Microbiological profiling of venous ulcers and predictors of poor outcome in venous ulcer infections

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Abstract

Background: Venous ulcer is the long-term resultant morbidity of chronic venous disease. While it is believed to be of non-infectious aetiology, unlike diabetic foot ulcers, wound healing can be delayed in case of colonizing bacteria, and polymicrobial flora can further hinder the process.

Aim -To identify the bacterial profile of infection in chronic venous ulcers and its significance in managing chronic venous ulcers.

Material and Methods: This prospective observational study included 150 patients with Chronic Venous Leg Ulcers (CVLU) who had undergone treatment at our institution. A wound swab for culture was taken from the venous ulcer for microbiological sampling. Antibiotics were prescribed asper the culture sensitivity of bacteria. Treatment protocols for managing all venous ulcers were four-layer compression bandages with weekly dressing changes. The endpoint was the healing of the ulcer. The statistical significance of the association of microbiological sampling in diagnosing and managing chronic venous ulcers and secondary objectives was assessed using the chi-square test with "Fisher's exact test".

Results: The mean size of the ulcer was 12.08 mm. Microbiological culture yielded growth of organisms in 137 (91%) patients, and of these, 78 (56.9%) were monomicrobial, and 59 (43.1%) were polymicrobial. There were 69 patients harbouring multi-drug resistant bacteria, which included Staphylococcus aureus (MRSA) (n=33) and Pseudomonas aeruginosa (n=36). Patients with evidence of infection showed a lower partial and complete response rate. Staphylococcus aureus, Pseudomonas spp (all species), and Klebsiella spp infections reduced early response rates compared to overall responses.

Conclusion: A positive culture from the wound decreases the chances of and prolongs wound healing time. Early and appropriate antibiotic therapy in culture-positive patients hastens the time of healing.

Keywords: Chronic venous insufficiency, Wound infection, Venous ulcer, Phlebology, Surgery

Key Messages: A positive culture from the wound decreases the chances of wound healing. Staphylococcus aureus, Pseudomonas spp and Klebsiella spp or multi-drug resistant organisms reduce the possibility of healing and increase the healing time. Early and appropriate antibiotic therapy in culture-positive patients hastens the time of healing.

Introduction

Venous disease, with its resultant chronic venous insufficiency, is common in our society and results in long-term morbidity for the patient. The end stage of this disease process is the venous ulcer[1,2,3,4]. According to the revised Clinical-Etiology-Anatomy-Pathophysiology (CEAP) classification of chronic venous disease (2004), a venous ulcer is defined as a full-thickness defect of the skin, most frequently involving the ankle region, that fails to heal spontaneously and is a result of chronic venous disease[5]. Various studies show impaired healing in venous ulcers is the effect of a background inflammatory process resulting in elevated proteases, including collagenases and matrix metalloproteases 2 and 9 (MMP-2 and 9) in the local tissue[6,7]. Since venous ulcers are of inflammatory origin due to stasis and ambulatory venous hypertension, these are usually treated with compression, elevation and surgery. Infection is not generally considered a significant

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factor affecting wound healing in venous ulcers. Chronic wounds of the lower extremities are more prone to microbial invasion, leading to complications like cellulitis, soft tissue infection, ascending infection, non-healing, and delayed healing[8]. Diabetic foot ulcers are commonly associated with infections. A broad spectrum of bacteria, including anaerobes, infects these wounds[9]. Several studies have been done on diabetic foot infections, which have shown that most mild infections are monomicrobial and are caused by *Staphylococcus aureus* and Streptococcus spp(all species). Severe infections are usually polymicrobial and caused by *Pseudomonas* spp. Escherichia coli, Klebsiella spp[10,11]. Treatment of the infective foci with antibiotics plays a significant role in healing diabetic ulcers. Though bacteria are isolated on the culture of a venous ulcer, they do not show any evident signs of local tissue damage like in a diabetic ulcer. There is little data on the microbiological profiling of infection in chronic venous ulcers and how these infections affect healing, especially in India. Though the mainstay of treatment for diabetic ulcers is antibiotics, antibiotics are rarely prescribed for venous ulcers. Most venous ulcers are believed to heal with adequate compression or early operative intervention.

Our study aims to identify the bacterial infection profile in chronic venous ulcers and its significance in managing them.

