ORIGINAL ARTICLE **Mirror of Research in Veterinary Sciences and Animals MRVSA/ Open Access DOAJ**

Mirror of Research in Veterinary Sciences and Animals
(MRVSA) / Journal homepage: http://mrvsa.com/
E-ISSN 2307-8073; 2520-324X (Print)

Amniotic membranes as biological dressings for treatment of severe experimental burns in rabbit

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ARTICLE INFO
Received: 08.09.2017
Revised: 28 .09. 2017
Accepted: 17.011. 2017
Publish online: 09.12.2017

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Abstract

Burns have estimated as one of the most destructive conditions in emergency medicine affecting the population in developed and developing countries, lead to physical and chronic disabilities due to psychological scars. Various natural and synthetic materials have been used for treatment and coverage of burn wound. Nonetheless, disadvantages associated with these materials are including high price which prohibits their widespread use, especially in developing countries. Among all, human amniotic membrane (AM) is an excellent candidate for use in cellular therapy and regenerative medicine. Moreover, it is the only easily available and cost-free coverage. This study intends to determine the healing effect of amniotic biological dressing in the regeneration of experimental induced severe burn wounds in the rabbit. Four male rabbits randomly divided into two equal groups. All rabbits were exposed to 3rd-degree burn wound using a hot plate the first group left without treatment and consider as control, while the second group (treatment group) was treated with prepared biological dressing amniotic membrane. Amniotic membrane collected from an elective human caesarean delivery. The donor was screened and was seronegative for hepatitis B and C, syphilis and human immunodeficiency virus. Amniotic membrane was prepared by separating it from chorion of the placenta. Processing of the amniotic membrane was carried out under sterile conditions using an antibiotic cocktail. After 21 days of therapy, a skin biopsy was collected from the burned areas and examined for histological evaluation. Application of amniotic biological dressing resulted in complete healing of the burn wounds and absence of inflammation after 14th days. Re-epithelialization was prominent in the treatment groups in compare to non-AM treatment group. In the treated group, epidermis exhibited well-structured layers without any crusting. There were spindle-shaped fibroblasts in a fascicular pattern, oriented parallel to the epithelial surface with eosinophilic collagen matrix. In conclusion, this study approved that amniotic membrane as an available and inexpensive biological product revealed to be a suitable substitute in the healing of burn wounds especially when dressing form was applied directly after burning. For the author's knowledge, this is the first study regarding the application of amniotic membrane in the treatment of burns injuries in Iraq. The authors recommend moving the results of this preliminary studies to clinical studies after standardized the method of preparation of amniotic membrane dressing.

To cite this article: Al Salihi K A; Fatima Atiya Kareem; Hayder Merhej Watban ; Noor AbdulHadi Hasan . (2017). Amniotic membranes as biological dressings for treatment of severe experimental burns in rabbit. MRVSA. 6; (3): 1-12. DOI: 10.22428/mrvsa. 2307-8073.2016. 00631.x

Keywords: Amniotic membrane; Burn; Wound; Healing; Rabbit.

Introduction

The biggest structure of the body part is the skin. It plays many important functions such as protection, thermoregulation, metabolic functions, and sensation. The integument protection roles include its action as a physical barrier to prevent the losing of body fluids and hinder the entry of toxic materials. Moreover, different cell types of the dermis have immunological function against the invading microorganisms. The mechanisms of the skin as a thermoregulatory to preserve body heat are vasodilatation and sweating in reaction to heat and decrease the body temperature. There are two principle regions in skin, the epidermis, and the dermis, individually responsible for a particular role of the total function of the skin. The subcutaneous connective tissue is adhered to the dermis layer and stored the adipose tissue that is as the superficial fascia of gross anatomy.

