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

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Dedication

الاهداء

إلى معلم البشــرية وهاديها ، إلى النور المبين ، إلى رســول الله صلى الله عليه وآله وسلم ...

وإلى من رضــا الله برضــاهما ، إلى أمي وأبي ، إلى ســندي في شدتي ورخائي ...

إلى الـذين ينظرون إلي بعين المحبـة والاحترام ، إلى أخوتي وأخواتي

إلى من تـابعني ونصـــحني ووجهني حتى أتممت بحثي ، إلى أستاذتي الفاضلة ..

إلى من هم لولاهم لما كنا نكمل حياتنا ... الحشد الشعبي المقدس

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نتقدم بالشكر الجزيل لأساتذتنا في جامعة المثنى / كلية الطب البيطري لجهودهم المتميزة خلال سنين الدراسة ولتوجيهاتهم لنا أثناء كتابة البحث فجزاهم الله خيراً عن العلم وأهله ...

كما نسجل شكرنا وتقديرنا إلى أستاذتنا المشرفة الدكتورة (كريمة عاكول الصالحي) لما أحاطتنا به من رعاية علمية ، وما بذلته من جهد ووقت ثمين ، ومنحتنا ثقة وإيمان بالعمل ...

ونتقدم بالشكر الجزيل ووافر الامتنان للجنة المناقشة التي تحملت عناء قراءة البحث وتعضيدها لما قد يعتريها من زلل أو خلل فجزاهم الله خيراً عن العلم وأهله ...

وإلى جميع أساتذتنا في جامعة المثنى / كلية الطب البيطري.. ونتقدم بالشكر الجزيل لكل من مد يد العون من التوجيه وتوفير المصادر ...

إلى كل هؤلاء شكراً جزيلا

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 used for treatment and coverage of burns wounds. 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 to regenerate the experimental induced severe burn wounds in the rabbit.

Materials and Methods

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 was 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 comprising 400 ml of saline 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. After 21 days of therapy, a skin biopsy was collected from the burned areas and examined for histological evaluation.

Results &Conclusions

Application of amniotic biological dressing resulted in complete healing of the burn wounds and absence of in inflammation after 14th days. Re-epithelialization was prominent in the treatment groups in compare to non-treated 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, 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.

الخلاصة

تعتبر الحالات الناتجة من الحروق من اشد الاصابات التي تواجه طب الطوارئ في الدول النامية والمتقدمة وتتسبب في الاعاقات الفيزيائية الدائمية وكذلك معاناة المرضى من الاثار النفسية المزمنة . لقد استخدمت مواد طبيعية وصناعية مختلفة لمعالجة وتغطية الجروح الناتجة من الحروق. ومع ذلك، فإن الاثار الجانبية المرتبطة مع هذه المواد تشمل عدم كفائتها في العلاج وتركها لأثار الحروق , وارتفاع أسعارها والتي تقلل من استخدامها على نطاق واسع، ولا سيما في البلدان النامية. ومن بين كل هذه المواد ، يعتبر الغشاء الأمنيوسي البشري المادة المثالية في العلاج الخلوي والطب التجديدي. ويعتبر علاوة على ذلك، المادة الوحيدة المتاح الحصول عليها بسهولة وبدون تكاليف. وعليه فان هذه الدراسة صممت لتحديد التأثير العلاجي لغشاء الأمنيوسي البشري البايولوجي (ضمادات الأمنيوتيك البيولوجية) في شفاء الحروق الشديدة التجريبية في حيوانات التجارب (الارانب) . استخدمت في هذه التجربة اربعة أرانب ذكور, وقسمت عشوائيا إلى مجموعتين متساوية. تم احداث حروق الدرجة الثالثة في جميع الأرانب باستخدام صفيحة ساخنة وتركت المجموعة الأولى دون علاج واعتبرت بمثابة مجموعة السيطرة، في حين تم معالجة حيوانات المجموعة الثانية (مجموعة العلاج) بضماد غشاء الأمنيوسي البشري وهو الغشاء الذي يحيط بالجنين والذي تم جمعه من عمليات الولادة القيصرية. بعد ان تم التاكد من خلو المتبرعين من التهاب الكبد الفايروسي Bو C ومرض الزهري وفيروس نقص المناعة البشرية. تم إعداد الغشاء الذي يحيط بالجنين عن طريق فصله عن اغشية المشيمة الاخرى . وتم تحضير هذا الغشاء تحت ظروف معقمة وبطريقة خاصة وباستخدام كوكتيل المضادات الحيوية . وبعد 21 يوما من العلاج، تم جمع خزعة من جلد المنطقة المحروقة وتم فحصها للتقييم النسيجي. بعد 14 يوما ,اظهرت جروح الحروق والتي عولجت بالأمنيوسي البشري الشفاء الكامل مع غياب علامات الالتهاب.

وكانت عملية إعادة البطانة الظهارية من العلامات المميزة في مجموعة حيوانات التجربة مقارنة بمجموعة السيطرة .وفي الفحص النسيجي, اظهرت المجموعة المعالجة طبقات بشرة متكاملة ومتناسقة الترتيب مع غياب الندبة وتميزت بوجود الخلايا الليفية المغزلية الشكل والمنتظمة بشكل موازي لطبقة السطح الظهاري للاديمة.

وفي الخلاصة , اظهرت هذه الدراسة كفاءة الغشاء الأمنيوسي البشري الذي يحيط بالجنين , بالعلاج البايولوجي للحروق . وتشير هذه الدراسة ايضا الى امكانية اعتبار الغشاء الأمنيوسي البشري المتوفر والرخيص الثمن كعلاج بديل ومناسب لشفاء الجروح الناتجة من الحروق خاصة بعد استعماله مباشرة على المنطقة المحروقة حديثا. لمعرفة الباحثون , فان هذه الدراسة الاولى من نوعها في العراق الخاصة باسخدام الغشاء الذي يحيط بالجنين في علاج إصابات الحروق. ويوصي الباحثون بتطبيق نتائج هذه الدراسات الأولية للدراسات السريرية بعد توحيد طريقة وتوفير الامكانيات الخاصة بتصنيع ضمادات الغشاء الأمنيوسي البشري.

CONTENTS

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 the 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 to the skin and the underlying dermal tissues that called burns. 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).

Aims of the study

Review of the literature reveals no previous publication about the application of amniotic membrane as a biological dermal dressing for burns injuries in Iraq. In addition, there are an increasing number of burns percentages between Iraqi population due to war and terrorist action.

Therefore, this study intends to determine the healing ability of human amniotic membrane in the treatment of experimentally created full skin thickness burns in the animal model (rabbits).

