcers are often associated with vascular disease and restricted peripheral blood flow, which may render

systemically acting antibiotics less effective. By achieving very high localized concentrations of antibiotic, gentamycin may be used to overcome these concerns if used locally**.**

In case of the preformed biofilm, ciprofloxacin showed the best results in biofilm removal. Concentrations ranging from 0.5-2 MIC caused removal of 50% of an already formed biofilm in five (62.5 %) of the tested isolates, followed by gentamycin where the MIC caused the removal of 50% of an already formed biofilm in only four (50%) of the tested isolates. However, amoxicillin-clavulanic acid showed the least results in biofilm removal (Figure 4). In addition, the effect of MIC of the tested antibiotics on a preformed biofilm showed that there is no fixed pattern for the effect of antibiotics on this preformed biofilm but it was noticed that the bacterial behavior in response to the different antibiotics applied is strain dependant, which requires further investigations. These results suggest that ciprofloxacin is able to penetrate staphylococcal biofilm with a strain-specific efficiency, as suggested by the high strain-to-strain variation, probably due to chemical and physical heterogeneity of staphylococcal biofilms. This difficulty in biofim removal with antibiotics alone led Krespi YP *et al*. (2010) to use ciprofloxacin in combination with different types of lasers to remove more than 80% of Staphylococcal biofilm cells. Other investigators used other approaches to treat biofilm-associated staphylococcal infections such as cell-wall degrading enzymes (Son J *et al*. 2009).

From this study we can conclude that once biofilms are formed they are very difficult to be removed even by applying multiples of the MIC of the tested antibiotics. And it was found that there is a high strain-to-strain variation in the behavior towards the tested antibiotics, so it is advised to study each strain separately.

The majority of skin pathogenic and non pathogenic biofilm research is performed *in vitro*. Although *in vitro* assays have several advantages, including lower cost, and the ability to control the number of bacteria, they do not take into consideration the effect of wound fluid, growth factors, proteases, and antimicrobial peptides (Davis *et al.*, 2008a). In this study the rat model used was anticipated to help clarify the role of bacterial biofilms in diabetic wound infection and healing.

In *in vivo* studies of ulcer healing, surgical ulcers were usually induced on the chest or back of a rat or a mouse. With the aim of mimicking more closely the clinical condition of diabetic foot ulcers, the ulcers were created on the feet of alloxan-diabetic rats. The animal model was probably suitable for *in vivo* studies of ulcers in response to different medications. In this study, the diabetic rat foot ulcer model was created by removal of full-thickness skin. This artificially created ulcer is different from the ulcer in the human diabetic foot which usually results from local pressure. Human diabetic foot ulcers usually extend beyond the surface boundaries and this subcutaneous extension does not occur in this animal model (Lau *et al*., 2008).

The authors previously demonstrated that pathogenic wound bacteria *in vitro* can form a mature biofilm within 10 hours. Results of an *in vivo* study have shown that occluding wounds following inoculation with pathogenic bacteria facilitates the development of biofilms after 24 hours. The authors believe that biofilms are likely to be present in chronic wounds and additional strategies to disturb or eliminate them are sorely needed (Davis *et al*., 2008b).

Studies made by Dr. Akiyama's group demonstrate biofilm-like structures in a mouse wound model and one in human skin. Using confocal laser scanning microscopy they showed colonies of bacteria surrounded by glycocalyx. These studies firmly established that biofilm-like structures are present in the murine wound infection model (Akiyama *et al*., 2004)

The management of chronic wounds such as those found in diabetic feet or leg ulcers is placing an increasing burden on health service systems (Krouskop, 2002). Chronic wounds, however, have an unfortunate tendency of healing very slowly. Any measurement technique used for the purpose of establishing healing progress therefore has to be very precise in order to capture small changes in a wound's dimensions.

The 'gold standard' for area measurement is the practice of tracing the perimeter of a wound through a double layer of a flexible transparent sheet material (Keast, 2004). While the layer in contact with the wounds is discarded, the upper layer with the tracing is then measured by either placing it on metric graph paper, planimeters or by a second round of tracing using a digitizing tablet (Thawer, 2002). This contact making method can be painful to the patient and may risk infection. In practice the use of unsuitably thick or exhausted marker pens and the high degree of dexterity required by the clinician performing the tracing significantly reduce the theoretical precision of the method and errors up to 25% have been reported (Lagan, 2000).