Materials and Methods:

This prospective observational study was done on a cohort of patients with Chronic Venous Leg Ulcers(CVLU) undergoing treatment at our tertiary referral centre institution between October 2017 and May2019. The study was approved by the institutional ethics committee (IEC-AIMS-2017-GENSSURG-380) and has been performed according to the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Based on the results from an earlier publication concerning our primary objectives observed in the existing literature, with 95% confidence and 20% allowable error, the minimum sample size was 135[12]. The study included all the patients with CEAP Category - 6 who presented to the Surgery department with chronic venous ulcers for evaluation and management. Patients diagnosed with ulcers due to arterial insufficiency, neuropathy, diabetes, tuberculosis, leprosy, trauma, decubitus, and skin ulcers due to cancer, radiation and vasculitis were excluded. The diagnosis was confirmed by complete clinical examination, handheld Doppler study, and Duplex studies of the superficial and deep venous system. A wedge biopsy for histological diagnosis was performed in ulcers persisting for more than six months to rule out a malignant change in the ulcer. The size of the ulcer was measured by taking an impression on a transparency sheet and then superimposing it over graph paper. A microbiological sample was taken in all the patients before any antibiotic was started. The wound was cleaned with saline to remove surface contaminants, causing conflicting reports of mixed surface bacterial flora. Slough and necrotic tissue were removed before sampling. A swab for culture was taken from viable tissue displaying signs of infection by rotating the swab or by Z-shaped motion. The swabs were sent to the Microbiology laboratory without any delay. Blood agar and MacConkey agar were used for culture. The culture plates were then incubated overnight at 35 degrees Celsius. Gram staining was also performed with the swabs received. The plates were examined for growth on the next day, and the growth was graded as heavy, medium and scanty based on the distribution of colonies along the primary inoculum and secondary and tertiary streak lines. Antibiotic susceptibility testing for the culture isolates was performed using Kirby Bauer's disc diffusion method. Early management of ulcers without clinical and haematological evidence of active infection was the application of a four-layer compression bandage, which was reapplied every five days, ensuring patient compliance. Patients who showed evidence of spreading cellulitis received empirical broad-spectrum antibiotic therapy till bacteriological culture result was available. Antibiotics were otherwise initiated based on antibiotic susceptibility reports. Repeat culture was done once in two weeks, during the third revisit of the patient for reapplication of 4-layer bandage, to confirm a sterile wound and to stop or continue antibiotics. The antibiotic was continued for 7 to 10 more days if the culture returned positive at two weeks and stopped if it was negative. The four-layer bandage application was continued till the ulcer healed. Ulcer size was measured during the revision of the four-layer bandage application. The observational study's endpoint was the ulcer's status after 90 days of management.

Definitions used:

Refractory ulcer - Ulcer that failed to heal after 90 days of preliminary treatment.

Initial response -25% or more decrease in size of ulcer at four weeks

Complete response - Healed before 90 days

Bacterial colonization of an ulcer refers to a positive wound culture with no clinical signs of infection.

Ulcer infection is considered in a patient with a positive culture and clinical signs and symptoms of infection – pus, redness, fever, swelling, and throbbing pain.

Multi-drug resistant organism (MDR)-Organisms having resistance to 3 or more antibiotics

Statistical details:

Statistical Analysis was performed using IBM SPSS for Windows version 20.0 (IBM Corp., Armonk, N.Y, U.S.A). Categorical variables were expressed using frequency and percentage, and numerical variables using mean and standard deviation. The statistical significance of the association of microbiological sampling in diagnosing and managing chronic venous ulcers and secondary objectives was assessed using the chi-square test with "Fisher's exact test". A log-rank test was applied to determine the survival time of infected and non-infected ulcers and the effect after giving antibiotics.

Results:

The study included 150 patients with a mean age of 57.93 years (SD: 12.93) and comprised 57% (n=86) of males. Age, gender and comorbidities did not significantly affect wound healing. (Table 1).

Table 1: Wound healing and demographics

Criteria		Healed (n=69)	Not healed (n=81)	P value
Mean Age		57.48±12.57	58.32±13.36	0.693
Gender	Male(n=86)	43 (62.3%)?	43 (53.1%)?	0.522
	Female(n=64)	26 (37.7%)?	38 (46.9%)?	
Diabetes	Positive	2 (2.9%)	2 (2.5%)	1
	Negative	67 (97.1%)	79 (97.5%)	

Table 2: Effect of culture positivity on wound healing

Groups	Total number	Early response	p value	Complete response	p value
Culture positive	137	67 (49%)	0.7318	58 (42%)	0.003
No growth	13	7 (54%)		11 (85%)	

The mean size of the ulcer was 12.08 mm (SD: 7.838; median - 12).