Review of literature

1. Pathophysiology of burns

A. Structure and functions of the skin

Normal skin consists of two layers, the epidermis and the dermis. The epidermis, which is derived from the embryonic ectoderm, is a layer of stratified squamous epithelium of several cell layers. The cells of the deepest "basal" layer of epidermis divide and produce the epidermal cells "keratinocytes" which gradually migrate to the upper layers and are eventually shed. As these cells move to the upper layers, they undergo morphological and biochemical changes "keratinisation" until eventually they turn to the dead horny layer "stratum corneum" of the epidermis. The epidermal turnover time i.e. time from basal layer till shedding normally takes around 50 days. The keratinized cells of the stratum corneum are devoid of nuclei, flattened and packed together providing a good protective barrier to the inner structures. The stratum corneum is thicker on palms and soles. The dermis, which forms the main bulk of the skin, consists of a layer of connective tissue composed of an interlacing network of collagen and elastin fibres that is responsible for the strength and elasticity of the skin. The dermis also contains blood and lymphatic vessels, nerves, sensory receptors, sweat and sebaceous glands and hair follicles. The dermis is thicker in certain areas such as palms, soles and dorsal aspects of the body and it is very thin in the eyelids, scrotum and penis. Beneath the dermis is the subcutaneous fat that separates the dermis from the underlying structures. The "eccrine" sweat glands, which are spread all over the body, lie deep in the dermis and their ducts pass through the epidermis to open on the surface to secrete water, electrolytes, urea and ammonia. The "apocrine" sweat glands are found in the axilla and groin. The wax glands of the ear and the milk glands of the breast are specialized types of these glands. Apocrine glands secrete an oily liquid containing proteins, carbohydrates, ammonia and lipids. The sebaceous glands are also spread all over the body, though more on the head and chest. Their oily secretion "sebum" pours into the hair follicles. Hair grows through these hair follicles which are small invaginations in the epidermis extending down to the dermis. Skin is an important physical barrier for the loss of body fluids and entry of microorganisms and toxic materials. In addition, various cell types in the dermis have immunological functions against invading agents. The thermoregulatory roles of the skin include vasoconstriction in response to cold in order to preserve body heat; and vasodilatation and perspiration in response to heat in order to cool down the body. Other functions of the skin include sensory perception, the protective role of melanin

against the destructive effect of the ultra violet (UV) light and the production of vitamin D through the action of UV light on dehydrocholesterol (Bousfield, 2002).

The body's response to burn injuries

Depending on the severity, burn injuries can lead to variable degrees of damage in the skin and adjacent tissues. Three zones of tissue damage have been described in response to burn trauma. The zone of coagulation is the area of maximum damage where irreversible tissue loss results from protein coagulation. Surrounding this zone is the zone of stasis which is characterised by decreased tissue perfusion. Around the area of low perfusion, there is a zone of hyperaemia where tissue perfusion is increased. The zone of stasis could be saved and zone of hyperaemia usually recovers unless it suffers further damage by later complications. (Hettiaratchy and Dziewulski , 2004).

In mild superficial burns, dermal capillary dilatation may cause redness, and fluid loss from the capillaries to the interstitial tissue may stimulate nerve endings and cause pain. In more severe burns, more capillary fluid accumulates in blisters formed in the dermis or at the junction with the epidermis leading to death of overlying epidermal cells. These cells will regenerate later from the adjacent epithelium. When the upper part of the dermis is also damaged regeneration takes a longer time. Deep dermal burns recover slowly resulting in thin skin. Destruction of the full thickness of skin and underlying tissue usually requires surgical intervention (Bousfield, 2002).

In addition to the local effects, more severe burn injuries cause systemic responses which could be life-threatening such as cardiovascular, respiratory, gastrointestinal, metabolic and immunological responses. Systemic hypotension and organ hypoperfusion could result from fluid loss due to increased capillary permeability as well as direct loss from the wound. When the burns size is less than 30% total body surface area (TBSA) the fluid leakage is limited to the site of injury. Haemolysis and a reduced life span of the red blood cells are responsible for anaemia after burn injury. Respiratory effects of burn injury include oedema of the airways, increased mucus production, reduced ciliary activity, bronchoconstriction and adult respiratory distress syndrome. Gastric dilatation and dysfunction of the intestines may occur. The metabolic effects of burn injury include disturbance of the thermoregulatory function of the skin, rapid breakdown of proteins and increased basal metabolic rate. Glucose tolerance is impaired and catecholamines and cortisol levels are raised. Lowered immunity is also observed in burns patients resulting from impairment of both cell mediated and humoral mechanisms (Bousfield, 2002; Hettiaratchy and Dziewulski , 2004).

Degrees of burn injuries

The depth of the injury depends on the intensity of the burning agent and the time of exposure. Depending on the depth of skin damage, burns are divided into 3 degrees: first degree (superficial) burns, second degree (partial thickness) burns, and third degree (full thickness) burns. Partial thickness burns are again subdivided into superficial and deep dermal burns. In superficial burns, only the surface epithelium is damaged with erythema, dry skin, slight oedema and mild pain. These burns heal in a few days. In partial thickness burns, both the epidermis and variable depths and structures of the dermis are damaged. In superficial partial thickness burns there is erythema, blisters, marked oedema, and pain. These burns spare the hair follicles and sweat and sebaceous glands and heal with mild or no scarring. Deep partial thickness burns involve deeper parts of the dermis with fewer blisters which may heal with scarring. In full thickness burns, all layers of the skin and variable depths of subcutaneous tissue are damaged and the skin function is lost. These burns are charred, brown or white in appearance and there is usually no pain. Full thickness burns heal with granulation tissue and scarring (Bousfield, 2002; Hettiaratchy and Dziewulski , 2004).

Mechanisms of burn injuries

Burns can be divided into several types according to the mechanism of injury (Bousfield, 2002; Hettiaratchy and Dziewulski , 2004; Fernandez-Morales *et al*. ,1997).

1. Scalds: Injuries due to exposure of the skin to hot fluids such as water, tea, milk etc.

Most scald burns are superficial but boiling fluids can cause full thickness burn. Boiling fat causes more severe burns because of its higher temperature

2. Flame burns: Injuries caused by direct contact with flames from gas, kerosene and electric equipment, open fires and other sources of flames. Flash burns are caused by momentary exposure of the skin to flames such as those produced by a high voltage electric current. Flame burns tend to be deeper than scald burns and they may be associated with inhalation injury.
3. Contact burns: Injuries caused by contact with hot objects such as cooking and heating equipment, hot kitchen utensils, hot ground and other hot objects. Friction burns may occur when skin is sheared against another surface such as in road traffic accidents.
4. Chemical burns: Injuries caused by exposure of the skin and mucous membranes to corrosive agents such as acids and alkalis, bleaches, domestic

cleaners, cement, napalm and phosphorus. The severity of injury depends on the nature and concentration of the chemical and exposure time, but alkalis usually penetrate deeper into tissues and cause more severe burns than acids.

5. Electrical burns: Injuries produced when an electric current travels through tissue while the body is earthed. A variable amount of heat and resultant tissue damage is produced by the current depending on the voltage and tissue resistance. Contact with high voltage of 1000 volts or more often produces extensive softtissue and bone necrosis while contact with very high voltages of 70,000 volts or more is invariably fatal (Hettiaratchy and Dziewulski , 2004). Contact with domestic 240 volts alternating current produces deep burns at the sites of entry and exit of the current. Electrical burns may be accompanied by arrhythmias and other injuries due to violent propulsion of the patient.

Global epidemiology of burn injuries

The true incidence of all burn injuries is difficult to estimate from hospital-based studies and indeed, the majority of published studies, which are based on admissions, do not report burn incidence. As minor injuries are less likely to attend hospitals, burns are probably more common than figures calculated from hospital-based reports and studies. A population-based study in 1997 in Spain estimates that 23% of the population interviewed had ever suffered at least one burn of any degree not necessarily requiring medical attention (Fernandez-Morales *et al.*, 1997). Another survey among rural students in China reports an annual (home and medically treated) burn incidence of 5%. A Turkish survey based on a 10-year recall of burns reports that 10-year prevalence of burns in the population was 12.6%. The estimation of the incidence of medically reported injuries is usually based on patients attending health facilities (emergency departments and burn units) in a particular area. Therefore such estimates may not be taken for national estimates but reflects incidence in the study area. A review of published papers in the developed countries in the late 1980s found that the reported incidence of all medically reported burns by different studies ranged from 200-400 per 100,000 per year. More recent papers from individual countries around the world report similar estimates. For example in Netherlands, data based on emergency department and hospital admissions suggest that the incidence of medically reported burn injuries was 420 per 100,000 per year in 1994. A study reporting on all medically reported burn injuries during 1991-2004 in Lithuania estimates the incidence as 260 per 100,000 population per year in this country. Hospital-based studies from Norway and Iran report incidence figures of 170 and 410 per 100,000 per year respectively (Salas *et al.*, 2005).

