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Freshly Collected Amniotic Membrane as a Novel Cell Therapy for Treating Diabetic Non-Healing Ulcers

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Abstract

Introduction: Freshly collected amniotic membrane have been successfully used for treating diabetic foot ulcers (DFUs) since 1999. Freshly collected amniotic membrane is supposed to contain an array of growth factors, cytokines, and different types of potent cells that can participate in wound healing mechanism and act as a novel cell therapy model.

Method: Patients with confirmed diagnosis of diabetes, on oral anti-diabetic medication and insulin therapy presented with non-healing ulcers were enrolled in the study following the inclusion and exclusion criteria. Out of 9 patients, 4 patients (mean age 51 yrs, all male) were selected for the study. Human amniotic membrane was collected from healthy mothers undergoing caesarean section after their informed. Donor screening for ABO Rh blood grouping, and serological tests for HIV-I/II, Hepatitis B & C, VDRL, CMV and Syphilis were carried out before the collection of the amniotic membrane. After collection, the amniotic membranes were irrigated thoroughly with normal saline and any blood clots were removed manually. Screening of the patients included blood tests and checking for HIV-I/II, Hepatitis B & C, VDRL, CMV, malaria and toxoplasmosis. At baseline visit, all patients provided their informed consent. The wounds were thoroughly washed with normal saline, followed by the application of freshly collected human amniotic membrane (amnion or chorionic part). The amnion side was applied in ulcers where epithelialisation was required and the chorion side where vascularization was needed. Patient visit was scheduled after every 5 to 6 days and the interval between each visit increased after the first couple of months to 8 to 9 days approximately.

Results: No graft rejection was observed in any of the four patients in any of the visits. In the later part of the study, the duration of next patient visit was intentionally increased to observe any rejection or adverse events in patients. Patient follow up was successfully extended from 5-6 to 8-9 days approximately. At each visit, the wound size was measured which showed epithelialization, appearance of granulation tissue and reduction in the overall wound size. The wound region in all the four patients showed less discharge, lowering of pain, local oedema and induration. No adverse events like fever, inflammation, discharge or rejection were reported.

Conclusion: Freshly collected amniotic membrane can be an effective economical, biological dressing model for the treatment of diabetic non-healing ulcers demonstrating favourable clinical outcomes and good patient tolerance.

Keywords: Diabetic Foot Ulcer (DFU); Freshly Collected Human Amniotic Membrane

Introduction

The prevalence of diabetes in India is 8.7% among those aged 20 to 70, therefore making it an important healthcare concern as per a report in 2024. Among other comorbidities in diabetes, the most frequent one is diabetic foot ulcer (DFU) and chronic non-healing ulcers. It is estimated that 4.5% of diabetic people are affected with DFUs which is characterized by loss and degradation of tissue, ulceration, and infection [1]. The current standard of treating diabetic non healing ulcers and DFUs is debridement depending upon the extent of necrotic tissues followed by antibiotic and antimicrobial topical ointments including amputation in extreme cases such as gangrene. Maggot therapy is also followed in some parts of the world, along with hyperbaric oxygen therapy, negative pressure wound therapy, and moist dressing. With newer concepts in wound dressing models, application of dehydrated human amniotic membrane or dHAM is gaining popularity also. There has been till date three commercially available amnion or amnion and chorion products that have been used in randomized controlled trials which are Amnioexcel from Derma Sciences, EpiFix and Grafix of Mimdex and Osiris Therapeutics. Following dehydration, these dHAM were cryopreserved before its application in DFU patients [2].

However, there are several disadvantages of using dry and processed amniotic membrane as they are mainly used for covering the exudation and prevent the ulcer area from getting infected without any actual cell therapy-based support. The use of placental membranes to treat wounds has been in existence for the last 100 years [3]. Many previous studies have selectively shown that human amniotic membrane can be very effective in treating DFUs and diabetic wounds as it helps in rapid promotion of wound healing. One such study where PURION, a dehydrated processed human amnion and chorionic membrane was used to treat DFU has shown that it can stimulate adipose derived mesenchymal stem cells and growth factors which has shown to contribute towards healing [4].

Based on our previous understanding and contribution in using freshly collected properly screened amniotic membranes and amniotic fluids for treating chronic and non-healing ulcers in more than 100 patients at the backdrop of different clinical conditions has proved to be an effective cell therapy model and has shown that it might be an alternate wound healing treatment option to standard wound care model [5].

The primary objective of the study was to assess the safety of the freshly collected amniotic membrane application in a subset of diabetic patients with non-healing ulcers by looking into the wound reduction size from baseline studies along with any membrane rejection, infection and the physical characteristics of the wound like odour, presence of any exudate or discharge, and the appearance of granulation tissue and re-epithelialization. The secondary objective of the study was to assess the efficacy of the amniotic membrane dressing model, subjective pain index at every patient visit and the overall well-being of the patients.