Culture yielded microbial growth in 137 (91%) patients, and the remaining 13 (8.7%) had no microbial growth (Table 2).

Table 3: Microbiological profile of the culture-positive venous ulcers

Groups	Total number (n=137)	Partial response	P value	Complete response	P value
Polymicrobial	59	25 (42%)	0.167	18 (31%)	0.019
Monomicrobial	78	42 (54%)		40 (51%)	

On analysing the bacterial profile of the 137 positive cultures, 78(56.9%) were monomicrobial, and 59(43.1%) were polymicrobial (Table 3).

There were a total of 206 bacterial species isolated from 137 positive cultures. The common isolates were Staphylococcus aureus (n=62; 30%), Pseudomonas aeruginosa (n=49; 23%), Streptococcus species (n=19; 9%), Klebsiella species (n=13; 6.1%) and Escherichia coli (n=13; 6.1%). MDR bacteria were found in 84 isolates from 69 patients. Among the significant MDRs, nine S.aureus isolates were MRSA, and 24% of the Pseudomonas spp were MDR. (Table 4).

Table 4: Distribution of bacterial organisms isolated from study population

Organism(n=206)	Number	MDR
Staphylococcus aureus	62 (30.1%)	29(46%)
Pseudomonas aeruginosa	49 (23%)	12 (24.5%)
Streptococcus species	19 (8.9%)	2(10.5%)
Klebsiella species	13 (6.1%)	4 (30.7%)
Escherichia coli	13 (6.1%)	13 (100%)
Enterobacter species	7 (3.2%)	3 (42.9%
Proteus species	6 (2.8%)	6 (100%)
Beta hemolytic streptococci	6 (2.8%)	2 (33%)
Enterococcus species	6 (2.8%)	2 (33%)
Coagulase negative Staphylococcus	5 (2.3%)	2 (40%)
Acinetobacter species	4 (1.8%)	2 (50%)
Citrobacter koseri	4 (1.8%)	1 (25%)
Providencia stuartii	2 (0.93%)	2 (100%)
Diphteroids	2 (0.93%)	2 (100%)
Aeromonas species	2 (0.93%)	nil
Shewanella algae	1 (0.47%)	nil
Serratia species	1 (0.47%)	nil
Myroides species	1 (0.47%)	1 (100%)
Morganella species	1 (0.47%)	1 (100%)
Achromobacter denitificans	1 (0.47%)	nil
Other Gram positive cocci	1 (0.47%)	nil

Infection of CVLU by *Staphylococcus*, *Klebsiella* and *Pseudomonas* resulted in a significant delay and often non-healing of these venous ulcers. *Staphylococcus aureus* was the most frequently isolated gram-positive organism (30.1%) (Table 4).

All Methicillin-resistant Staphylococcus Aureus (MRSA) were susceptible to vancomycin and linezolid, while MDR Pseudomonas spp were susceptible to meropenem and colistimethate sodium. A complete response to treatment was seen in 69 (46%) patients after 90 days.

A partial early response was seen in 74 patients after four weeks of management. Culture-negative patients had better and earlier healing. A patient with a culture-negative ulcer with early partial response had a higher complete response rate (Table 2). In the culture-positive patients, those growing monomicrobial isolates healed faster than polymicrobial isolates. (Table 3) Patients with evidence of infection showed a significantly lower rate of partial and complete response (Table 2 and 3). The early response of patients with polymicrobial infection did not differ from those with monomicrobial infection. However, polymicrobial infection negatively influenced the complete response rate despite no significant impact on early response (Table 3).The isolation of MDR bacteria from the ulcer significantly affected the healing of venous ulcers and delayed the healing (Table 5).