A diversity of causes like radiation, caustic elements, electricity, and heat can make damage and injuries called burns to the skin and the underlying dermal tissues. Burns occur in variable degrees of damage to the skin and nearby tissues depending on the severity of the causative agent. Three zones of tissue damage occur due to response to burn trauma. The zone of coagulation is extreme damage part with irreversible tissue loss that occurs due to protein coagulation. Bounding this region is the ischemic or stasis zone with low tissue effusion and enclosed by recovery zone of hyperemia that suffers furthers damage and complication in severe burns degree (Bousfield, 2002). In minor superficial injuries, dilatation of dermal vessels may lead to redness and escape of fluids from vessels to interstitial space that may stimulate nerve ending and result pain (Bousfield, 2002; Hettiaratchy *et al.,* 2004). In severe burns, plenty of fluids congregates and make bubbles in the dermis or/ at the connection with the epidermis layer resulting in the death of covering epidermal cell that may regenerate later from the neighboring epithelium. However, in severe burns damage of the dermis upper part, destruction of skin full thickness and underlying tissue lead to delay in regeneration process and usually requires surgical intervention (Hettiaratchy *et al.,* 2004). Severe burns also cause life- threat systemic responses such as cardiovascular, gastrointestinal, respiratory, metabolic and immunological responses ((Bousfield, 2002; Hettiaratchy *et al.*, 2004). Burns are divided into three degrees depending on the depth of the skin damage. The first-degree (superficial) burns heal in few days and characterize by damage to the surface epithelium with local redness, dry skin, mild edema and minor pain. The second-degree (partial thickness) burns take a long time to heal and cause to damage to both epidermises and variable depths and structures of the dermis layers. Moreover, Erythema, blisters, noticeable swelling, and pain are common in superficial partial thickness burns that heal with minor or no scar tissue and reserve hair follicles and sweat and sebaceous glands. Meanwhile, the deep partial thickness burns comprise deeper regions of the dermis with fewer swellings which may restore with scarring. The third degree (full thickness) burns characterize by the damage of skin all layers with variable depths of subcutaneous tissue and loss of skin functions. Moreover, full thickness burns reveal carbonized and black or gray-white appearance without pain and heal with granulation tissue and scarring. The actual incidence of burns injuries is difficult to assess from hospital-based studies because a large number of mild cases did not request hospital treatment. The percentages of burns injures have been reported in different countries worldwide and were 23%, 5% and 12.6 in Spain, China and Turkey respectively (Fernandez-Morales *et al.,* 1997; Jia *et al*., 2005; Kara *et al.,* 2008). Universally, there were more than 7.1 million fire-associated unintended burns with 310 death (69% females and 31% males) in 2004, according to the WHO (WHO, Summary. 2004).

Available published documents concerning burns in Iraq are scarce. Besides, the estimated burns death were 3.390 with equal rate to 12.3 per 100.000 per year, according to WHO 2004 estimation. This rate is greater than the universal rate (WHO. Annual incidence, 2004). Carini *et al.*, (2004)

reported the Italian Red Cross a short period (45 days) experience regarding 1.350 burn patients in Baghdad. There were 48 (23 males and 25 females) patients that admitted officially with 27% mortality rate (13% males and 40% female). The war and terrorist related burns injuries increased in the last few years in Iraq that lead to increasing the number of admitted patients suffering from burns. (Mousa, 1997). The burned patients suffer from clean open local burn injuries initially and face three main dangers. These dangerous are shock, sepsis, and contractures (William and Douglas, 1952). Merely once restoration has been done for the blood-dynamic and cardiovascular system and respiratory consistency repaired, should care be focused on the burns injuries itself.

Various traditional approaches have used to treat burns injuries comprises accurate surgical elimination (debridement) of loose, infected necrotic tissue. The presence of eschar forms in both partial and full thickness burns impact the healing and encourage the colonization of microorganism on the surface of the burns wound. The chief aims of burns management are to speed tissue healing and to hinder the infection (Salas *et al.,* 2005). Burns topical applications are the most important component of wound care in the hospital. Until now, there is no topical application available, whether alone or in combination has the features of ideal therapeutic effects on the burns wound.

