Efficacy of Freshly Collected Amniotic Membrane Local Application in Wound Management.

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Abstract: Wound management is most important and challenging job in medical field as we know there are regulatory guidelines for wound management. Wound has two major type’s acute and chronic. Acute wound subdivided into post-operative wound, abrasion, contusion, superficial burn which heal with conventional line of treatment. We are concerned about non-healing wound. Chronic wound is defined as the wound which is more than 3 months old and not responding to treatment. The causes for chronic wound are numerous, underlining chronic infective diseases, nutritional deprivation, and arterial or venous blood impermanent, post radiation injury, immunocompromised status of patient, diabetes. Chronic wound not respond always to conventional line of treatment so what option we have? As we know numerous new strategies are employed to manage wound. Amongst them are hyperbaric oxygen therapy, electric stimulation of skin, maggots’ therapy, leech therapy, vacuum. Science based on three important pillars 1. Pharma 2. Biology 3. Medical devices and equipment’s. The cell therapy including stem cell is most important fourth pillar. In day to day practice division are engage into finding new molecules to combat bacteria, viruses, fungi. To implement one new molecule required billion of dollar irrespective to short life of that molecule. Stem cell are most important therapeutic approach for wound management including acute as well as chronic wound. Globally conducted clinical trials shows new hope to new therapeutic approach based on stem cell.

In this article, we are working on using pregnancy specific biological substances—placenta, amniotic membrane, Wharton’s jelly, umbilical split cord as local dressing for nonhealing ulcer irrespective underlining causes of diseases. Under guidance of Dr. Niranjan Bhattacharya who coined term pregnancy specific biological substances. He published book pregnancy specific biological substances in international journals so I will carried out this terminology further with underlining his reference. Also bone marrow derived MSC, adipose tissue derived MSC are used for local application, systemic transplantation, and infiltration surrounding wound edges. Stem cell work on two principle which are regeneration and repair. As per Paul Martin et al. We are using freshly collected amniotic membrane after screening for HIV 1 & 2, Hepatitis B & C, VDRL, cytomegalovirus & Donor consent, hospital ethical committee approval. Our research project going on in government hospital, Kolkata, India

Keywords: non-healing wound, regenerative medicine, stem cell, Amniotic membrane.

METHODS AND MATERIALS:

We are using pregnancy specific biological substances (placenta, amniotic membrane, amniotic fluid, Wharton’s jelly, and umbilical vein) for biological dressing of nonhealing wound as well as for acute wound as local application. The research granted by West Bengal health department, Kolkata, India in various government hospitals in Kolkata. We enrol indoor as well as outdoor patient under strict guidance of centenary professor. Dr. Andrew Burd and HOD Dr. Niranjan Bhattacharya both belongs to school of tropical medicine, Kolkata, India. Consent should be taken before caesarean section, explaining her in native language which she understand, audio-visual consent and written consent taken but before selecting donor, detailed strict prehistory of congenital diseases, personal history of addiction, family history taken to ruled out congenital, genetic diseases. Meconium stain, preeclampsia and eclampsia, prematurity conditions are excluded and the pregnancy specific biological substances from these LSCS not taken. Freshly collected amniotic membrane, amniotic fluid, placenta used for biological dressing of wound. After collection of placenta placental blood screened for HIV 1 & 2, Hepatitis B & C, VDRL, CMV, negative blood for these tests are selected. Placenta with amniotic membrane is collected in perfectly sterilised tray
and deliver to operation theatre with following aseptic technique. Patient also investigated for routinely for HIV 1 &2,hepatitis B & C,VDRL.bedside examination of patient is carried out before applying placenta to ruled out underlining cause of nonhealing wound as well as chronic diseases, detail family ,personal history taken. Local examination carried out size of ulcer, shape, edges, anatomic location, type, cause, duration are noted before application. Other clinical parameters like ABI (ankle brachial index), peripheral arterial pulsation, and oxygen pressure, bacterial index (swab and tissue biopsy) carried out. Infraopopliteal Doppler study of arterial and venous study carried out for suspicious arterial or venous circulation deprived condition patient. chronic wound patients are selected which undergone conventional management of ulcer protocols but wound wasn’t healed, these patient are not able to spend money for conventional treatment same about acute wound case patient also, these selected patients are from poor economical group, as our duty we explain them about treatment modalities available and also pros and cons of applying pregnancy specific biological substances for wound dressing. After written as well as audio-visual consent taken, we enrol them for study.