Table 5: Significance of culture positivity and wound healing

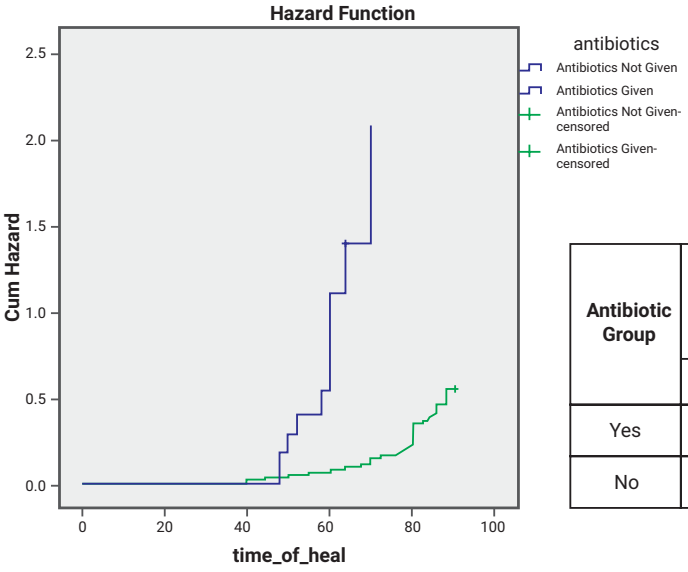
Parameter	Culture Positive (n=137)	Culture Negative (n=13)	Chi Square	pvalue	Significance
Decrease in size (n=74)	67 (90.54%)	7 (9.45%)	0.116	0.733	p>0.05
Same size (n=76)	70 (92.11%)	6 (7.89%)			
Healed (n=69)	58 (84.06%)	11 (15.94%)	8.514	0.007	p<0.05
Not healed (n= 81)	79 (97.53%)	2 (2.47%)			
Mean decrease in size after 4 weeks. Mean(cm2)±SD	22.08±25.7	47.9±25.8		0.001	p<0.05
Mean duration of healing in days)±SD	(n=58) 74.62±12.818	(n=11) 59.09±9.81		0.0001	<0.05
MDR in culture of patients with Healed wound (n=69)	21 (30.43%)		0.284	0.027	p<0.05
MDR in culture in patients with non-healing(n=81)	39 (48.15%)				

Table 6: Wound healing rates comparison for various bacteria isolated

Organism	Association of decrease in size of ulcer after 4 weeks				Healing rates			
	No change in size	Decrease in size	Chi-square p value	Significant	Not healed	Healed	p value	Significant
Staphylococcus (n=62)	44 (57.9%)	18 (24.3%)	0.0001	p value <0.05	54 (66.7%)	8 (11.6%)	0.0001	p value <0.05
Pseudomonas (n=49)	20 (26.3%)	29 (39.2%)	0.093	pvalue >0.05	20 (25.7%)	29 (42%)	0.024	
Klebsiella (n=13)	2 (2.6%)	11 (14.9%)	0.008	p value <0.05	4 (5%)	9 (13%)	0.0079	
Coagulase Negative Staphylococcus (n=5)	2 (2.63%)	3 (4.1%)	0.679	p value >0.05	4 (5%)	1 (1.4%)	0.375	p value >0.05
Enterobacter (n=7)	4 (5.3%)	3 (4.1%)	0.726		4 (5%)	3 (4.4%)	1	
Proteus (n=6)	3 (3.9%)	3 (4.1%)	1		1 (1.3%)	5 (7.2%)	0.095	
Streptococcus (n=19)	10 (13.2%)	9 (12.2%)	0.855		13 (16%)	6 (8.7%)	0.177	
Enterococcus (n=6)	2 (2.7%)	4 (14.9%)	0.681		3 (3.7%)	3 (4.3%)	0.641	
E coli (n=13)	8 (10.5%)	5 (6.8%)	0.421		8 (9.9%)	5 (7.2%)	0.568	
Aeromonas (n=2)	2 (2.6%)	0 (0%)	0.497		2 (2.5%)	0 (0%)	0.5	
Citrobacter (n=4)	2 (2.6%)	2 (2.7%)	1		3 (3.7%)	1 (1.4%)	0.625	
Providentia Stuartii (n=2)	2 (2.6%)	0 (0%)	0.16		2 (2.5%)	0 (0%)	0.5	
Acinetobacter Baumani (n=4)	2 (2.6%)	2 (2.7%)	1		2 (2.5%)	2 (2.9%)	1	
Schwanella Algae, Serratia, Myroide, Diphteroids, Morganella, Achromobacter denitrifican (1each)	1 (1.3%)	0 (0%)	0.322		1 (1.2%)	0 (0%)	1	
Beta haemolytic streptococci (n=6)	4 (5.3%)	2 (2.7%)	0.424		4 (5%)	2 (2.9%)	0.687	
Mixed (n=58)	33 (43.4%)	25 (33.8%)	0.226		40 (49.4%)	18 (22.8%)	0.004	p value <0.05