The inclusion and exclusion criteria of the studies are mentioned as below

Inclusion criteria

- Diabetic patients confirmed with hyperglycaemia through blood test (confirmed through HbA1c ≥ 6.5 or fasting above 120 mg/dl or post prandial above 150 mg/dl) with evidence of non-healing ulcers for more than 1 month.
- Patients on Oral anti-diabetic therapy including Insulin.
- Diabetic foot ulcer patients or patients with lower limbs, upper limbs, venous ulcers, facial and body ulcers are eligible for this study.
- All patients screened negative for HIV-1, 2, Hepatitis-B, C, VDRL, Toxoplasmosis, Herpes infection, renal and lipid profile, thyroid profile, hepatic profile, CBC, blood sugar and autoimmune disorders.
- Age group anywhere between 12 years to 70 years including both male and female

Exclusion criteria

- Non adherence to study regulations.
- Drinking, smoking or any substance abuse.
- Comatose patients, Tuberculosis, HIV and any other immune compromised patients.

Study Methods

Materials and methods

Initial study was conducted at the OPD of several public hospitals in Kolkata. Patients were recruited after referred to the Department of Regenerative Medicine and Translational Science, Vidyasagar State General Hospital, Behala and MR Bangur Hospital outpatient ward as per the government order. Out of 9 patients, four patients were recruited for the current case series after satisfying all the inclusion/exclusion criteria. All patients gave their informed consent before enrolling for the treatment. Diabetic patients having chronic non-healing ulcers were treated with the application of freshly collected and screened amniotic membrane. The studies were conducted after obtaining necessary ethics committee clearance. Records and details of the individual patient will be kept in the OPD facility of each hospital respectively for further reference and research related work. All patients were screened for HIV-I & II, Hepatitis "B" & "C", VDRL, toxoplasmosis, herpes infection, &, hepatic profile, profile for autoimmune diseases, TC, DC, ESR, haemoglobin, blood sugar; of both donor and recipient.

Selection of donor

Potential donors or mothers were identified at the Obstetrics and Gynaecology Department of designated hospitals. Along with demographic details, the medical histories were also collected from the potential donors and were screened for HIV-I/II, Hepatitis B & C, VDRL, CMV and Syphilis including ABO Rh blood grouping. Women with uncomplicated pregnancies, absence of clinical chorioamnionitis, and no signs of obstetric or systemic infection were considered eligible. Donors with a history of malignancy, severe pre-eclampsia, gestational infection, hepatitis, HIV, or other transmissible diseases were excluded. Amniotic membrane was collected only from healthy mothers who underwent elective caesarean section (LSCS) at full-term gestation (≥ 37 weeks) under aseptic environment in the O.T. All eligible mothers were counselled before the collection of amniotic membrane and everyone provided their informed consent.

Application of amniotic membrane

Patients were selected based on the inclusion and exclusion criteria. Patients with chronic non-healing lower-limb ulcers associated with Type 2 diabetes mellitus (HBbA1c confirmed through screening ≥ 6,5) and individuals ≥18 years with ulcers persisting for more than four weeks showing no significant improvement with standard care were eligible for the study. The ulcer gradation was carried out following the European Pressure Ulcer Advisory Panel (EPUAP), National Pressure Injury Advisory Panel (NPUAP) Classification NPUAP/EPUAP from Grade I to Grade IV. All the four patients had Grade IV ulcers as per the NPUAP/EPUAP gradation scale. From baseline, all four patients were on anti-diabetic therapy and injectable antibiotic therapy (See the details in the chart below).

After the collection, the amniotic membrane was taken to the surgery unit for application in patients in a closed sterile container. The amniotic membrane was washed thoroughly with normal saline so as to remove any blood clots. All the four patients provided their informed consent before the application of the amniotic membrane. The collection and application of the amniotic membrane was conducted after receiving the necessary permission from the institutional ethics committee (IEC Ref. No: CREC-STM/411 dated on 21.12.2017). Wounds were first thoroughly washed with normal saline to remove debris, foreign particles, and other contaminants. Based on the nature of the wound, the amniotic membrane was separated into the translucent amnion and coarse chorionic layer through an incision. The amnion side was applied in superficial ulcers for epithelialisation and the chorionic side was applied where vascularisation was required. Accordingly, the amnion and the chorion were cut as per wound size and shape under aseptic conditions and the wound bed was covered up to the skin margin. Sterile gauzes were applied over the whole wound surface to cover the wound appropriately along with sterile bandage. After dressing with amniotic membrane, next visit was scheduled on the 5th to 7th day. In case of any foul discharge from the wound, patients were asked to contact the research team immediately. Redressing is done with normal saline and amniotic membrane and the same procedure was followed in the subsequent patient visits. Weekly dressing with amniotic membrane was carried out successively for the next 4 months, keeping an account of the wound size, reduced signs of inflammation like redness, discharge, pain, and local edema, or induration if present. All patients were on oral anti-diabetic therapy and antibiotics were continued till the patient visit no.3. All clinical assessment outcomes were evaluated by independent evaluators.