PROCEDURES:

1. First step of lavage by normal saline of wound bed to remove debris, foreign particles, and bacteria. We washed thoroughly.

2. Simple debridement – debridement done with sterile gauge or surgical blade, scissor to remove necrosed tissue .then again thoroughly washed with normal saline following aseptic techniques.

3. Cotyledon application – this is not compulsory step, as per requirement pieces of cotyledon of placenta rub over wound bed if wound is grossly contaminated.

4. Pouring of amniotic fluid over wound which was collected in 20 ml syringe previously.

5. Application of amniotic membrane – amniotic membrane has two surfaces amnion and chorion that separated by making small cut and pulling two part apart, Glistening surface is amnion and rough one is chorion.for epithelisation we apply amnion surface and for angiogenesis we apply chorion .by measuring size of wound ,we cut amniotic membrane of that size with less manipulation. Then apply over wound bed.

6. Applying glycerine tulle over amniotic membrane graft. Dressed it by applying sterile gauge pieces over it and lastly bandaging.

After first dressing, check dressing on post 4 Th day if it is exudated, foul smelling then Change it immediately otherwise change it after 7 days.

We are coined term pregnancy specific biological substances because we are using combinely placenta, amniotic membrane, amniotic fluid, Wharton’s jelly or singularly as local application but science behind it is regeneration and repair of wound by stem cell and growth factor. Before going in detail first we know in detail about pregnancy specific biological substances.

AMNIOTIC MEMBRANE: full term amniotic membrane comprise 1300 to 1500 cm² in surface area .amniotic membrane content two surfaces amnion which is bathed in amniotic fluid and contain epithelial cells ,based on basement membrane ,second layer is chorion continue with decidual layer of maternal side. Chorion is rich in collagen .amniotic membrane have growth factor ,cytokines.

Human placenta is most important organ for foetus development and survival of foetus, which develop in few days after fertilisation procedure. Placenta is 10 -15 micrometre thick in size.AM surrounds, protects foetus and content amniotic fluid, and it is easily detached from chorionic because highly flexible. AM has two types of cells amnion epithelial cells which derived from embryonic ectoderm and amnion mesenchymal cells derived from embryonic mesoderm.AM is thin, tough, transparent, avascular composite membrane which composed three major layers: a single epithelial layer, a thick basement membrane, an avascular mesenchyme which consist abundant collagen [8-10].amniotic epithelial cell layer is single layer of flat, cuboidal and columnar cells which directly have contact with amniotic fluid. This is potent source for amniotic MSC (AMSC) which can be isolated for regenerative use, this layer doesn’t have nerves, muscles, lymphatic’s supply [7, 11].HAE cells and AMSC derived from amniotic membrane express pluripotency and potent source for stem cells.Amniotic mesoderm layer consist macrophages and fibroblast –like mesenchymal cells [7].