The basic fundamental in the treatment of burns are to control of pain and infection, accelerate healing and prevent trauma that converts the superficial burns to deep tissue injury. There is a range of different natural and artificial substances that use for transitory burns wound dressing; though, they are related with disadvantages including a high price, which decrease their extensive use, particularly in the developing countries. Allograft, xenograft and human amnion membrane have used as dermal biological skin substitutes and considered as an excellent standard for temporary covering of burns. These biological alternatives should own particular characteristics features that promote the healing of the burns wound. It should cling and inhibit bacterial colonization on the burned surface; decrease loose of fluids, microelements and proteins from the burned surface; allow good permeability of fluids and airs from the surface of the burn area to the surrounding tissue; reduce the opportunity of keloids formation during burns healing process and reduce the pain (Jones *et al.,* 2002). Human amniotic membranes are between all dermal substitutes that are available with very low cost and have all the golden properties of dermal substitutes that enable it to be the ideal skin substitute, particularly in developing countries. The amniotic membrane is originated from the internal layer of the placenta and is built of adjoined amnion and chorion membranes. Amniotic membrane is a metabolically active structure and repeatedly remodels the extracellular matrix via procedures directed by paracrine growth factors. Moreover, neither blood vessels nor direct blood supply is presented in the amniotic membranes (Dua *et al.,* 2004). The mechanisms of nutrients providing of the amniotic membranes are via directly diffusion out of the amniotic fluid or from the underlying decidua (Niknejad *et al.,* 2008). Amniotic membrane is a multilayer membrane comprising of five specific layers; these include epithelium, basement membrane, dense layer, fibroblast layer and intermediate or spongy layer. Various studies used amniotic membrane as a wound dressing or surgical patch and placed on a huge burns injuries that allowed to stay for 3 to seven weeks (Artz *et al.,* 1972). Boss, (1979) and Rinastiti *et al.,* (2006) mentioned to used amniotic membrane as a biological covering material to speed the wound repairing and reform the damaged tissue. Also, it is free to obtain from the maternity delivering ward and be better than allograft and xenograft. Amniotic membrane aids the burns wound healing process and diminish morbidity due to its ability to inhibit heat and water loss from the injury surface and to operates as a barrier against bacterial colonization. The advantages of amniotic membrane wound dressing are its ability to encourage the epithelization, inhibits fluid,

protein, heat and energy loss and the best unique wound cover next to the patient's skin (harberal *et al.*, 1987).

Review of the literature reveals scarce publication about the application of amniotic membrane as a biological dermal dressing for burns injuries in Iraq, with increasing number of burns percentage between Iraqi population due to war and terrorist action. Therefore, the goal of this study was to determine the healing ability of human amniotic membrane in the treatment of experimentally created full skin thickness burns in the animal model (rabbits).

Material and Methods

Amniotic membrane Collection and harvesting

This study approved by the research committee/college of veterinary medicine / Al Muthanna University. The amniotic membrane collected from a selective human caesarean delivery at Samawah maternity hospital/ Al Muthanna governorate and the donor were signed a consent form. The donor was screened and was seronegative for hepatitis B and C, syphilis and human immunodeficiency virus. Amniotic membrane washed with Phosphate buffered saline supplemented with an antibiotic cocktail (PBS-ABC) comprising 400 ml of PBS containing 1,200,000 IU benzathine penicillin and 100 ml of metronidazole to use as a decontaminant for Gram-negative, Gram-positive bacteria and fungi and storage medium. Amniotic membrane was gently separated from chorion of the placenta under the sterile condition and washed three times with PBS-ABC. Later on, the amniotic membrane was cut into 5 cm X 5cm pieces and flattened on a gauze covered with Vaseline and Povidone iodine and kept in deep freeze until use (Figure.1).