Method of evaluation of treatment benefit and follow-up studies

Patients were evaluated by weekly monitoring of size and depth of ulcer, signs of reduction in inflammation, epithelialisation, appearance of granulation tissue, systemic effect-like sense of well-

being, weight gain, improvements inco-morbidities and odour. At each weekly visit, a gross wound size of the patients was measured by surface area, expressed in centimetre. Based on the gross wound measurements and their characteristics, the wound was graded following the NPUAP/EPUAP classification. Photographs were taken to assess the gross nature of the wound healing at each patient visit. If, after 4 months no improvement was documented, then re-evaluation of patient by vascular surgeon, dermatologist, diabetologist, and other experts were done and ulcer was re-evaluated and reschedule of program is fixed. Follow up of the patients were conducted to assess the short-term healing of wound.

Case I

A 46-year-old male daily wage worker was admitted on 28th March 2022 with cellulitis and a DFU in the setting of uncontrolled diabetes and poor compliance with medications. He reported recurrent severe pain, up to 9/10. Laboratory findings showed anaemia (Hb 9.5-10 g/dl), leukocytosis (WBC 13,100/mm^3, neutrophils 86%), ESR 46 mm/hr, and persistently elevated blood glucose (FBS 166-316 mg/dl; PPBS 330-400 mg/dl). Management included broad-spectrum antibiotics (Ceftriaxone, Amoxyclav, Meropenem, Linezolid), insulin (Glargine and sliding scale, Janumet XR), Chymoral Forte, and analgesics. The ulcer, initially 11 × 8 × 2 cm, was stage III with necrotic slough, macerated edges, scant exudate, moderate odour, and pitting edema. A total of 11 amniotic membrane dressings were performed between April 13 and 21 June, 2022. By mid-May, wounds showed progressive granulation, epithelialization, and reduction in odour. In the follow-up study by 03 July, 2022 the ulcer size had reduced to 9.8 × 5.7 × 1.4 cm with healthy red-pink granulation tissue, no exudate, and pain decreased to 3/10. Healing was steady under amniotic membrane therapy and glycaemic control, and surgical intervention was avoided.