Globally, according to the WHO there were more than 7.1 million fire-related unintentional burns (X00-X09) in 2004, equivalent to a global incidence risk of 110 per 100,000 per year. The lowest incidence is reported for the Americas with 19 and the highest for South East Asia with 243 per 100,000 per year per year. It has to be said that these estimates do not include scalds, contact, chemical and electrical burns, which are not separately reported in the WHO statistics. Scalds and contact burns are important contributors in overall morbidity from burns especially in children although fire-related burns are responsible for the majority of burns deaths. The WHO estimates that 310,000 people died in fires in 2004; 69% of them were females and 31% were males, which amounts to a global mortality rate of 4.8 per 100,000 per year. The highest mortality is observed in the countries of south east Asia with 11.1 deaths per 100,000 per year and the lowest the Americas with 0.9 deaths per 100,000 per year. Fire statistics from the Fire and Rescue Services reports 489 fatalities in fires throughout the UK in the year ending September 2005 which was the lowest since1959. A two year population-based study from the northwest of England estimates mortality rate of burn as 0.5 per 100,000 per year. In the United States, the total number of deaths from unintentional burns during 2006 was 3,202 which is equal to a crude mortality rate is 1.1 per 100,000 population. Mortality rate in some other countries are reported as 1.8 per 100,000 per year in Korea, 2.3 in Chile, 4.5 in Iran, 8.0 in Lithuania and 15.1 in India. An important indicator of the outcome of burn management is in-hospital mortality which is likely to be related to case mix. Studies involving all burns and all age groups have reported variable in-hospital mortality rates including 2% in Australia; 3% in Sweden and Taiwan; 4% in Portugal, United States and 6% in UK; 7% in Netherlands; 8% in Korea; 12% in Malawi; 14% in Turkey; 20% in Iran; 20% in Lithuania; 22% in Zimbabwe; 27% in Sri Lanka and 52% in India (WHO and UNICEF,2008).

Risk factors for burns in children

Young children aged 0-5 years are generally considered one of the risk groups for burns and comprise approximately one third of burn injuries around the world. However, the reasons for the vulnerability of these children to burn injuries are not well established. The WHO report on child injuries in 2008 states that “while the existing data identify children and young people as a high-risk population for burns, information on mechanisms and causal factors is largely missing WHO and UNICEF,2008). Broadly speaking, factors related to the child and the family and housing conditions are generally thought to be important in causation of childhood burns. Identification of factors that put these children at a greater risk for burn injuries has been subject of several case-control studies.

The situation of burns wound in Iraq

Published data regarding burns in Iraq including Kurdistan are scarce. The WHO estimates that there were 3, 390 fire-related deaths in 2004 in Iraq which is equivalent to a death rate of 12.3 per 100,000 per year, which is higher than the global rate (WHO, 2004). A study reporting on 45 days experience of the Italian Red Cross in Baghdad in 2004 reports that during that short period 1,350 burn patients visited the hospital of which 48 (23 males & 25 females) were admitted (Carini *et al.*, 2005). There is no further data about the non-admitted patients but there is some analysis about the 48 admissions. The mortality was 27% among these admitted patients (13% in males and 40% in females). Eight percent of the admissions were for intentional self-harm burns and the rest were accidental including two war-related burn injuries. The majority of these admitted patients were burnt at home. An earlier study about 127 admissions reports that 46% of the admissions were male and 54% were female; the mean age was 20 years; 63% were flame injuries and 22% were scalds; and that 84% of burns happened at home. A retrospective analysis of patients admitted to the burns centre in Erbil province recently published in a local journal, provides some descriptive analysis about burn admissions in Iraqi Kurdistan. The study, comprised 54%females, children up to 12 years of age 50% and children 0-6 years 38% of the burn admissions. The most common mechanism of injury was scalds accounting for 48% of all burns (68 % in children) and flame injuries accounting for 47% of all burns (61% in adults). The TBSA burnt was more than 20% in 39% of all patients and 58% of adults. Winter was the commonest season for burns and the overall mortality was 21% (36% in adults). Another study on childhood burns (0-12 years) highlights the problem of burns amongst children in the region. There were similar numbers of males and females in this study where scalds accounted for 79% of burns and flame injuries accounted for 19%. Home was the commonest place where childhood burns occurred (75%) and winter was the commonest season (38%). The mean TBSA burnt was 12% and in-hospital mortality was 12% (65% of them caused by flame injuries). The researcher is not aware of other epidemiological studies on burn injuries in Iraqi Kurdistan. The print media regularly report on individual cases of suicide of young women by self-burning and the women's organizations have being trying to highlight the problem of self-burning which, according to them, is becoming more evident and, in absence of scientific research, appears to a local observer to be the commonest way of suicide amongst the Kurdish women. According to statistics data obtained from the Directorate of Civil Defence in Sulaymaniyah, which is the department in charge of fire and accident rescue response, there were 1461 fires throughout the year 2008 in the province of Sulaymaniyah. The majority of these fires (30%) were caused by electricity problems followed by leakages and explosions of cooking gas cylinders

(20%). These statistics report 25 deaths and 159 injuries during 2008 but it is not clear how many of these causalities were due to fires as the statistics includes in addition to fires 20 instances of floods and one bomb explosion. However, according to the department officer, around 20 fatalities were due to burring in fires on the scene. According to the practitioners in the field, burn injuries have been a cause of concern for the health department not only because of the large number of patients but also because of the cost and logistics required to maintain provision of appropriate care for the victims. Indeed this may be the reason why the burns centre has been run or supported by international agencies for the most part of its existence. In absence of detailed data on such an important public health issue, the need for further studies is quite evident. Comprehensive epidemiological studies are required to collect detailed information about burn injuries in order to be able to provide reliable analyses about the incidence, mechanisms, risk factors, circumstances, outcomes and other epidemiological features of burn injuries. Such information could provide a better insight to the problem and furnish grounds for evidence-based planning for future interventions. The main part of the current study aims to achieve this goal. In addition and since small children are globally reported to be at a higher risk, the case-control part 40of the study aims to investigate the risk factors of burn injuries amongst pre-school children aged 0-5 years (Fernandez-Morales *et al.*, 1997; Jia *et al.,* 2005; Kara *et al.*, 2008).

Treatment of burns

The standard treatment for deep partial-thickness and full-thickness burns is early excision and grafting. However, this is not always possible due to the paucity of autologus donor site available in patients with massive burn injuries1 or due to the patient's general condition. Besides, determining which burn will heal in 3 weeks (that will not need E&G) is challenging and not possible in the first few days. In these cases, temporary skin substitutes play an important role to provide transient physiologic wound closure. Several materials have been used for this purpose; yet some are not available in large quantity (such as cadaver skin) and most are very expensive (e.g. Biobrane, INTEGRA). The main principles in the treatment of minor burns are control of pain, control of infection, promote healing, and decreasing repeated trauma to the burn surface that may injure the damaged epithelium and convert a superficial burn to a deeper tissue injury. All of these goals can be achieved by using amniotic membrane dressing. It effectively relieves pain, protects from secondary wound infection, promotes healing, adheres well to the wound, is easily applicable and most importantly, is economical (Fairbairn *et al.*, 2014).