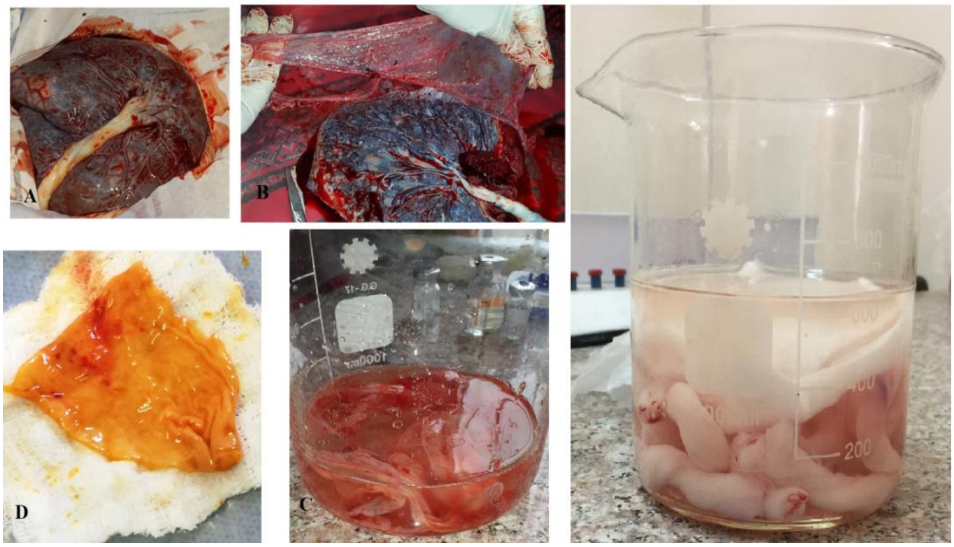

Figure.1: Shows the procedures in the collection of human amniotic membrane

Experimental animals

This study was done between 2nd December 2016 and 30 January 2017. Four male rabbits randomly divided into two equal groups. All experimental animal used according to international guidelines for animal care. Each animal kept separately in one cage and maintained under

controlled environmental conditions and freely accessed by water and food. Ketamine (15mg/Kg) and Xylocaine (1.1 mg/Kg) were injected intramuscularly to calm the rabbits. The hairs on the back shaved, and povidone iodine solution was used to cleanse the skin. Then, it was dried off with sterile water and exposed to a typical 3rd-degree burn wound using a hot plate (Figure.2). Burns induction were done according to method described previously by other researcher (Hojati *et al.*, 2004; Hosseini *et al.*, 2007;Manafi *et al.*, 2009; Amini *et al.*, 2010; Hazrati *et al.*, 2010; Mehrabani *et al.*, 2015; Tanideh *et al.*, 2015; Tanideh *et al.*, 2014). No treatment was used for the wounds in the first group and consider as control, while the second group (treatment group) treated with prepared biological dressing human amniotic membrane. The wounds were under interval day of dressing and were examined daily for any changes in the color, shape, smell, discharge and scar separation. After 21 days of therapy, a skin biopsy was collected from the burned areas and kept in 10% formalin. Skin biopsy was processed routinely and embedded in paraffin. Then, 5-4 mm thick section was prepared and stained with Hematoxylin & and eosin (H& E). The prepared tissue sections examined under light microscope and images were captured using a digital camera, and histological evaluation was done.

Figure. 2: shows the procedures in the induction of the burns wound and method of treatment and healing procedures.

Results

A. Gross pathological observations

Treatment group

The size of the wounds of the first group was started in decreasing at 3 days. The wound areas continued to decrease and recover. Moreover, the closure of the wound occurred peripherally. At 7 and 14 days, the wound became small, and healing scar tissue was occurred, which attached to the center of the wound, but it was free at the peripheral. At 21 day, prominent wound healing observed in compare to control group and the wounds completely healed leaving the normal slightly pink skin in the area, but no hairs were growing in the area. At one month, the hair was growing in the area (Figure. 3).

Non-treated (control) group

The border of the wound become very dry at 3day. Moreover, elevated lesion and no reduction in the size observed at 3 days. At 7 and 14 days, a dense crust was attached firmly to the wound, and the wounds were bleeding and appeared as brown leathery area. At 21 days the wound still large in compare to treated group. The wound decreased in size at 49 days and left a scar like tissue. No normal hair was grown back on the areas (Figure. 4).