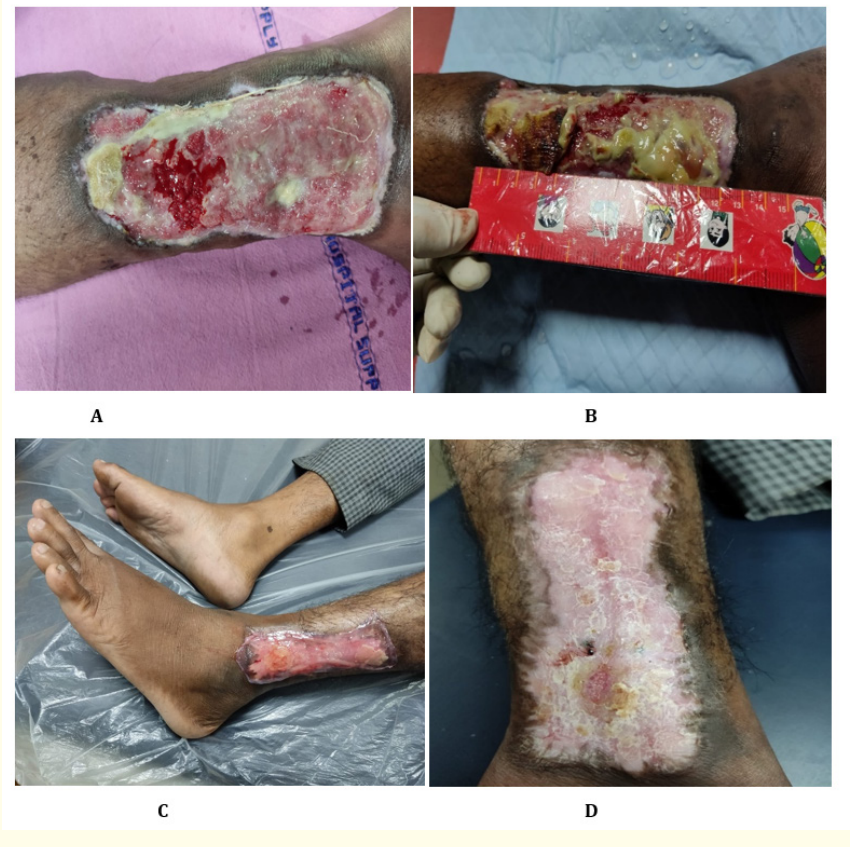

Figure 1: A. Dressing of the Diabetic ulcer with amniotic membrane on April 13, 2022 at baseline. B. Shows subsequent dressing of the diabetic wound and its measurement. C. Corresponds to the dressing of the diabetic wound after 2 months and D. Shows the appearance of epithelialization and the rate of healing.

Table 1: Treatment and Improvement Table.

Date	Treatment/Medications	Wound Size (l × b × depth) cm	Wound Base and Progress	Odor	Pain (0-10)	Stage
13-30 Apr 2022	1st-4th Amniotic Membrane dressings + injectable antibiotics (Ceftriaxone, Amoxyclav, Linezolid, Diclofenac, Pantoprazole) + Insulin (sliding scale)	11 × 8 × 2	Ulcer with slough, moderate exudate, foul odour; early improvement with dressings	Foul	9	IV
07 May 2022	5th AM dressing + PIPZO, Paracetamol, Chymoral Forte, Glargine 14 units + Insulin SS	11 × 7.9 × 2	Stage III ulcer with slough, moderate odour, slight improvement	Moderate	9	
08-12 May 2022	6th-7th AM dressings + antibiotics continued + Insulin	11 × 7.4 × 2	Granulation tissue present; moderate odour; slight improvement	Moderate	7	
19 May 2022	8th AM dressing + Glargine HS, Insulin 14-14-14	11 × 6.8 × 1.8	Granulation + slough, contractures forming; slight improvement	Moderate	7	
28 May 2022	9th AM dressing + Linezolid 600, Insulin regimen continued	10.6 × 6.8 × 1.7	Granulation + epithelialization, edges regular; improvement	Moderate	7	
09 June 2022	10th AM dressing + ongoing medications	10 × 6 × 1.8	Continued improvement with granulation tissue	Moderate	6	
21 June 2022	11th AM dressing + ongoing medications	9.8 × 6 × 1.5	Healthy granulation + epithelialization; no odour	None	4	
03 July 2022	Follow up study	9.8 × 5.7 × 1.4	Epithelization visible with no odour	None	3	

AM: Amniotic Membrane: PPZIO: Tazaobactam.

Case Study II

A 45-year-old male was admitted with A diabetic ulcer on the right leg. He had uncontrolled diabetes for 5 years with poor compliance to oral hypoglycaemics. On admission, fasting glucose was 428 mg/dl and postprandial 642 mg/dl. Laboratory investigations showed anaemia (Hb 9.5 g/dl), leukocytosis (19,160/mm^3), neutrophilia (88%), ESR 56 mm/hr, and mild renal impairment (creatinine 1.5 mg/dl). Management included intravenous Linezolid, Meropenem, insulin (sliding scale and Glargine), Chymoral Forte, and analgesics. The right foot ulcer below the great toe measured 4×6×3 cm, Stage IV, with necrotic tissue, eschar, macerated surrounding skin, moderate sanguineous exudate, and foul odour. Pain was mild (2-3/10) with probable diabetic neuropathy. Amniotic membrane therapy was initiated on 10th June, 2024 and continued till the month of August, 2024. Early granulation tissue was observed after two applications, though necrotic tissue persisted around the edges of the wound bed.

Table 2: Treatment and Improvement Table.

Date	Treatment/Medications	Wound Size (l × b × depth) cm	Wound Base & Progress	Odor	Pain (0-10)	Stage
16-31 May 2022	Initial management with Linezolid, Meropenem, Voveran, Pan 40, P650, Chymoral Forte; Insulin sliding scale; Glargine (12-14 units HS); Soluble insulin 12-12-12, later 14-14-14; Rantac; Tab Linezolid 600 BD	4.3 × 6.5 × 3.1	Cellulitis left leg and gangrenous diabetic foot ulcer; necrotic tissue with eschar, slough, and macerated wound edges.	Moderate	2	IV
10 June 2022	1st Amniotic Membrane dressing + ongoing medications	4 × 6 × 3	Necrotic tissue with thick black eschar, yellow slough, and some granulation tissue. Sanguineous wound bed. Moist with moderate exudate.	Moderate	2	
19 June 2022	2nd Amniotic Membrane dressing + continued antibiotics and insulin regimen	4 × 6 × 3	Similar wound dimensions; moderate necrotic tissue persists with eschar and slough, but slight granulation increase noted. Slight improvement observed.	Moderate	3	
28 June 2022	3rd Amniotic Membrane dressing + continued antibiotics and insulin regimen	4 × 4.5 × 2	Healthy granulation + epithelialization; no odour	None	2	
8 Jul 2022	4th Amniotic Membrane dressing + continued antibiotics and insulin regimen	3.8 × 4.3 × 2	Improvement was observed with initiation of re epithelization	None	2	
22 Jul 2022	5th Amniotic Membrane dressing + continued antibiotics and insulin regimen	3.6 × 4.3 × 2	Epithelization was visible with no discharge and infection	None	3	
02 August 2022	Follow Up	3.4 × 4.0 × 1.7	Wound bed was dry with visible formation of epithelization	None	2	