Photography has the advantage of avoiding contact with the patient (Smith, 1992; Stacey, 1991). Wound pictures are usually measured on a computer screen where they can also be enlarged so that the tracing process is freed from time restrictions and physical demands. Although area measurements

produced this way tend to be more precise than those obtained by transparency tracings the method depends on the photography skills of the clinician who has to combine a frame-filling wound picture together with a reference scale in a single image taken perpendicular to the wound site (Solomon, 1995).

Digital photography was the way used in the wound area measurement in this study, where the wound and the metric reference scale were included in the same picture as shown in Figure (2). This gave us the advantage of avoiding direct contact with the wound and allowed us to enlarge the wound picture together with scale on the computer screen in order to increase the accuracy of wound area measurement.

In this study bacterial biofilm colonization in an animal model was obtained, where the wounds were inoculated with the selected isolate and left untreated, swabs were taken to ensure the presence of the bacteria in the wound of the control group through out the study period. This was also made by Davis *et al*. (2008a) who observed colonies of bacteria firmly attached to and on the wound surface, encased in an amorphous substance; they also observed the polymorphnuclear cells on the surface of the biofilm, but not within the biofilm. Histologically, bacterial biofilms are tissue-like structures resembling multicellular eukaryotic tissues. According to the histology text book edited by Leon Weiss (1988) a tissue is a multicellular aggregate of similar cells. In line with this definition bacterial biofilm can be described as a "bacterial tissue" within the host tissue.

In order to show a physiological difference between the planktonic and biofilm bacteria *in vivo*, we studied the efficacy of two antibiotics on two bacterial isolates in the rat model. The treatment with ciprofloxacin or amoxicillin-clavulanic acid was extremely effective in eradicating the organisms that were not attached to the wound surface (3-4 days). However, after allowing the biofilm formation, this treatment was not able to eradicate the staphylococcal infection at such a short time because the biofilm bacteria were more persistent, fulfilling one of the criteria for biofilm-associated diseases, which is reduced susceptibility. These results were also demonstrated by Davis *et al*. (2008a) who studied the effect of two topical antibiotics on *S. aureus* infected wound healing, where the antibiotics were not able to eradicate bacteria that were allowed to form biofilm.

The concordance of *in vitro* and *in vivo* findings observed in the staphylococcal isolates tested using this biofilm test methodology is encouraging. This concordance suggests that the test could be useful in clinical practice and extensive studies especially needed to eradicate the increasingly frequent chronic staphylococcal infections and in particular those associated with the diabetic foot ulcers.

The use of an *in vivo* model in this study supports the hypothesis that staphylococcal biofilm are more difficult to eradicate and may play an important role in acute and chronic diabetic foot infections. We have provided evidence that biofilms play a role in diabetic foot infections. A new paradigm has arisen; it is not sufficient to think of bacteria in infections as unicellular independent pathogens, rather it is now important to understand that they form multicellular tissue like structures (Davis *et al.*, 2008a).

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5. References:

1. Agarwal, G., Kapil, A., Kabra, S. K., Das, B. K., Dwivedi, S. N. 2005. In vitro efficacy of ciprofloxacin and gentamicin against a biofilm of Pseudomonas aeruginosa and its free-living forms. The National Medical Journal of India. 18(4): 184-186.
2. Akiyama, H., Oono, T., Saito, M, and Iwatsuki, K. 2004. Assessment of cadexomer iodine against *Staphylococcus aureus* biofilm in vivo and in vitro using confocal laser scanning microscopy. J Dermatol. 31: 529-34.
3. Amorena, B., Gracia, E., Monzon, M., Leiva, J., Oteiza, C., Pérez, M., Alabart, J., and Hernández-Yago, J. 1999. Antibiotic susceptibility assay for *Staphylococcus aureus* in biofilms developed in vitro. J Antimicrob Chemothr. 44:43-55
4. Arciola, C. R., Baldassarri, L, and Montanaro, L. 2001. Presence of icaA and icaD Genes and slime production in a collection of staphylococcal strains from catheter-associated infections. J. Clin. Microbiol. 39: 2151-2156.
5. Aulivola, B., Hile, C. N., Hamdan, A. D., Sheahan, M. G., Veralid, J. R., Skillman, J. J., Campbell, D. R., Scovel, S. D., Logerfo, F. W., and Pomposelli, F. B. 2004. Major lower extremity amputation: outcome of a modern series. Arch Surg. 139: 395-399.
6. Baskin, H., Dogan, Y., Bahar, I. H., and Yulug, N. 2002. Effect of subminimal inhibitory concentrations of three fluoroquinolones on adherence of uropathogenic strains of *Escherichia coli*. Int. J. Antimicrob. Agents 19:79–82.
7. Berry, V., Page, R., Satterfield, J., Singley, C., Straub, R., and Woodnutt, G. 2000. Comparative efficacy of gemifloxacin in experimental models