AM is potent carrier of embryonic stem cells, we can isolate embryonic stem cells from it and it is useful source of ESC application. Amniotic cells connect each other by desmosomes, tight junction between cells occlude the lateral intercellular spaces and limit paracellular transport [12]. The basal lamina is the potent source mainly of proteoglycans like heparin sulphate which is one of
the major proteoglycan in the gingiva. Amniotic epithelium  Intracellular cytoskeletal filaments such as actin,αactinin,spectrin,ezrin,cytokeratins,vimentin, desmoplakin plays important role in structural integration and modulation of cell shape of healing tissue [13].spongy layer of stromal portion of amnion content proteoglycans and glycoproteins which form non fibrillar network along with a collagen [14]. AECs secrete collagen type III and IV with noncollagenous glycoproteins ( laminins, nidogen, fibronectin and vitronectin) within the basement membrane Which Act As adhesion ligands, play role of transmitting signals , interacting at cell surface receptors [15]. Laminin and its isoforms 2, 4, 5, 6, 7, 10, 11 mostly help in cell differentiation, cell shape and movement, maintenance of tissue phenotypes, help in tissue survival via cell surface receptors such as integrin’s and dystroglycans [16].laminin5 in amnion membrane helps in cellular adhesions of gingival cells, invasive growth of fibroblasts and angiogenesis in the early phase of wound healing. The stroma contains mitogenic factors, anti-inflammatory proteins and natural inhibitors to proteases and anti-scarring properties that help in wound to heal in a much faster way [17-19]. In addition, Human AM Matrix contains ample growth factor such as basic-fibroblast growth factor (b-FGF), transforming growth factor-beta (TGF-β), keratinocyte growth factor (KGF), epidermal derived growth factor (EDGF), nidogen growth factor (NGF) all of them enhance regeneration [12]. Growth factors help in providing natural healing environment and mimic the stem cell niche for ex vivo growth [16].the main ligands of basement membrane integrin α6/β4 helps in the construction of the heme desmosome like structure favours the adhesion and anchoring of ESCs to the healing wound. Migration of epithelial cells, help in adhesion of basal epithelial cells, enhance epithelial differentiation as well as prevents apoptosis [20, 21]. Amnion membrane express stem cell markers like hepatocytes nuclear factor-3β (HNF3β), nestin, nanog, octamer-binding transcription factor (OCT)-4[22, 23]. The stem cell markers like epidermal marker CA125 [24], general epithelial markers vimentin and cytokeratin’s are present in large amount in the amniotic epithelial cells [25]. human amniotic mesenchymal cells express CD44 and desmin, CK19/ vimentin, AECs express TNF alpha, NGF, BDNF, noggin and activin [26, 27]. Human MSC have a characteristic cell surface phenotype markers of CD90+, CD73+,CD105+, CD44+, HLA I+,

CD45, CD34, CD11b, HLA II [28]. marker expression pattern of amnion derived cells show that amnion derived cells can be differentiated into epithelial or mesenchymal cells but remained as undifferentiated stem cells [13]. Wound Healing Mechanism by amnion membrane is divided as follows:

- Immunomodulation due to AMSCs.
- Antibacterial, Antifungal, Antiviral
- Pain Reduction.
- Anti-Inflammatory And Anti-Scarring
- Tissue reparative activities with enhanced bone remodelling, osteogenesis and chondrogenesis
- Speed fibro genesis and angiogenesis
- Increased extracellular matrix deposition

We selected patient randomly, criteria for selection is nonhealing wound (ulcer), acute or chronic, different aetiology ,we have outdoor clinic in different government hospitals,Kolkata.10 patients
enrolled from bangur government hospital, 4 patient from vidyasagar hospital, 4 patient from Shambhunath pandit hospital. Amongst them, some of came first time in outdoor department, some of them are admitted patient. Out of 18 patients, 8 patients were diabetic, 1 patient diagnosed gangrene of right lower limb, 1 pt. have multibicialary leprosy, 3 pt. were ischemic venous and arterial disease, and 4 were traumatic injury. One young male ,age 24 came to outdoor with right lower limb gangrene with foul smelling ,we immediately admit him ,routine investigation complete blood count ,blood sugar, liver profile ,kidney profile and infrapelileal Doppler study of right leg carried out. Infrapelileal arterial and venous study shows no blood flow in anterior tibial,posterior tibial, dorsalis pedis arteries.immediately we advise him for below knee amputation, patient was not physically fit for surgery , decided to continued routine line of treatment with anti-inflammatory drugs, intravenous antibiotics with amniotic membrane dressing. Firstly we washed wound with normal saline then we poured amniotic membrane over wound ,lastly application of amniotic membrane dressing, covering whole gangrene part.at time of first dressing ,we noted that tissue is macerated ,moist, foul smelling ,no sensation, no maggots. We kept dressing for 7 days.

At time of next dressing, we found maggots, foul smelling is less compare to previous dressing, and during dressing he feels sensation pain. Subsequently after 7 days he was dressed with amniotic membrane, at later stage we noted that the redness, granulation started near demarcation line of gangrene which was drawn at first dressing and Demarcation line was improved and lowered.

Picture no 3, shows a patient wound treated by amniotic membrane he has traumatic injury to foot, known case of diabetes after debridement amniotic membrane applied. Only three dressings are done, 7 days apart, treatment is going on.