Staphylococcal infection significantly delayed the early response rate compared to the overall response (p< 0.0001). The rate of complete response rate was also delayed significantly in these patients (p< 0.001). Wounds infected with *Pseudomonas* spp showed a slightly better early partial response that did not attain statistical significance (p<0.093). However, these wounds had an inferior complete response rate, and the result was statistically significant (p<0.024). Klebsiella infection of the wound significantly lowered the early partial and complete responses (Table 6).

All the *E. coli* isolated in our study were MDR. Non-healing was present in 8/13 (61.5%) patients having *E. coli* isolates. However, this data did not achieve statistical significance. (Table 6) In our study, all the patients with a positive culture received antibiotics for ulcer healing. However, this was confounding since we could not prove the non-healing of ulcers in infected vs. non-infected venous ulcers. A hazard chart was prepared, showing that patients with non-infected wounds (60.417 ±3.099 days) healed better and faster than those with infected wounds (83.53±0.954 days). The addition of antibiotics improved healing rates in infected ulcers. However, the rate of healing was slower in such infected ulcers (figure 1)



Antibiotic Group	No of patients In the study (N=150)	No of patients healed after giving antibiotics (n=69)	Mean Duration of healing (Days)	95% Confidence		p value
				Lower	Upper	
Yes	**137**	58	83.53±0.954	81.667	85.405	<0.001
No	**13**	11	60.417±3.099	54.342	66.491	

Figure 1: Effect of antibiotics on time of healing

Discussion

Venous ulcers are the most common ulcers of the lower limb, resulting in very high morbidity, and are also an economic burden to the patient. Effect on Health-related quality of life (HRQL) is comparable to diabetes mellitus and cardiovascular disease [4].

Our study was in contradiction with many Western studies concerning the gender affected. Our study showed a male preponderance, with males constituting 57.3% of the study population[13,14,15,16,17]. In India, the demography of venous ulcers seems to be different with male preponderance. Studies by Kota AA et al. and Alamelu et al. from India confirm this fact [18,19]. We also found in our study that males had an earlier and better healing rate of venous ulcers even if the ulcer was infected. Diabetes in the patient had no significant effect in healing a venous ulcer in our study.

In their study, Nelson et al. found that most venous ulcers are inflammatory, while infection was attributable to a diabetic ulcer[20]. However, a large percentage of venous ulcers returned a positive culture. These ulcers had a decreased chance of healing (p<0.01), and the time taken for wound healing was prolonged (p< 0.0001).

Monomicrobial isolates constituted 56.9%, while 43.1% were polymicrobial in the venous ulcers of our patients. The bacterial flora found in non-healing ulcers changed with the age of the ulcer.

Staphylococci and streptococci were typically found in new ulcers, while gram-negative - -mixed flora were often found in old ulcers[18,19]. Conventional culture methods underestimate the occurrence of highly virulent organisms such as *Pseudomonas*. Our study had *Staphylococcus aureus* (29.1%) and *Pseudomonas* (23%) species as the most frequently isolated organisms. Enterobacteriaceae comprised21% of the isolates.

The role bacteria play in healing non-clinically infected venous ulcers needs to be better defined.

Though many *studies* have found no statistically significant relationship between individual bacterial

species and healing outcome, *Streptococcus agalactiae*, *Staphylococcus aureus*, *P aeruginosa*, coliforms and anaerobes have been associated with delayed healing in ulcers of larger areas in some studies[12,21,22]. In our series, we found that the Multi-drug resistance(MDR) of the bacteria cultured decreased the rate and possibility of ulcer healing($p<0.05$), similar to a study by Bowler et al[23].

Many authors accept that venous leg ulcers are colonized with multiple bacterial species without clinical signs of infection. However, the majority of studies have not measured healing outcomes. Hence, the relevance of bacterial infection in this context is uncertain. According to Davis,[12] ulcer size is a simpler parameter than bacterial burden or diversity to predict long-term healing. Though our study clearly shows delayed healing in infected venous ulcers, whether this is true is often masked by other confounders like age, gender and comorbidities. In our series, age and comorbidities like diabetes did not significantly affect healing rates, whereas male gender resulted in better healing.