of polynephritis and wound infection. J Antimicrob Chemother. 45: 87-93.
8. Bonaventura, G. D., Spedicato, I., D'Antonio, D., Robuffo, I., and Piccolomini, R. 2004. Biofilm Formation by *Stenotrophomonas maltophilia*: Modulation by Quinolones, Trimethoprim-Sulfamethoxazole, and Ceftazidime. Antimicrob. Agents Chemother. 48:151-160.
9. Cagni, A., Chuard, C., Vaudaux, P. E., Schrenzel, J., and Lew, D. P. 1995. Comparison of Sparfloxacin, Temafloxacin, and Ciprofloxacin for Prophylaxis and treatment of experimental foreign-body infection by methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. 39: 1655-1660.
10. Christensen, G. D., Simpson, W. A., Younger, J. J., Baddour, L. M., Barrett, F. F., Melton, D. M., and Beachey, E. H. 1985. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J. Clin. Microbiol. 22:996-1006.
11. Clinical and Laboratory Standards Institute (CLSI) (2003). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 6th ed. Approved standard. M7-A6. Wayne, PA: CLSI.
12. Davis, C., Martiner, L., and Kirsner, R. 2006. The diabetic foot: The importance of biofilms and wound bed preparation. Current Diabetes Reports. 6: 439-445.
13. Davis, S. C., Ricotti, C., Cazzaniga, A., Welsh, E., Eaglstein, W. H., and Mertz, P. M. 2008a. Microscopic and physiologic evidence for biofilm-associated wound colonization in vivo. Wound Rep Reg. 16:23-29
14. Davis, C. S., and Mertz, P. M. 2008b.Determining the effect of an Oak Bark Formulation on Methicillin-resistant Staphylococcus aureus and wound healing in porcine wound models.
15. Diniz, S. F., Amorim, F. P. L. G., Cavalcante-Neto, F. F., Bocca, A. L., Batista, A. C., Simm, G. E. P. M., and Silva, T. A. 2008. Alloxan-induced diabetes delays repair in a rat model of closed tibial fracture. Braz. J. Med. Biol. Res., 41(5): 373-379.
16. Gotz, F. 2002. *Staphylococcus* and biofilms. Mol. Microbiol. 43:1367-1378.
17. Griffis, C. D., Metcalfe, S., Bowling, F. L., Boulton, A. J. M., and Armstrong, D. G. 2009. The use of gentamicin-impregnated foam in the management of Diabetic foot infections: a promising delivery system. Expert opinion on drug delivery. 6 (6): 639-642.
18. Kandemir, O., Akbay, E., Sahin, E., Milcan, A., and Gen, R. 2007. Risk factors of infection of the diabetic foot with multi-antibiotic resistant microorganisms. J. infect. 54: 439-445.
19. Keast, D. H., Bowering, C. K., Evans, A. W., Mackean, G. L., Burrows, C., and D'Souza, L. 2004. A proposed assessment framework for developing best practice recommendations for wound assessment Wound Repair and Regeneration. MEASURE. 12, s1-s17.
20. Krespi, Y. P., Kizhner, V., Nistico, L., Hall-Stoodley, L., Stoodley, P. 2010. Laser disruption and killing of methicillin-resistant *Staphylococcus aureus*. In Press.
21. Krouskop, T. A., Baker, R., and Wilson, M. S., 2002. A non contact wound measurement system, J Rehabil Res Dev. 39: 337-346.
22. Kugelberg, E., Norstrom, T., Petersen, T. K., Petersen, T. K., Duvold, T. 2005. Establishment of a superfacial skin infection model in mice by using *Staphylococcus aureus* and *Streptococcus pyogens*. Antimicorb. Agents Chemother. 49: 3435-3441.
23. Lagan, K. M., Dusoir, A. E., McDonough, S. M., and Baxter, G. D. 2000. Wound measurement: the comparative reliability of direct versus photographic tracings analyzed by planimetry versus digitizing techniques Arch Phys Med Rehabil, 81, 1110-1116.
24. Lau, T.W., Sahota, D.S., Lau, C.H., Chan, C.M., Lam, F.C., Ho, Y.Y., Fung, K.P., Lau, C.B.S., Leung, P.C. 2008. An in vivo Investigation on the Wound-Healing Effect of two Medicinal Herbs Using an Animal Model with Foot Ulcer Eur Surg Res. 41:15-23
25. Lau, T. W., Lam, F. F. Y., Lau, K. M., Chan, Y. W., Lee, K.M., Sahota, D. S., Ho, Y. Y., Fung, K. P., Leung, P. C., and Lau, C. B. S. 2009. Pharmacological investigation on the wound healing effects of Radix Rehmanniae in an animal model of diabetic foot ulcer. Jo urnal of Ethnopharmacology. 123: 155-162.
26. Morikawa, K., Nonaka, M., Yoshikawa, Y., and Torii, I. 2005. Synergistic effect of fosfamycin and arbekacin on a methicillin-resistant *Staphylococcus aureus*-induced biofilm in a rat model. Int. J antimicrob agents. 25(1): 44-50.
27. Saginur, R., St Denis M., Ferris, W., Aaron, S. D., Chan, F., Lee, C., and Ramotar, K. 2006. Multiple combination bactericidal testing of staphylococcal biofilms from implant-associated infections. Antimicrob. Agents Chemother. 50: 55-61.
28. Smith, D. J., Bhat, S., and Bulgrin, J. P. 1992. Video image analysis of wound repair. Wounds. 4: 6-15.
29. Solomon, C., Munro, A. R., Van Rij, A. M., and Christie, R. 1995. The use of video image analysis