Picture no 3 traumatic injury of foot, dressing done with amniotic membrane (A), (B), (C). improvement in wound healing after application of amniotic membrane as local coverage of wound, necrotised and devitalised tissue surface of wound will convert into healthy granulation tissue.as we observe, in all patients were improved gradually. Firstly we removed devitalised tissue by sharp
As I stated above, amniotic membrane contained mesenchymal stem cell and growth factors which eventually help in healing of wound. Many peoples used irradiated, cryopreserved amniotic membrane as wound coverage. Manipulation of amnion lose epithelial cells. Repair and regeneration processes are not separate entity. Repair is defined as transplanted or endogenous stem cell released bioactive molecules like growth factor and cytokines which trigger native cell of injured organ or tissue to restore and re-established function and structure of that organ or tissue. Regeneration is defined as process in which stem cell travel to injured side, Trans differentiate or differentiated themselves to functional cells of that organ or tissue to restore and re-established function and structure of that organ or tissue. We have one male patient, 35 years old, history of traumatic injury over left foot on ankle joint, he was checked in outpatient department, as our standard protocols of wound management established by institute, we washed wound with normal saline, apply amniotic membrane dressing over wound bed and dressed as usual. Patient was discharged and told him to come after 7 days, patient is not addicted to drugs, alcohol, tobacco etc. But we noted that he has poor background, the people surrounding his native place found diseased due to nutritional deprivation. He is also nutritional deprived. We dressed wound with amniotic membrane up to 21 days. We noticed debridement, then we apply amniotic membrane. We noted that colour of wound bed changes, size reduced in length and breadth, wound contracts, pain reduced. Notable thing is that oedema surrounding wound bed reduced, skin wrinkled and peels off. All cases improved somehow as per severity of underlined health status of patient, we continued intravenous antibiotics, anti-inflammatory drugs. One patient was operated for varicose vein of rt lower extremity, stripping of vein was done but he is laundry worker, standing job so after surgery 2 years later he was developed rt lower leg venous ulcer, at first time we washed wound with normal saline, pour amniotic fluid and dressed with amniotic membrane, we can’t apply compression stockings at that time, subsequently we noticed size of ulcer was reduced, edges of ulcer move inwards, healing started, later on we advised him to wear compression stockings(STUVA) at 40 years. Male was diagnosed diabetes type 2 on oral hypoglycaemic drugs. He was came in outpatient department with recent complaint of nonhealing wound over left sole of foot with diabetic underlined. Patient told that he have traumatic injury after that wound developed and not healed, immediately we advised him for investigation for blood sugar fasting and post prandial, complete blood count, Hepatitis B and HIV, after all essential permission granted by ethical committee and patients consent, we planned and suggest him to came after couple of days for amniotic dressing, at the time of dressing, we used freshly collected amniotic membrane and placenta by lower segment caesarean section after screening for HIV I & 2, Hepatitis B & C, CMV, VDRL. Firstly we washed wound with normal saline, clean it by sterile gauge piece then we took small piece of cotyledon of placenta and rubbed over wound bed two times, we poured amniotic membrane over wound and lastly we apply amniotic membrane as wound coverage. Glycerine tulle applied over amniotic membrane and dressed with gauge and bandaged. We checked dressing for alternate day for foul smelling or oozing, as per protocols established by department if there will be oozing or foul smelling then it need to be changed dressing otherwise kept it for 7 days, there was not foul smelling or oozing so we changed dressing after 7 days, on next dressing we saw that granulation was started, wound margin moves inside, oedema surrounding wound reduced, dramatically pain subside, depth of wound slightly reduced oozing stopped. Patient told us that he feels better and happy. We continued dressing scheduled up to 1 month and 7 days apart. With amniotic membrane dressing we continued oral hypoglycaemic drugs but we never used antibiotics. He was 35 years male patient, has history of traumatic injury over right lower limb at lateral malleolus, he was examined, saw large circular wound over right lateral malleolus of lower limb with centrally devitalised and necrosed tissue. Firstly we washed wound with normal saline then we removed necrosed tissue, after doing debridement again we washed wound with normal saline, pour amniotic fluid and lastly amniotic membrane covering and dressed, bandaged. Subsequently we do dressing with amniotic membrane 7 days apart, at post 21 days after first dressing, we noticed that size of wound reduced, colour changed to pink, pain subside, no oozing, no slough but most important thing is that we noticed that there was excessive growth of granulation tissue, more than our expectation. We see that granulation tissue is more, we noticed that oedema surrounding wound tissue is reduced, skin peels off. Patient feels happy and comfortable. We doesn’t prescribe antibiotic medication to patient neither we used local antimicrobial drugs for dressing purpose but it’s our auto surprise that wound healed without conventional line of treatment support. We continued medicine for diabetes, blood pressure, its most important property of stem cell that stem cell travels to injured side, homing in concerned organ or tissue, Trans differentiate themselves and regenerate and restore structure and function of tissue or organ.
there was improvement in wound healing but it’s very slow, as we expected. Every time when we opened dressing, we found it was contaminated with dust, urine. Apart from our advice, patient not taking care of that. Patient wound wasn’t recovered as we expected.