Amniotic membrane

A. History of application of amniotic membrane

Prior to the realisation of its medical and surgical applications, human amnion was the focus of myth and superstition. Being born with the fetal membranes or "caul" intact was considered extremely lucky. Children were gifted with life-long happiness, the ability to see spirits, and protection from death by arms and drowning. The magical powers of the caul were not confined to the original bearer and could be transferred by inheritance or legitimate sale. As a result, the trade of caul amulets became extremely popular, particularly between seafaring men during the 1800s at the time of the Napoleonic War.1 In 1910, Davis reported on early experience with fetal membranes in skin transplantation. Over the last century, the beneficial effects of amnion have been applied to burns, chronic vascular and diabetic ulcers, dural defects, intra-abdominal adhesions, peritoneal reconstruction, genital reconstruction, hip arthroplasty, tendon repair, nerve repair, microvascular grafts, corneal repair, intraoral reconstruction and reconstruction of the nasal lining and tympanic membrane. More recently amnion has been shown to be a viable source of stem cells with a potentially exciting future in tissue engineering and regenerative medicine. Although many of these roles are of historical interest only, an awareness of this history is an important pre-requisite for future development and innovation. It is the purpose of this article to review past and present applications of human amnion relevant to plastic surgery and how it may contribute to our future (Sedighi *et al.,* 2015).

B. Anatomy and physiology

Amnion forms during the transition of the morula into the blastocyst at approximately 7-days following fertilisation. Amnion is between 0.02 and 0.05 mm thick and consists of five distinct layers: (1) epithelium, (2) basement membrane, (3) compact layer, (4) fibroblast layer, (5) spongy layer (Figure. 1). The innermost epithelium consists of a single layer of cells in direct contact with amniotic fluid. Microvilli at the apical surface of these cells play an important role in amniotic fluid homeostasis. The basement membrane border of the cells contains blunt projections that inter-digitate with similar processes in the basement membrane, forming a densely adherent bond. The basement membrane is a thin layer composed of reticular fibers. The compact, fibroblast and spongy layers are referred to as the amniotic mesenchyme and originate from the primary extra-embryonic mesoderm of the blastocyst. The mesenchyme contains collagen Ie VII and noncollagenous

proteins such as elastin, laminin, fibronectin and vitronectin. The compact layer is composed of a dense network of fibers and is almost entirely free from cells. Abundant type I, II and III collagen and elastin within this layer endow amnion with tensile strength and elasticity. These properties help protect the fetus from mechanical stress and desiccation. The fibroblast layer is the thickest layer and is composed of a loose fibroblast network within a matrix of reticulin. The outermost spongy layer represents the transitional layer between amnion and chorion and is composed of bundles of reticulin within a background of mucin. The two layers are loosely adherent, allowing a degree of gliding during gestation and easy separation by blunt dissection during harvest. In spite of being devoid of vascularity, nerves, muscles and lymphatics, amnion is highly metabolically active. Oxygen and nutrients are obtained by diffusion from amniotic fluid and chorionic vasculature. The epithelial layer is a source of prostaglandins, particularly prostaglandin-E2, and is thought to play an important role in the initiation and maintenance of uterine contractions. The epithelium also contains human chorionic gonadotrophin receptors that regulate prostaglandin production and activity. Epithelial cells manufacture multiple vasoactive peptides, growth factors, cytokines and extracellular matrix (ECM) proteins. These biological factors may reside in the epithelium or may be transported and accumulated in the mesenchyme where they act as a reservoir from which the amnion exerts its therapeutic effects following transplantation (Dua *et al.*, 2004; Koob *et al.*, 2014).

C. Mechanism of therapeutic effect

1. As a barrier and analgesic

The application of amnion to a wound bed prevents desiccation and excessive fluid loss and provides an analgesic effect by protecting exposed nerve ends from the environment.

2. As a non-immunogenic material

Several investigators have concluded that amniotic epithelial and mesenchymal cells lack HLA class A, B, DR and co-stimulatory molecules CD-40, CD-80 and CD-86.7 In contrast, others have shown the presence of class-1 and class-1b antigens in epithelial cells, mesenchymal cells and fibroblasts.8 Radiobiological studies suggest that although amnion cells retain the ability to synthesise HLA, they do not express HLA-A, B, C or DR antigens of b-2 microglobulin on the cell surface.9 Mesenchymal stromal cells may inhibit the maturation of peripheral blood monocytes into antigenpresenting dendritic cells.

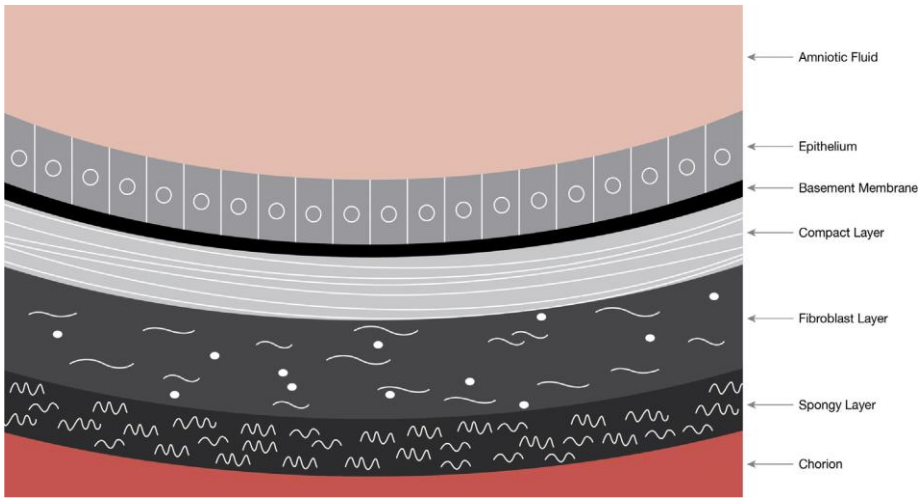

Figure. 1: Structure of amniotic membrane (Koob *et al.*, 2014).

3. As a promoter of epithelialisation and an inhibitor of fibrosis and scar

Amniotic epithelial and mesenchymal cells contain epidermal growth factor (EGF), keratinocyte growth factor (KGF), keratinocyte growth factor receptor (KGFR), hepatocyte growth factor (HGF), and hepatocyte growth factor receptor (HGFR). These growth factors are responsible for proliferation, migration and differentiation of epithelial cells and the promotion of epithelialisation.10 Basic fibroblast growth factor (bFGF), and transforming growth factor (TGF) -b1, b2, b3 have also been demonstrated in amnion cells. bFGF is a pro-angiogenic factor and plays a role in the formation of granulation tissue through the proliferation of fibroblasts. The TGF-b family is responsible for the synthesis and deposition of ECM proteins and the regulation and transformation of fibroblasts into myofibroblasts. Mesenchymal hyaluronic acid may inhibit TGF-b and the generation of excessive fibrosis and scar. This may explain the beneficial effect amnion has on scar formation and why fetal wound healing is essentially scarless.

4. As an anti-inflammatory and anti-bacterial

Amniotic epithelial cells contain interleukin 10 (IL-10) that down-regulates the expression of Th1 cytokines, major histocompatibility complex (MHC) class II antigens and costimulatory molecules on macrophages. IL-10 also enhances B-cell survival, proliferation and antibody production and has been shown to inhibit the production of proinflammatory cytokines such as interferon-g, IL-2, IL-3, tumour necrosis factor-a (TNF-a), and granulocyte macrophage colony stimulating factor

(GM-CSF). Other anti-inflammatory mediators such as IL-1 receptor antagonist and tissue inhibitors of metalloproteinase-1, 2, 3, 4 (TIMPs) have also been found in amniotic cells. Amniotic fluid contains lysozymes and immunoglobulins.13 In vitro experiments confirm reduced viability of group-A and group-B Streptococcus, Staphylococcus aureus and Staphylococcus saprophyticus in the presence of amnion. Amnion has also been shown to produce human-beta-3-defensin. These antimicrobial peptides are implicated in the resistance of epithelial surfaces to microbial colonisation and have been shown to be upregulated in inflamed amnion. Amnion epithelial cells can be induced to express intercellular adhesion molecule-1 (ICAM-1) by pro-inflammatory cytokines such as tumour necrosis factor-a (TNF-a) and IL-1b. ICAM-1 has a role in the attraction and adhesion of leukocytes andmay also have a role in signal transduction in pro-inflammatory pathways resulting in the recruitment of inflammatory mediators such as macrophages and granulocytes (Sedighi *et al.*, 2015).