NA: Not Applicable.

Figure 2: A. Show the application of amniotic membrane application in patient no.2 at baseline or patient visit no.1. B. Shows the measurement of the wound bed in subsequent dressing and C. Shows the epithelialization after three months of amniotic membrane dressing with some necrotic tissues at the edges.

Case Study III

A 45-year-old male daily wage worker presented with maggot-infested ulcers and cellulitis of the left leg following untreated abrasions, progressing to early gangrene. On admission the patient was reported to be diabetic (Fasting blood glucose was 210 mg/dl) and reported episodic pain ranging from 3-8/10. Laboratory results showed anaemia (Hb 7.7 g/dl), normal WBC count (7,800/mm^3), ESR 28 mm/hr, and normal renal parameters. Treatment included antibiotics (Ceftriaxone, Amikacin, later Levofloxacin), analgesics, oral antidiabetic therapy (Metformin) paracetamol, and 12 serial amniotic membrane dressings between April 8 and June 25, 2022. Initially the ulcer (7×3×0.6 cm) were Stage IV with necrotic slough, serosanguineous discharge, and moderate odour. Over successive dressings, wounds showed granulation, epithelialization, and contraction, with odour resolution and pain reduction to 4/10. By late May, ulcers demonstrated healthy red-pink granulation tissue, absence of exudate, and stable improvement, avoiding surgical intervention or amputation.

Table 3: Treatment and Improvement Table.

Date	Treatment/Medications	Wound Size (l × b × depth) cm	Wound Base and Progress	Odor	Pain (0-10)	Stage
08-30 Apr 2022	1st-5th AM dressings + injectable antibiotics (Ceftriaxone, Amikacin, Diclofenac, Ranitidine, Paracetamol) + Oral anti diabetic therapy (Metformin)	Approx. 7×3×0.6	Early granulation and epithelialization; wounds dry, serosanguineous drainage	Moderate	8	IV
07 May 2022	6th & 7th AM + switched to Levofloxacin, B-complex + Metformin	Approx 6×4×0.3	Dry medium wounds, serosanguineous with moderate odour; firmly adherent base	Moderate	8	
14 May 2022	8th AM dressing + Metformin	6×4×0.1	Wounds improving, firm granulation; dry; moderate odour	Moderate	7	
22 May 2022	9th AM dressing + Metformin	6×4 (depth N/A)	Granulation and epithelialization; wounds dry with scant exudates, contractures forming	Moderate	7	
02 June 2022	10th AM dressing + Metformin	6×4 (depth N/A)	Red/pink tissue, regular edges, no exudates, improving	None	5	
14 June 2022	11th AM dressing + Metformin	5.8 × 3.6 (depth N/A)	Continued improvement	None	4	
25 June 2022	12th AM dressing + Metformin	5.6 × 3.3 (depth N/A)	Healthy red-pink tissue, contractures, no exudates, improving	None	4	
11th July 2022	Follow up studies (Oral anti-diabetes continued)	5.3 × 3.5 (depth N/A)	Healthy red and pink tissue characteristics of granulation and re-epithelization	None	2	

AM: Amniotic membrane; N/A: Not applicable as over the course of treatment the depth dimension became negligible for both the ulcers.

Figure 3: A. Show the first amniotic membrane dressing of the patient at baseline or patient visit no.1, B. Amniotic membrane dressing of the patient in the month of May, 2022 with the disappearance of the wound bed C. Reepithelialization and presence of some granulation tissue at the base and D. Complete healing of the wound with some necrotic tissue.