RESULTS:
We have weekly outdoor in three different government hospital so each time, there was mixture of old as well as new cases. But I was took 18 patients which were recovered and have follow up regularly. Out of 18 patients one patient has dry gangrene of RT lower limb, amputed below knee, due to unavoidable circumstances amputation was not done immediately so we include him in our trial with conventional line of treatment continued. We observed that line of demarcation was lowered and there was reestablishment of blood circulation. Later on ultimately amputated, surgeon who done amputation told us that there was profuse bleeding in spite of completely block of infrapopliteal arteries shown in Colour Doppler study of lower limbs, done before enrollement. 16 patients shows complete healing of wound.

DISCUSSION:
As per our observation in small cohort of study, all our patients are satisfactorily improved. Out of 18 patients 16 patients are rid of non-healing wound. Many of them have underlined diabetic, ischemic, leprosy etiological causes, apart from wound most important thing was noted that patient have got systemic improvement as well. Freshly collected placenta application have cytokines, growth factors, and stem cell component cocktails. We all know, the global wastage of pregnancy specific biological substances like placenta, amniotic membrane, and umbilical cord. Amniotic membrane is most efficient alternative for management of wound compare to other costly antimicrobial dressings. It’s not new for us that use of amniotic membrane in corneal surgery as graft. Burn wound patient also benefited by application of amniotic membrane. We concerned about fresh amniotic membrane collected by caesarean section and we used it immediately, the use of amniotic membrane within 4 hours of collection will have cytokines which lost in preservation of it. It is potent source MSC which isolated in vitro for clinical application. immediate result we noted after application of amniotic membrane is pain reduced in intensity, it is due to trypsin, chymotrypsin like substances. We noted after multiple application of chorion surface of amniotic membrane over wound bed formed excessive granulation tissue(proud flesh) which is bright red in colour. we all know excessive granulation tissue inhibit wound epithelisation. after excessive granulation tissue formed we stopped application of chorionic surface and starting application of amnion surface. We noted excessive granulation tissue formed in wound which on bony Prominence. We never used any medication for granulation to subside, ultimately it was healed.

CONCLUSION
As per our study we noted freshly collected amniotic membrane application over chronic wound, have miraculous effect in healing of wound irrespective underlined causes. Amniotic membrane have three major layers amnion, thick basement membrane and chorionic. Amnion is derived from embryonic ectoderm, thick basement membrane derived from embryonic mesoderm. Amniotic membrane is potent source of epithelial stem cells. Mesenchymal stem cells, endothelial stem cells. Embryonic stem cells are isolated in vitro From amniotic membrane. It is potent reservoir of growth factors, cytokines. we prefer to used freshly collected amniotic membrane for clinical application if we preserved it then we loss epidermal growth factor(EGF), vascular endothelial growth factor(VEGF), keratinocyte growth factor(KGF), fibroblast growth factor (FGF), transforming growth factor (TGFβ).these factor play crucial role in wound healing apart from them amniotic membrane act like structure as scaffold or temporary wound covering material. it is our small cohort of study, we need to conduct double blind, randomised placebo controlled trials which based on freshly collected amniotic membrane clinical application for wound healing as well as other clinical applications.

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