5. As a regulator of angiogenesis

The angiogenic influence of amnion is uncertain. The presence of platelet derived growth factor (PDGF) and vascular endothelial derived growth factor (VEGF) are suggestive of a pro-angiogenic role. bFGF may have an even greater pro-angiogenic influence than PDGF and VEGF. However, a large amount of ophthalmological research contends that it is the ability of amnion to suppress angiogenesis that renders it useful in corneal healing. The expression of tissue inhibitors of metalloproteinase (TIMP- 1, 2, 3, 4), thromboplastin-1 and endostatin in amniotic cells supports these claims (Mohammadi *et al.,* 2011).

Amnion collection and processing

Elective cesarean section donors undergo rigorous serological screening for human immunodeficiency virus-1/2, Hepatitis B, Hepatitis C, human T-cell lymphotrophic virus, syphilis, cytomegalovirus, and tuberculosis. Following delivery, amnion is separated from the placenta by blunt dissection (Figure. 2). Once gross contaminants are removed, amnion is usually de-epithelialised to limit immunogenicity, sterilised to reduce risks of disease transmission, and preserved to improve longevity and convenience for storage. Improvements in processing have focused on preserving membrane architecture and growth factor content in order to optimise therapeutic effect. De-epithelialisation can be performed by mechanical scraping or exposure to chemicals. It is uncertain how these protocols affect the levels of growth factors and ECM proteins. Koizumi et al. showed that, although amnion denuded of its epitheliumcontained EGF, TGF-a, KGF, HGF, bFGF, TGFb1, and TGF-b2, protein

levels were reduced in comparison to samples with intact epithelium. Whether this is clinically significant is uncertain. Neurotransmitters, neurotrophic factors and neuropeptides are concentrated in the epithelium and therefore amnion with intact epithelium may be superior when applied to neural injury. In contrast, denuded amnion results in superior cell adhesion, migration and proliferation and therefore may be preferable when applied to acute and chronic wounds. As the majority of clinical applications concern wound healing, the use of denuded amnion has greater representation in the literature.

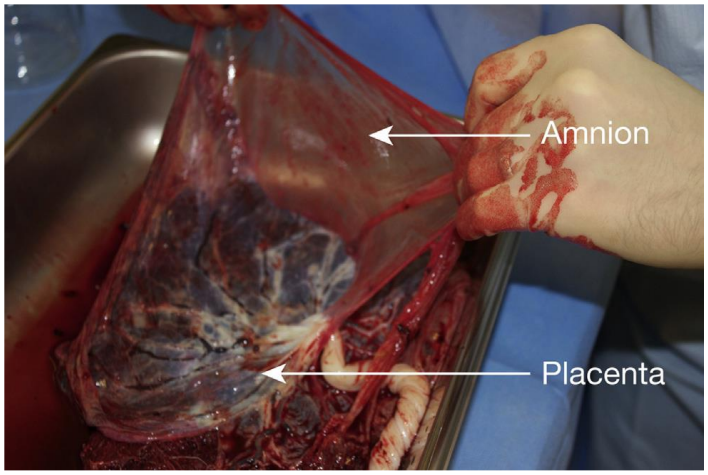

Figure 2 Amnion being bluntly dissected from human placenta (Fairbairn et al., 2014)

Developed in the late 1980s, cryopreservation in glycerol is the most widely used preservation technique. Antibacterials and anti-fungals are often added before freezing at _80 _C. Cells are devitalised although not sterilised. Viable bacteria and viruses can be present following several months of storage. The effect on biological properties is uncertain. Thomasen et al. reported no detrimental impact on sterility, histological integrity or the availability of biological mediators. Amnion cryopreserved in 50% glycerol/ DMEM at _80_ for 1-month contained EGF, TGF-a, KGF, HGF, bFGF, TGF-b1, -b2, b3, KGFR and HGFR. Cryopreservation requires expensive equipment that may be unavailable for some institutions, particularly in developing nations. Lyophilisation is an alternative technique allowing storage of amnion at room temperature, obviating the requirement for deep freeze facilities and increasing surgeon convenience. Lyophilised membranes are commonly sterilised with gamma irradiation. Concerns exist regarding detrimental changes to membrane architecture and growth factor levels. Nakamura et al. reported no significant difference in tensile properties, tissue structure or ECM composition between lyophilised, gamma-irradiated and cryopreserved membrane. Lim et al. showed that

lyophilisation reduced the levels of several growth factors and ECM proteins although there was no appreciable difference in clinical performance when compared with cryopreserved samples. Other methods of preservation and sterilisation exist although these are less well accepted. The variation in processing within the literature makes it difficult to draw definitive conclusions on the optimal method. Variation also exists amongst commercially available products (Table. 1). Independent of processing technique, several donor specific factors can influence the biochemical composition of amniotic membrane. Fairbairn *et al.*, (2014) showed that in fresh, cryopreserved and lyophilised amnion, levels of bFGF, HGF, KGF and TGF-b1 were significantly lower in those membranes of greater chronological and gestational age. Researcher found significant differences in cytokine profiles between African Americans and Caucasians. Membrane architecture and growth factor profile can also vary depending on what area of amnion a specimen originates from. As a result, standardization of collection and processing may be essential if consistent therapeutic results are to be achieved. If consistent relationships between donor variables and biochemical profile exist, it may become possible to select certain varieties of amnion for specific clinical situations. Applications relevant to plastic surgery Broadly speaking, amnion has been applied as an alternative biological dressing or has in some way augmented reconstruction. Table 2 provides examples of experimental and clinical evidence supporting therapeutic benefit according to these categories. In spite of the large evidence base, there is a paucity of well-designed, randomized controlled trials testing amnion performance against gold standard alternatives (Fairbairn *et al.*, 2014).

Applications of amniotic membrane as biological wound dressing

Burns

The history of amnion use for the management of burns is extensive. The use of amnion for corneal burns and other ophthalmological epithelial defects is commonplace and has led to the development of several commercially available products (Table 1). Membranes have been used as overlay following standard autografting and microskin grafting and also in place of conventional dressings following superficial and mid-dermal burns, including cadaveric allograft and porcine xenograft (Fairbairn *et al.*, 2014).

Acute wounds

Amnion has been used as an alternative temporary biological dressing to protect exposed viscera in cases of congenital abdominal wall defects such as omphalocele

and gastroschiasis and also full thickness defects secondary to major trauma, infection or oncological resection. Amnion provides an alternative to the Bogota bag and can form part of a staged abdominal wall reconstruction with or without negative pressure therapy (NPT).

Table 1 Commercially available human amnion products.