Case IV

A 68-year-old male with a history of diabetes on oral antidiabetic drugs, was admitted on 17th May 2022 with ulcer cellulitis of the right hand caused by continuous shoe friction leading to pressure ulcers on the little finger, complicated by discharge and moderate odour. The wounds, persistent since November 2021, had not responded to routine dressings. Laboratory tests showed Hb 11.9 g/dl, WBC 7,500/mm^3, neutrophils 65%, ESR 48 mm/hr, and postprandial glucose 212 mg/dl, with normal renal function. Initial treatment included injectable Ceftriaxone, Metrogyl, Paracetamol 1 g, and insulin Glargine 10 units HS. The first amniotic membrane dressing (AMD) was applied on 27th May, and continued till August 2022. On 27th May, the wound measured 8 × 7 × 2 cm, Stage IV, with irregular borders, macerated tissue, medium exudate, serosanguineous base, thin slough, moderate odour, and chronic pain rated 8/10. By the final amniotic membrane dressing on 08 Aug, 2022, the wound size reduced to 5.7 × 3.8 × 1.1 cm and pain improved to 2/10. In the follow up study insulin therapy, and oral antibiotics were recommended.

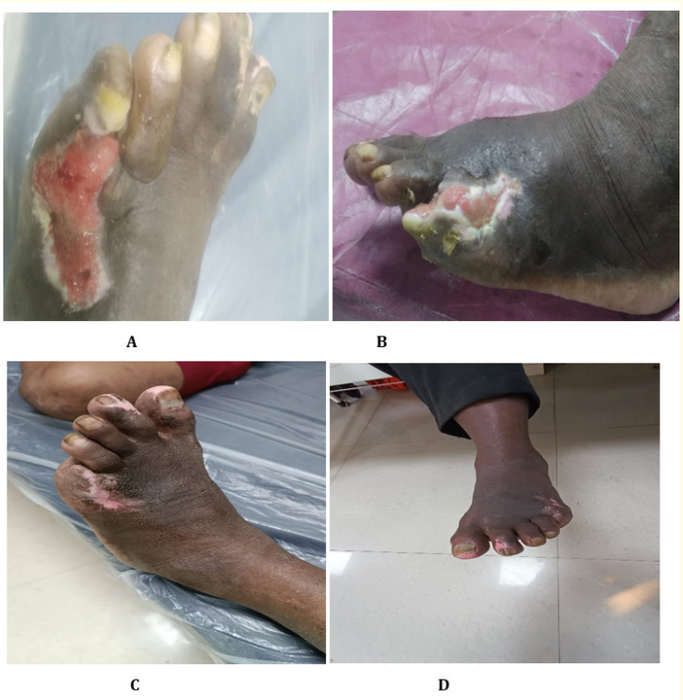

Figure 4: A. Dressing of the diabetic ulcer with amniotic membrane on April 27, 2022 at baseline or patient visit no.1. B. Diabetic wound and appearance of granulation tissue after one month dressing. C. Corresponds to the dressing of the diabetic wound after 2 months and shoes wound contracture along with re-epithelization, D. Shows the appearance of epithelial cells and a marked improvement in the epithelization and healing.

Table 4: Treatment and Improvement Table.

Date	Treatment/Medications	Wound Size (l × b × depth) cm	Wound Base and Progress	Odour	Pain (0-10)	Stage
27 May 2022	1st Amniotic membrane dressing + Injectable Ceftriaxone, Inj. Metrogyl, Inj. PCM 1 gm, Glargine 10 units HS	8 × 7 × 2	Large moist wound with slough; serosanguineous base; epithelialization islands forming; macerated/excoriated tissue	Moderate	8	IV
03 June 2022	2nd Amniotic membrane dressing + insulin and injectable antibiotics continued	8 × 6 × 1.5	Improving; beefy red granulation tissue with epithelialization; reduced necrotic slough; moist wound, regular edges	None	6	
08 June 2022	3rd Amniotic membrane dressing + insulin and injectable antibiotics continued	7.7 × 5.5 × 1.8	Improving; beefy red granulation tissue with epithelialization; reduced necrotic slough; moist wound, regular edges	Moderate	7	
16 June 2022	4th Amniotic membrane dressing + insulin and injectable antibiotics continued	7.9 × 5.5 × 1.5	Appearance of granulation tissue with epithelization	None	6	
26 June 2022	5th Amniotic membrane dressing + insulin and oral antibiotics continued	7.5 × 5.2 × 1.3	Appearance of granulation tissue with epithelization	None	6	
04 July 2022	6th Amniotic membrane dressing + insulin and oral antibiotics continued	6.8 × 4.8 × 1.1	Epithelization with no discharge and odour. Wound contracture started	None	5	
11 July 2022	7th Amniotic membrane dressing + insulin and oral antibiotics continued	6.2 × 4.6 × 1.2	Presence of Epithelization with no discharge and odour and contracture of the wound.	None	4	
19 Jul 2022	8th Amniotic membrane dressing + insulin and oral antibiotics continued	6.7 × 4.1 × 1.0	Presence of Epithelization with no discharge and odour and contracture of the wound.	None	5	
30 Jul 2022	9th Amniotic membrane dressing + insulin and oral antibiotics continued	6 × 4 × 1.1	Presence of Epithelization with no discharge and odour and contracture of the wound.	None	4	
08 Aug 2022	10th Amniotic membrane dressing + insulin and oral antibiotics continued	5.7 × 3.8 × 1.1	Large scale epithelization with no open wound.	None	2	
20 Aug 2022	Follow-up (Insulin + oral antibiotics)	5.3 × 3.4 × 1.0	The patient could walk freely and normally again. On inspection of the foot, almost complete epithelization was observed.	None	2	