Histopathological observations

The treated group revealed scarce inflammatory cell in the section of the healed burns skin at 21 days. And prominent reepithelization occurred which revealed well-structured epidermis layers with no crusting. The area was highly vascularized and also showed spindle-shaped fibroblasts and fibrocytes that were parallel to the epithelial surface of the eosinophilic collagen matrix. Moreover, regular epidermis layers were evident, and keratin layers were covered the healed skin (Figure.5).
The non-treated (Control) group revealed interstitial edema, the proliferation of fibroblasts, cleft between the epidermis layer and subcutaneous tissue. Besides, loose collagen matrix was also seen accompanied with hemorrhage. Intense infiltration of polymorphonuclear and lymphocyte cell were also observed with congested blood vessels. The monolayer of incomplete epidermal cells was also observed. The monolayer epidermis appeared as free necrotized edges characterized by vacuolation of cellular cytoplasm with the short migration of epidermal cell under the obvious crusting (Figure.6).

Figure.3: Healing features in the amniotic membrane treated group in compare to control group

Figure. 4: Healing features in the non -treated group (control group).

Discussion

The greatest common traumatic injuries worldwide are the burn. Its treatment cost the huge amount of traditional medical resources. Therefore, it is necessary to explore a suitable substance for covering of burns injuries that enhance, promote and facilitate the healing process. Moreover, this element should be freely available, effortlessly relevant, cheap and also can securing the wound injuries from infection and drying. Since 1910, amniotic membrane has been applied as burn dressing with inconstant success trials (Robson *et al.*, 1973).

The results of the current study revealed the obvious gross healing effects of the multiple amniotic membrane dressing on the burns injuries in the treated group in compare to control non-treated group. The amniotic dressing burns healed earlier, and the skin returned to its normal shape within 21 days after the treatment. The results of this study are compatible with the results of koob *et al.*, (2014), who approved that the human amniotic membrane is effective in the wound healing and help in reserve the growth factors, anti-inflammatory molecules, and inhibitors of metalloproteinases that play other essential roles in the injuries recovery.

The results of the histological observations of the current study approved acceptable reepithelization response, absences of inflammatory cells, and well-developed skin tissue that showed the normal skin structure with complete epidermis layers followed by the subcutaneous tissue, neovascularization, fibroblasts and fibrocytes and collagen matrix. These observations are compatible with the previous study that approved the successful application of amniotic membrane in the treatment of third-degree burn injury in an animal model (Anahita et al., 2015). Moreover, Anahita et al., (2015) approved the ability of the amniotic membrane to reduce the wound area, a decline of inflammatory cell infiltration and boosted epithelium after 21 days.