Manufacturer	Product	Membrane thickness	Indications for use	Processing technique
MiMedx (Marietta, Georgia)	AmnioFix membrane	50–100 microns	Dural reconstruction, spinal surgical barrier	Proprietary Purion process (dehydration and sterilization)
	AmnioFix Wrap	50–100 microns	Tendon and soft tissue inflammatory conditions	As above
	AmnioFix Injectable	N/A	Nerve and tendon repair	As above
	EpiFix	50–100 microns	Chronic and acute partial and full thickness wounds	As above
Bio-Tissue Inc (Miami, Florida)	Prokera corneal bandage	50–100 microns	Corneal erosion, infectious and inflammatory keratitis, herpes, superficial epithelial defects	Proprietary CryoTek process (cryopreservation)
	AmnioGraft	50–100 microns	Chemical burns, Pterygium, corneal defects, leaking glaucoma blebs, Stevens-Johnson syndrome, Strabismus	As above
	AmnioGuard	300–400 microns	Coverage of glaucoma drainage devices	As above
AcelaGraft Cellular Therapeutics (Cedar Knolls, New Jersey)	AcelaGraft	50–100 microns	General wound dressing and ophthalmic wounds	Deoxycholic acid, gel drying, electron beam irradiation

Table.1: shows the amniotic membrane products

Chronic wounds

Chronic wounds represent a major financial burden on healthcare services worldwide. Multiple studies have reported superior wound healing following the application of amnion to chronic leg ulcers of varying aetiology. Areas of pressure necrosis have also been treated although the suitability of amnion in these complex and often extensive wounds is most likely limited to only the most early and superficial cases. Amnion has also been applied to areas of stalled healing following large traumatic soft tissue loss in patients unfit for complex reconstruction. In each of these situations, amnion can be applied in conjunction with NPT (Jones et al., 2012).

Reconstruction Dural repair

Amnion has been used to reconstruct dural defects in the skull base and in cases of myelomeningocele. Water tight closure in these situations is essential in order to prevent CSF leak and potentially life threatening infection. Although synthetic materials are available in these situations, autologous solutions are preferred. In congenital anomalies such as myelomeningocele, amnion can be applied as an

autograft immediately or as a delayed procedure following storage. When soft tissue defects are large, amnion can form part of a layered closure under loco-regional or free tissue transfer. Amnion may support underlying neurological tissue through the production of neurotrophic factors such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and brain natriuretic peptide (BNP) (Fairbairn *et al.*, 2014)..

Tissue engineering and regenerative medicine

Amnion as a scaffold

Biological scaffolds require the presence of extracellular matrix proteins such as collagen, laminin and fibronectin. Adhesion molecules specific to these proteins facilitate cell adhesion, transmembrane receptor activation and intracellular signalling cascades that regulate cell migration, proliferation, differentiation and apoptosis. Ideal scaffolds are biocompatible, mechanically stable, flexible, resorbable at a rate consistent with repair and allow the incorporation of growth factors and genetic materials. Amnion basement membrane contains collagen III, IV and other glycoproteins such as laminin and fibronectin. Amnion scaffolds have been used to cultivate epithelial cells in vitro before in vivo transplantation. This has been used to reconstruct corneal surfaces following chemical burns, limbal stem cell deficiency and other related pathology. Amnion scaffolds seeded with human keratinocytes have generated living skin equivalents and have been successfully transplanted into an animal model. Denuded amnion has been used as a carrier matrix for chondrocytes and cartilage regeneration. Amnion seeded with human umbilical vein endothelial cells and human vascular smooth muscle cells has been rolled into a cell dense, mechanically stable, multi-layered blood vessel conduit. Although growth factor levels in denuded amnion may be reduced, several studies have suggested scaffolding function is more effective in the absence of epithelium. Due to the interference of hemidesmosome formation, amniotic epithelium may hinder uniform cell expansion.

Amnion as an alternative source of stem cells

The use of pluripotent embryonic stem cells (ESCs) is hindered by ethical controversy. Mesenchymal stem cells (MSCs) are a less controversial, non-embryonic source of multipotent cells. Bone marrow mesenchymal stem cells (BM-MSCs) are perhaps the gold standard adult multipotent cell. However, due to the invasive and painful nature of harvest, alternatives such as adipose derived

mesenchymal stem cells (AD-MSCs) have become popular. Adipose tissue is abundant, readily accessible with low morbidity, provides cell numbers and stem cell fractions that greatly exceed that of BM-MSCs, and have superior proliferation capacity and differentiation potential in vitro. Adipose derived stem cells can also be induced into pluripotent cells. These cells are reprogrammed into pluripotency by inducing the expression of transcription factors characteristic of undifferentiated embryonic stem cells. Several limitations of AD-MSCs exist. Cell populations are not homogenous. Considerable variations in phenotype, proliferative capacity and differentiation potential exist between and within individuals. Proliferative capacity and differentiation potential may decrease with donor age, a characteristic shared by all adult derived MSCs. The secretion of tumour promoting factors such as IL-6 and the pro-angiogenic effect of these cells have also raised concerns regarding malignant transformation. With regards to induced pluripotency, the persistence of source cell epigenetic memory may render these cells unstable and unpredictable. Amnion has advantages over all adult derived MSCs. Amnion supply is unlimited and is arguably more convenient to obtain than adipose tissue. Total cell number and stem cell fraction from amnion is thought to greatly exceed both BM-MSCs and AD-MSCs. In addition to amnion, placental tissue provides chorionic membrane, chorionic villi, maternal decidua, umbilical cord, umbilical cord blood and Whartons jelly (Lopez-Valladares *et al.,* 2010) These provide additional MSCs and also embryonic populations such as endothelial and haematopoietic stem cells. Proliferative capacity and differentiation potential of amnion derived cells is thought to exceed that of adipose tissue. Derivatives from all three germ layers such as adipogenic, osteogenic, chondrogenic, hepatic, pancreatic, cardiac, vascular and neural cells have been cultured and shown to possess reparative and functional capabilities. Placental cells of fetal origin (amnion, chorion, chrionic villi) may have greater differentiation potential than those of maternal origin (decidua). Fetal origins may also prevent age related reductions in proliferative and differentiation potential characteristic of adult cells. Due to a maximum gestational age of 9e10 months, it is also likely that amnion provides a population of cells that have accumulated less genetic damage than adult sources. It is currently uncertain whether amnion cells are truly pluripotent or whether multiple sub-populations of multipotent stem cells exist. The existence of multiple subpopulations is potentially problematic. Not unlike growth factor level, the proliferative and differentiation characteristics of these cells may vary according to membrane location, gestational and donor age, race and processing technique. In addition, different methods of culture, isolation and expansion may artificially select certain subpopulations and obscure true biological activity. Pluripotency is supported by the identification in amniotic cells of multiple molecular markers typically found on embryonic stem cells, such as octamer-4 (OCT-4), NANOG, sexdetermining Y-box-2 (SOX-2), Lefty-A, FGF-4, REX-1 and

teratocarcinoma derived growth factor-1 (TDGF-1). OCT-4 is responsible for the maintenance of pluripotency and it has been shown that the level of this marker decreases with increasing cellular differentiation. Embryonic stem cells are derived from the inner cell mass of the blastocyst, which in turn gives rise to the epiblast. The epiblast, from which the amnion is derived, gives rise to all 3 germ cell layers. It is therefore possible that amniotic cells retain epiblastic pluripotency. In addition, gastrulation plays an important role in the differentiation and determination of cell fate. Amnion forms prior to this phase and it is therefore possible that these cells are pluripotent (Russo *et al.*, 2012).

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).