Table 5: Summarizing Patient Demographics, Ulcer Details, Treatment Duration, And Outcomes.

Ulcer gradation and baseline characteristics					Baseline (n = 4)						After 4 months of amniotic membrane application (n = 4)					
Ulcer gradation (Based on NPUAP/ EPUAP)	**S. No.**	**Sex 7 age**	**Wound at initial visits (l × b × depth) cm)**	**Wound size after 4 months (l × b × depth) cm)**	**Nature/colour of the wound**	**Presence of exudate/ Drainage (Yes/No)**	**Wound base**	**Wound edges**	**Surrounding tissue health**	**Pain Index**	**Nature/ colour of the wound**	**Presence of exudate/ Drainage (Yes/ No)**	**Wound base**	**Wound edges**	**Surrounding tissue health**	**Pain Index**
IV	1	M	11 × 8 × 2	9.8 × 5.7 × 1.4	Full-thickness loss, bone/muscle exposed, slough/eschar present	Moderate serosanguineous or purulent	Ulcer with slough, moderate exudate, foul odour; early improvement with dressings	Often undermined, irregular	Indurated, inflamed	9	Global wound contracture observed	Mild serous	Epithelization visible with no odour	Moderately defined	Mildly inflammed	3
IV	2	M	4.3 × 6.5 × 3.1	3.4 × 4.0 × 1.7	Full-thickness loss, bone/muscle exposed, slough/eschar present	Moderate to large, serosanguineous or purulent	Cellulitis left leg and gangrenous diabetic foot ulcer; necrotic tissue with eschar, slough, and macerated wound edges.	Sometimes undermined, irregular	Inflamed, with atrophic around wound	2	Presence of global large wound contracture with no eschar	Mild serous	Wound bed was dry with visible formation of epithelization	Sometimes undermined, and more regular	Mild inflammation	2
IV	3	M	Approx. 7×3×0.6	5.3 × 3.5 (depth N/A)	Chronic non-healing ulcer with risk of infection	Large, serosanguineous or purulent	Early granulation and epithelialization; wounds dry, serosanguineous drainage	Irregular, may be attached or undermined	Erythema and inflammed	8	Global wound contracture observed	Scant serous	Healthy red and pink tissue characteristics of granulation and re-epithelization	Sometimes undermined, mostly defined	Mildly inflammed	2
IV	4	M	8 × 7 × 2	5.3 × 3.4 × 1.0	Ulcers due to injury and cuts	Moderate to large, serosanguineous or purulent	Large moist wound with slough; serosanguineous base; epithelialization islands forming; macerated/ excoriated tissue	Undermined at some areas, irregular	Erythema, Indurated and inflamed	8	Wound contracture with dry pink wound bed and white areas	Scant serous	The patient could walk freely and normally again. On inspection of the foot, almost complete epithelization was observed.	Undermined at some areas, well defined and mostly regular	Mildly inflammed	2

Discussion

The patient-specific outcomes in this case series demonstrated varied yet consistently positive responses to amniotic membrane dressings. Patient 1, who presented with a large stage IV ulcer (11 × 8 × 2 cm), showed progressive contraction and epithelialization after eleven amniotic membrane applications, with pain improving from 9/10 to 3/10 and the ulcer stage improving at follow-up. Whereas patient 2, with a Stage IV gangrenous ulcer (~4 × 6 × 3 cm), received six amniotic membrane applications that reduced necrotic tissue and promoted granulation, resulting in a wound size reduction to ~3.4 × 4 × 1.7 cm and stable pain scores of 2-3/10, indicating wound stabilization in a severe lesion. Patient 3 reported a stage IV ulcer (7 × 3 × 0.6 cm), demonstrating the most rapid improvement, with early epithelialization, contracture formation, and a reduction in pain from 8/10 to 4/10, highlighting the efficacy of amniotic membrane in superficial ulcers. Patient 4, with a large Stage IV necrotic ulcer (8 × 7 × 2 cm), exhibited slower healing, but repeated amniotic membrane applications reduced the wound to 5.3 × 3.4 × 1 cm, decreased pain from 8/10 to 5/10, and facilitated granulation and epithelialization, contributing to ulcer stabilization and tissue regeneration.