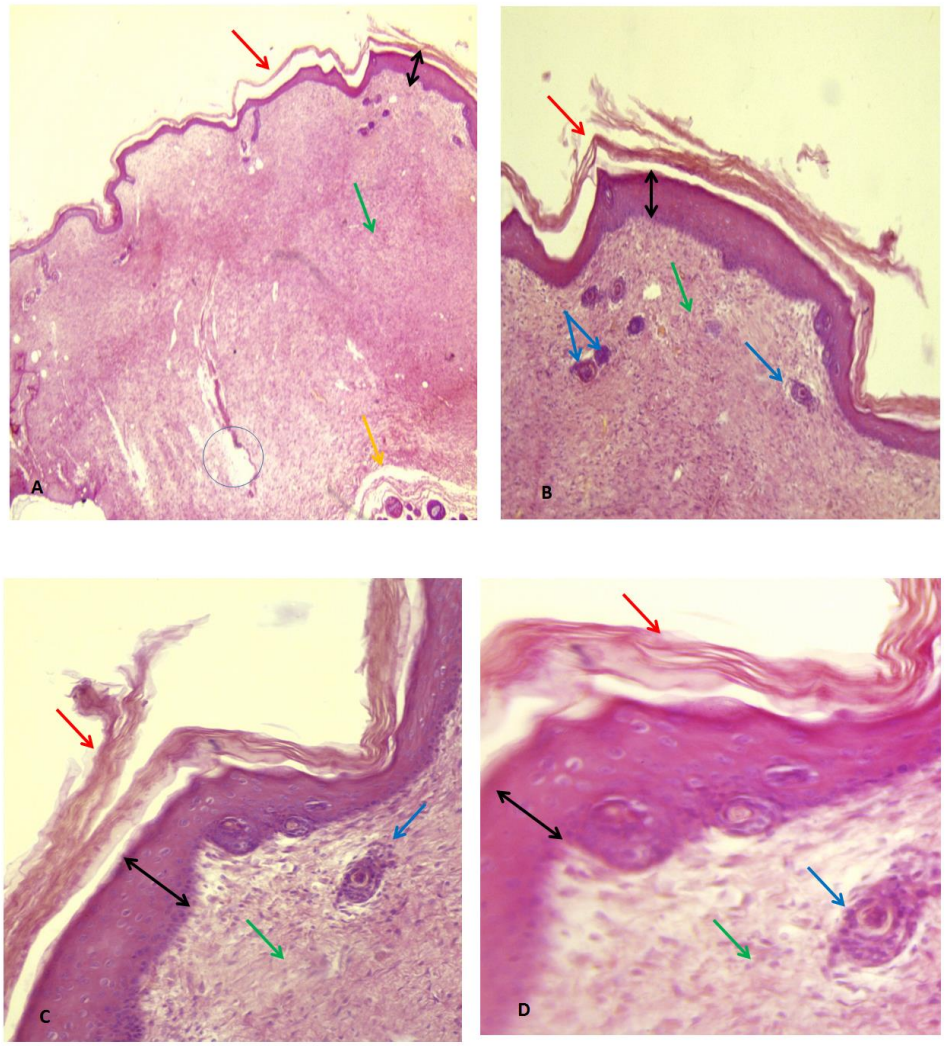

Figure 5: show the histological appearance in the amniotic membrane treated group. A. 4X, B. 10X, C. 20X, D. 40X (black two head arrow = full thickness epidermis, green arrow= fibrocytes; red arrow= keratin; orang circle= vacuolation in epidermis area; Blue arrow= blood vessels)

However, the results of this study are incompatible with the results reported by Loeffelbein *et al.*, (2014), who seen the satisfactory reepithelization effect in their experimental animals, but they concluded that human amniotic membrane had no relevant advantage over conventional dressings, and it can reduce the cost of the treatment. Meanwhile, the control group revealed incomplete monolayer epidermis that appeared as free necrotized edges characterized by vacuolation of cellular cytoplasm with the short migration of epidermal cell under the obvious crusting. These observations explain the fragile slight epithelization at this period that became prone to infection, sloughing and eventually the delay in the healing time and process of the burns injuries. These results are compatible with a previous study (Faten et al., 2016), who evaluated the Canine amniotic membrane as a biological dressing for treatment of a deep cutaneous wound in rabbits. They found that the non-dressing group revealed weak and minor epithelization layer, while the amniotic membrane dressed wounds showed the good rate of epithelization, fibroplasia, and

angiogenesis. They approved the beneficial effect of amniotic membrane in the wound healing in an animal model.

The histological study of the current study also revealed scarce inflammatory cell infiltration in the group of animal treated with the amniotic membrane in compared to the control group that showed Intense infiltration of polymorphonuclear and lymphocyte. The results of this study agree with studies that reported previously and approved the effectiveness of amniotic membrane as biological skin substitutes used in burn wounds, with the efficiency of maintaining low bacterial counts. It also has advantages of reducing the loss of protein, electrolytes, and fluids, decreasing the risk of infection, minimizing pain, acceleration of wound healing and excellent handling properties (Sukari Halim *et al.*, 2010). This study also approved the free availability of the amniotic membrane and also no any immunological complications, and hypersensitive reactions occurred in the treated animal during the treatment period. The most research mentioned only a few disadvantages of the amniotic membrane; these include the danger of the transmission of some viral infections such as VDRL, HIV, HCV and viral hepatitis (Mohammadi *et al.*, 2009; Mohammadi et al., 2013; Fairbairn *et al.*, 2014).