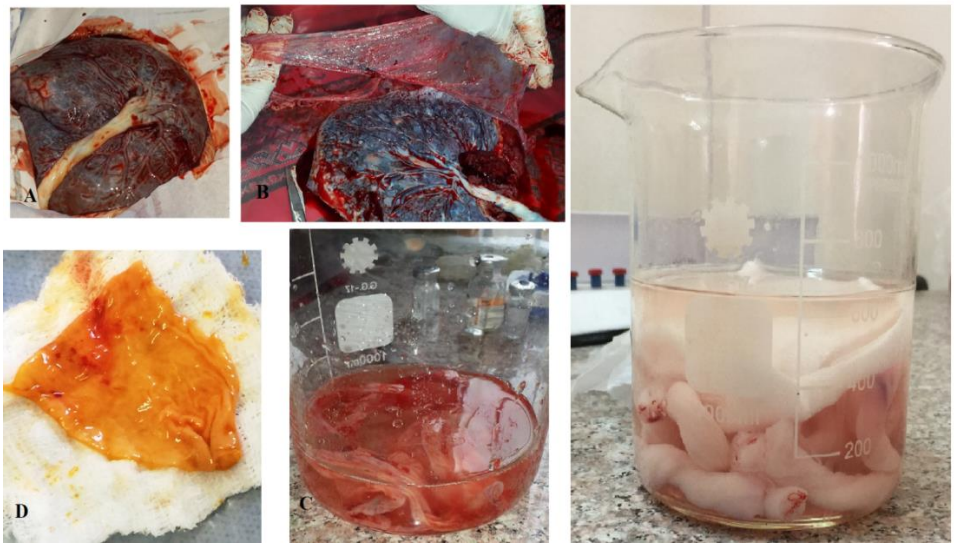

Figure.1: Shows the procedures in the collection of human amniotic membrane

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.

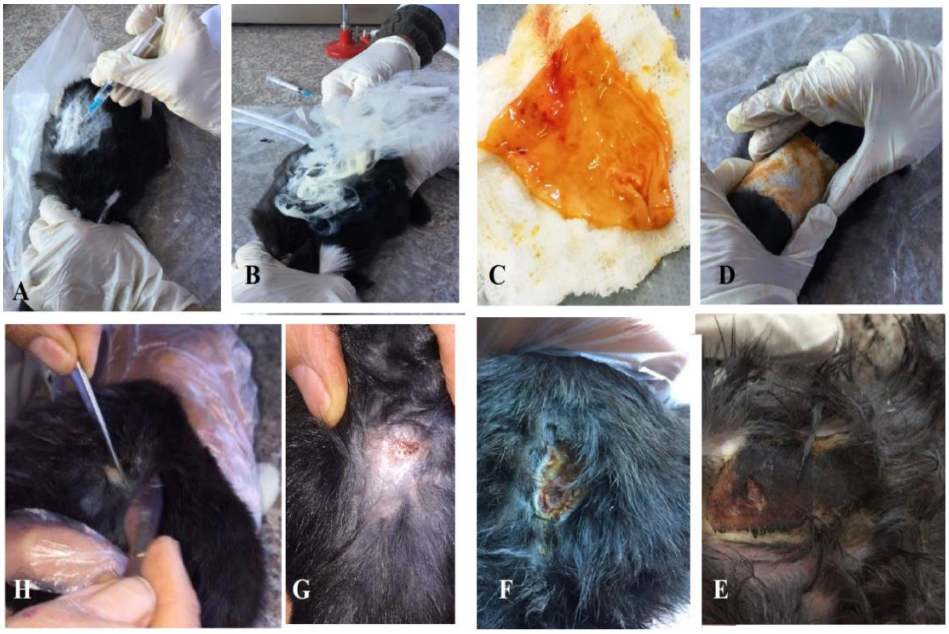

Figure. 2: shows the procedures in the induction of the burns wound and method of treatment and healing procedures.

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).

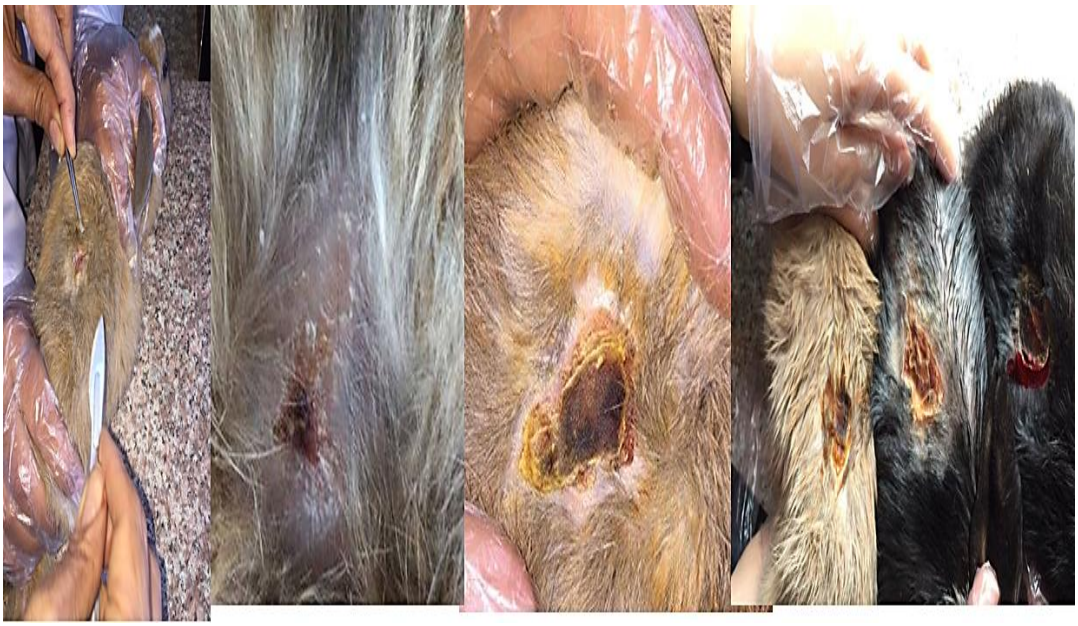

Figure.3: Healing features in the amniotic membrane treated group in compare to control group

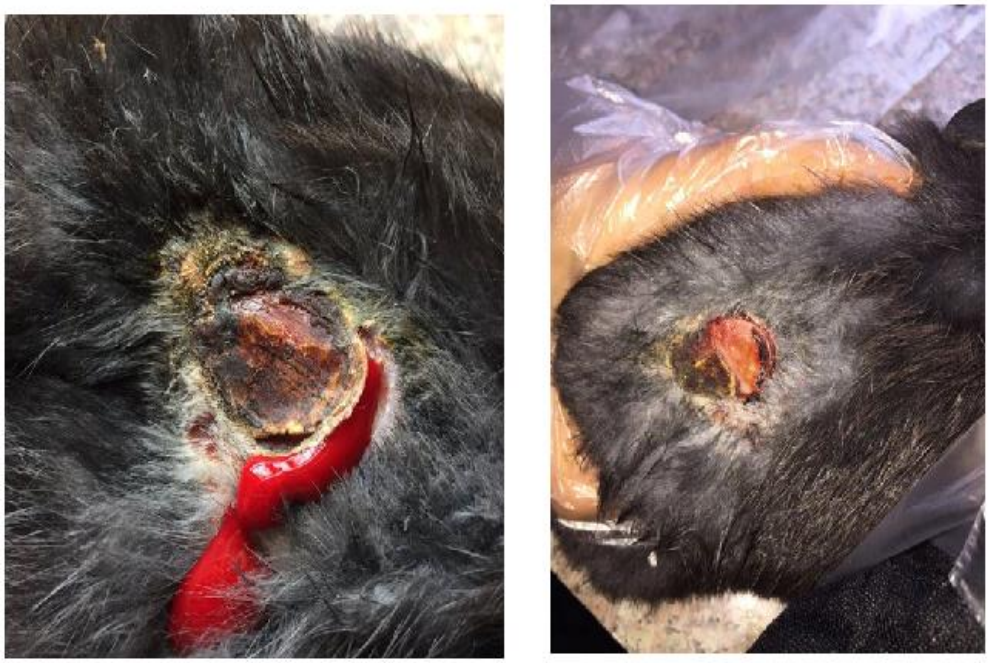

Figure. 4: Healing features in the non -treated group (control group).