The rate of healing among the four patients differed, as the wound size, infection status, clinical background, and the grade of the ulcer were quite different. However, from the data points provided above for each patient, it was evident that all the Stage IV ulcers demonstrated a rapid wound closure. However, in terms of overall improvement, all four patients showcased measurable improvement and pain reduction with amniotic membrane therapy.

The use of amniotic membrane dressings in this case series was consistently associated with progressive wound healing, reduction in pain, and improvement in the wound healing. In all the four patients, it is believed that the amniotic membrane served as a biological scaffold rich in growth factors and cytokines. Furthermore, the rate of epithelialization observed in all four patients supports the notion that amniotic membrane promotes epithelial migration due to the presence of human amniotic membrane epithelial cells (hAESCs), and also supports angiogenesis, and fibroblast proliferation, while simultaneously reducing inflammation and bacterial burden. This multifaceted action created a favourable wound environment, even in cases with deep necrosis or infection.

Also, amniotic membrane can help modulate the chronic inflammatory process by controlling the pro- and anti-inflammatory cytokines during the various stages of wound healing. During the course of treatment, none of the patients reported any infections or rejection indicating the suggested antimicrobial properties of the amniotic membrane. These outcomes reinforce amniotic membrane's utility as both a healing accelerator in superficial ulcers and a stabilizing agent in advanced, complex wounds.

The clinical application of amniotic membrane has evolved from its early ophthalmic use to widespread adoption in chronic wound management, including DFUs, largely facilitated by advances in cryopreservation and dehydration technologies [6-9]. Both cryopreserved and dehydrated amniotic membranes have demonstrated effectiveness in promoting epithelialization, reducing inflammation, and accelerating wound closure in chronic ulcers. However, these processing methods may compromise the structural integrity of the membrane and reduce the bioavailability of growth factors, cytokines, and extracellular matrix proteins essential for optimal tissue regeneration [10]. Dehydrated membranes, while advantageous for long-term storage and logistical convenience, may exhibit further reductions in biological activity compared to cryopreserved products due to more extensive processing.

In contrast, pioneering studies by Bhattacharya and colleagues have reintroduced the use of freshly collected amniotic membrane in chronic wound care, demonstrating its potential to retain higher levels of viable bioactive components, including endogenous stem cells, antimicrobial peptides, and native growth factors [5,11,12]. This enhanced biological profile may result in superior immunomodulatory and regenerative effects when compared with processed membranes. Although these studies remain limited in scale, they provide important insights into the therapeutic superiority of fresh amniotic membrane and highlight the need for large-scale, controlled comparative trials to establish its clinical efficacy, safety, and scalability alongside cryopreserved and dehydrated alternatives in diabetic ulcer management and regenerative medicine.

The study had few limitations. The case series had a small sample size (n = 4) and therefore it substantially restricted the generalizability of the findings. Also, there was a marginally high

variation in the ulcer size and the gradation group between the different ulcers. The study reported a short-term follow-up, as long-term follow-up studies would have given a more definite idea and picture of the rate of healing. Further evaluation, in the form of histopathology studies and cytokine studies, can confirm the findings more definitively. A larger randomized controlled trial would validate these findings, helping to establish an effective protocol for future studies and wound healing practices.

Conclusion

The current case series highlights that freshly collected and properly screened amniotic membranes can serve as a therapeutic purpose for the treatment of chronic non-healing diabetic wounds. All the four patients showed improvement in their clinical outcome with reduction in wound size, appearance of granulation tissue, pain reduction and the absence of graft rejection or infection as evident from the follow-up studies. The case series also shows similar results as per the previous reports of Bhattacharya., *et al*. in 1999, where more than 100 patients did not reported any adverse events in the follow-up studies [5]. However, further studies like histopathology and the role of pro- and anti-inflammatory cytokines are required to understand the in-depth mechanisms involved in healing of wounds after the application of human amniotic membrane in diabetic non-healing ulcers. Also, the sample size of the current case series was restricted to only four patients and therefore the results should be interpreted with caution as they might not truly represent the broader patient population. Further, the results of the above study are preliminary and there is a requirement for larger, randomized controlled studies to confirm the safety and effectiveness of freshly collected amniotic membranes before it can be used as a standard therapy for the treatment of DFUs and various chronic wounds.

Conflict of Interest

The author declares no conflict of interest in this work and supports the Calcutta School of Tropical Medicine for this work.

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