for the measurement of venous ulcers. Br J Dermatol. 133: 565-570.
30. Son, J., Lee, S., Jun, S. Y., Yoon, S. J., Kang, S. H., Paik, H. R., Kang, J. O., and Choi, Y. 2009. Antibacterial and biofilm removal activity of podoviridae *Staphylococcus aureus* bacteriophage SAP-2 and a derived recombinant cell-wall-degrading enzyme. Appl. Microbiol. Biotechnol. 86 (5): 1439-1449.
31. Stacey, M. C., Burnand, K. G., Layer, G. T., Pattison, M., and Browse, N. L. 1991. Measurement of the healing of venous ulcers. Aust N Z J Surg. 61: 844-848
32. Stepanovic, S., Vukovic, D., Dakic, I., Savic, B., and Svabic-Vlahovic, M. 2000. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J. of Microbiol. Methods. 40: 175-179
33. Sullivan, P. K., Conner-Kerr TA, Hamilton H, Smith EP, Tefertiller, C., and Webb, A. 2004. Assessment of Wound Bioburden Development in a Rat Acute Wound Model: Quantitative Swab versus Tissue Biopsy.
34. Thawer, H. A., Houghton, P. E., Woodbury, M. G., Keast, D., and Campbell, K. 2002. A comparison of computer-assisted and manual wound size measurement, Ostomy Wound Management, 48, 46-53
35. Tre-Hardy, M., Vanderbist F, Traore, H., and Devleeschouwer, M. J. 2008. In vitro activity of antibiotic combinations against *Pseudomonas aeruginosa* biofilm and planktonic cultures. Int. J. Antimicrob. Agents. 31:329-336.
36. Weiss, L., (ed.). 1988. Cell and tissue biology: a textbook of histology. 6th edn. Urban and Schwarzenberg. 115pp.
37. Wilcox, M. H., Finch, R. G., Smith, D. G. E., Williams, P., and Denyer, S. P. 1991. Effects of carbon dioxide and sub-lethal levels of antibiotics on adherence of coagulase-negative staphylococci to polystyrene and silicone rubber. J. Antimicrob. Chemother. 27:577–587.
38. Wrobel, J. S., Mayfield, J. A., and Reiber, G. E. 2001. Geographic variation of lower-extremity major amputation in individuals with and without diabetes in the Medicare population. Diabetes care. 24: 860-864.

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