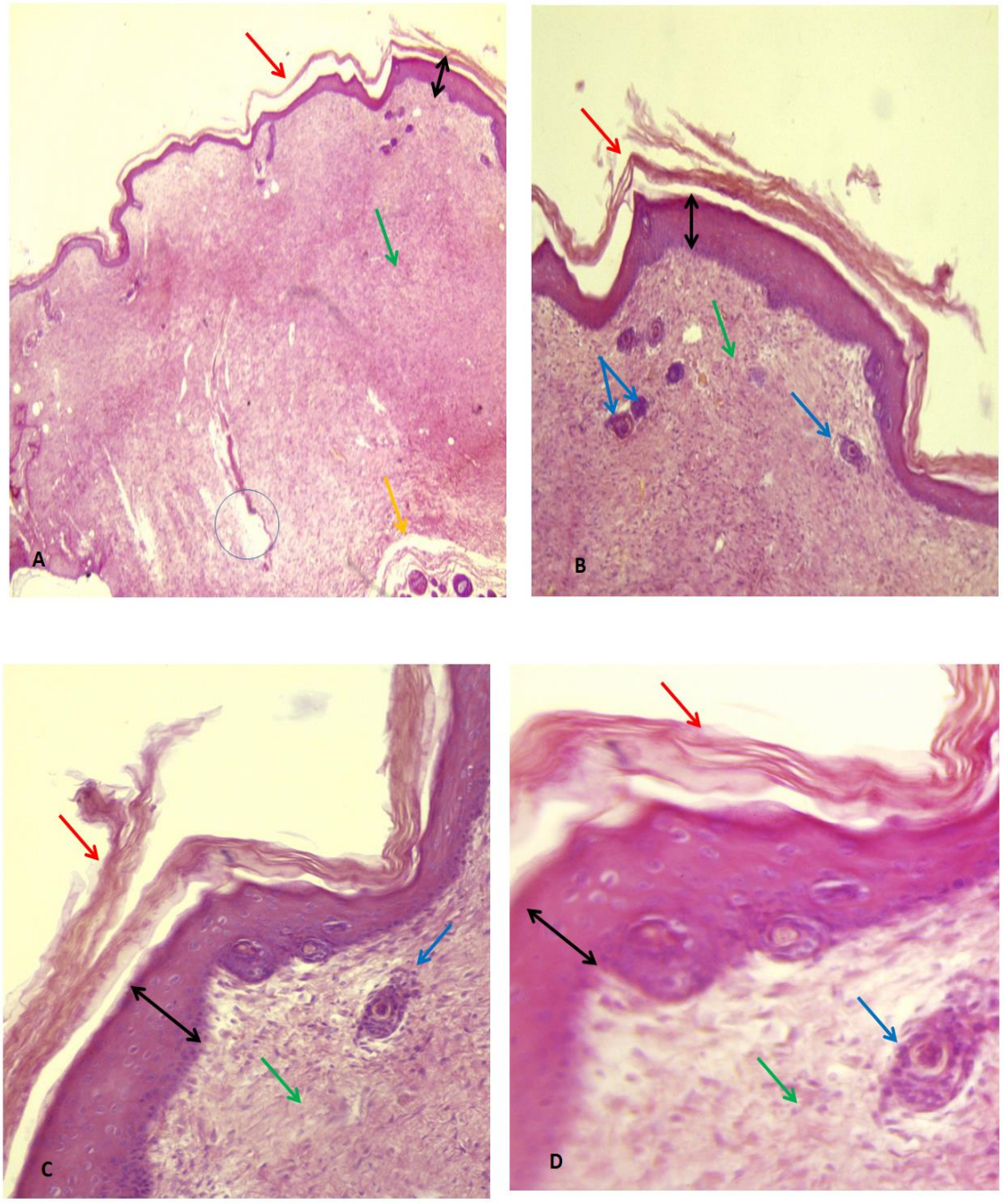

Figure 5: show the histological appearance in the amniotic membrane treated group

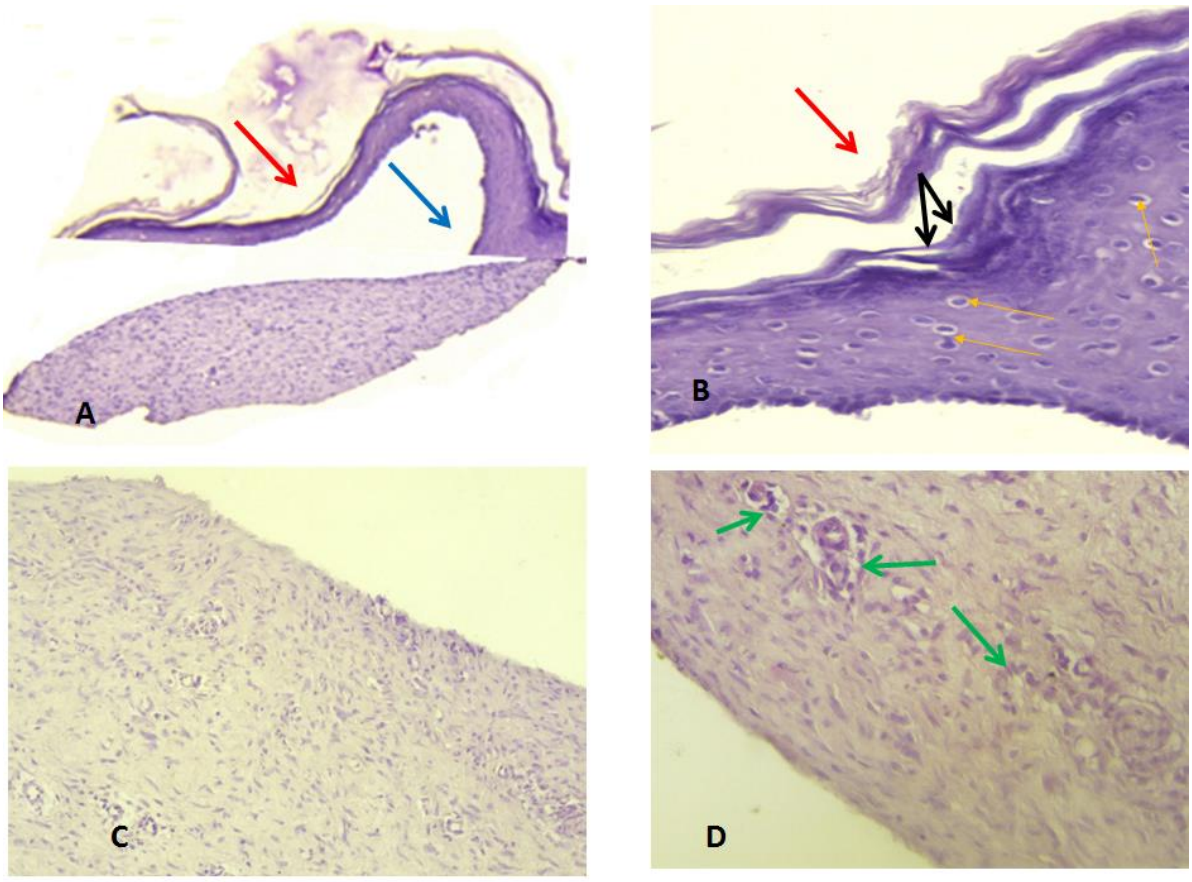

Figure 5: show the histological appearance in the amniotic membrane treated group

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. However, the results of this study are incompatible with the results reported by Sukari *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 *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). 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.

Conclusions

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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