The choice of antibiotic was determined by multidrug resistance, which was a statistically significant factor ($p<0.05$) to achieve healing status. MDR bacteria were isolated in 30.43% of patients with ulcer healing and 48.15% with non-healed ulcers. Almost half (46.7%) of Staphylococcus isolated were multi-drug resistant. Though 80.64% (50 out of 62) *S.aureus* were resistant to penicillin, only 14.5% (9 out of 62) were MRSA (Methicillin Resistant Staphylococcus aureus). MDR was seen in 24.5% of Pseudomonas isolated. Judicious selection of antibiotics is necessary and must be correlated with outcome.

Bowler and Davis et al. have reported that aerobic pathogens such as *S.aureus, P.aeruginosa*, Group A streptococci and *Klebsiella* spp were associated with significant infection and delayed healing[12,23]. In the present study, 29.1% of organisms isolated were *Staphylococcus aureus*, 23% Pseudomonas and Klebsiella 8.7%. In our study, Staphylococcus aureus, Pseudomonas, and Klebsiella increased the healing time with a significant p-value (<0.05).

When bacteria colonize a venous ulcer, significant infection occurs only when there is progression to a "Critical colonization"[24].There is no simple test that can differentiate colonization from infection. Bacterial early colonization of venous ulcers is not considered to affect healing adversely. Symptoms are also challenging to elicit due to lipodermato-sclerosis, erythema and pain, which may already exist[24]. Staphylococcus and streptococcus are commonly found in new ulcers, while gram-negative mixed flora is often found in old ulcers[21,22]. Early aggressive systemic antimicrobial therapy is justified in infections by aerobic pathogens like Group A β-haemolytic streptococci, Staphylococcus aureus, and Pseudomonas aeruginosa, as they tend to be associated with significant infection and delayed healing[23,25].Other Streptococci, Staphylococci and anaerobes may also be associated with infection clinically. Most other bacterial colonization of wounds is not considered to affect healing adversely. Topical antiseptics may benefit individual patients but are not routinely recommended in treating venous leg ulcers because frequent use leads to bacterial resistance. Systemic antibiotics are indicated in the presence of locally spreading cellulitis or other signs of infection only[26]. Assessment of bacterial flora provided by microbiologists should focus on the correct choice of antibiotics to reduce antibiotic resistance, health care cost and side effects of antibiotics[27].

Antibiotics may significantly reduce the healing time[10,11,23,27]. Our study shows a significant delay in healing for patients who received antibiotics, which seems contradictory (figure 1). Culture-negative patients who did not receive antibiotics healed faster. If a culture-positive patient had not received antibiotics, the ulcer may have taken longer or not healed. However, this fact could not be proved due to ethical considerations, as patients with a positive culture were routinely started on culture-sensitive antibiotics. Since the patients with a negative culture healed faster, it can be assumed that the healing rate will be hastened once the wound infection is controlled with culture-sensitive antibiotics. Inappropriate antibiotics may promote the growth of a resistant organism population in the venous ulcer. Tailoring antimicrobial therapy per the culture sensitivity report is advisable to improve patient outcomes.

Study of the role of biofilm in non-healing ulcers, molecular analysis of wound microflora and fungal analysis of wound microflora are the limitations of this study and avenues for future research.

Conclusion

A positive culture from the wound decreases the chances and prolongs the healing time. In the first four weeks, a positive culture significantly reduces the size of the ulcer. The presence of multi-drug-resistant organisms decreases the chance of healing. The presence of Staphylococcus aureus, Pseudomonas and Klebsiella increases the time of healing in the long term and the short term. Early antibiotic therapy in culture-positive patients hastens the time of healing. When there is a good reduction in ulcer size at four weeks, the ulcer is likely to heal, but if there is no

change in the first four weeks, the ulcer is unlikely to heal and requires additional modalities of treatment to ensure ulcer healing. Age and sex have no significant association with healing status. In a diabetic patient with a venous ulcer, there is no substantial change in ulcer healing.

Recommendations

Early cultures taken should determine the antibiotic regimen in patients with venous ulcers. If the ulcer continues to be nonhealing even after 4 weeks, advanced dressings should be considered since the wound is unlikely to heal with routine measures.

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