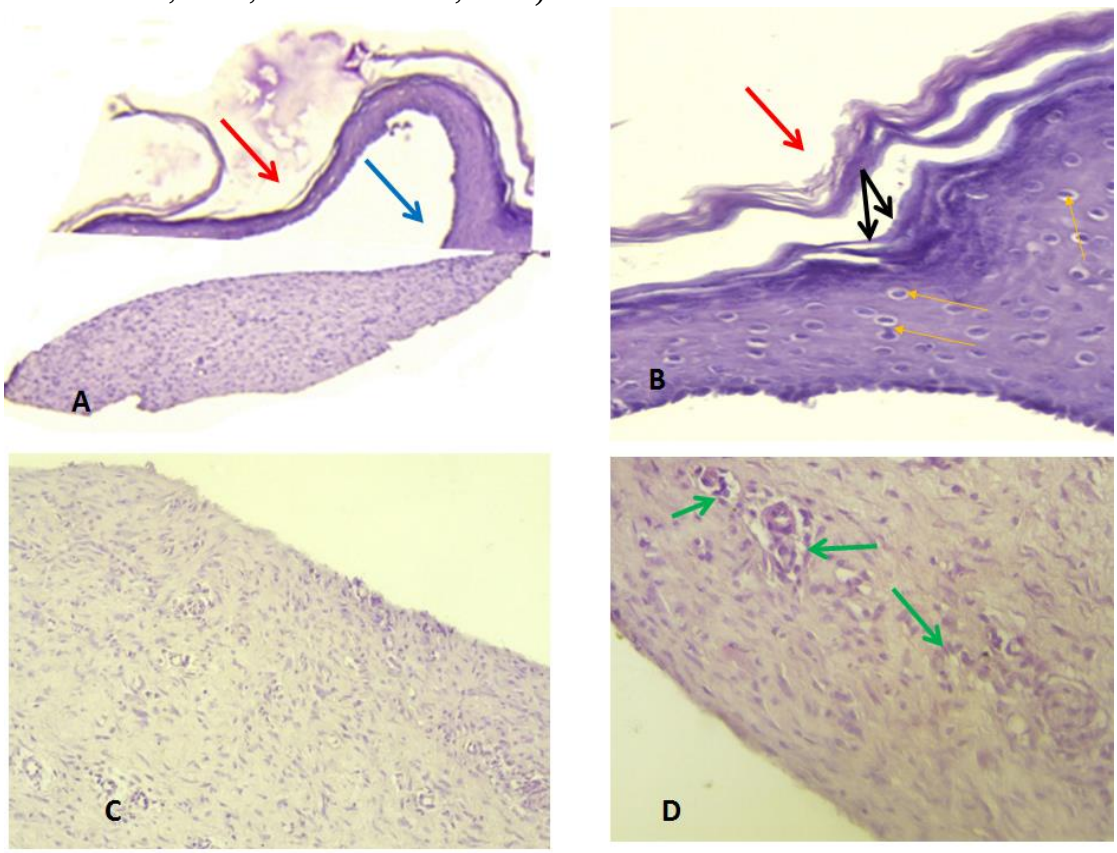

Figure 6: show the histological appearance in the non-treated (Control) group. (Red arrow= sloughed keratin and epidermis layer, Green arrow =interstitial edema, the proliferation of fibroblasts &loose collagen matrix, black arrow& blue arrow=cleft between the epidermis layer and subcutaneous tissue. Orang arrow = vacuolation of keratinocytes of cellular cytoplasm). A. 4X, B. 10X, C. 20X, D. 40X.

However, these disadvantages can be solved by selection procedures of amniotic membranes from the patient who are negative for VDRL, HIV, HCV and HBS. Therefore, sexually transmitted diseases, endometritis, premature rupture of membranes should be avoided, and the selected placenta should not contaminate with meconium and express normal color and smell.

In conclusion, this study approved the successful application of human amniotic membrane dressing to treat the experimentally inducted third-degree burns injuries in the rabbit. The recovered skin revealed excellent gross healing features and well re-epithelized dermal tissue. The easily and freely collected of amniotic membrane are one of the advantages of the investment of this dressing that act in reducing the cost of treatment in compare to the traditional method of treatment of burns injuries. Besides, amniotic membrane has another advantage including the promotion and speed the recovery and antibacterial activity. For the author's knowledge, this is the first study regarding the application of amniotic membrane in the treatment of burns injuries in Iraq. The authors recommend moving the results of this preliminary studies to clinical studies after standardized the method of preparation of amniotic membrane dressing.

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