Comparative evaluation of bovine pericardial membrane and amniotic membrane in wounds skin healing in rabbits

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Summary

The present study aimed to compare between the amniotic and pericardial membranes for the wound dressing models. Ten local breed rabbits have been used in the current study. They were divided into two main equal groups: Experiment (Amniotic and pericardial membranes) and control group. According to healing periods each group was subdivided into four subgroups (7, 14, 21 and 28) days post wounding. Specimens of amniotic membranes were collected from full term pregnant cows, and pericardium was obtained from abattoir immediately after slaughtering. The study was conducted on skin wound for experiment application site as full-thickness skin wounds (3×3 cm) were done on the dorsal thoracic sides. Histopathological evaluation of study depended on the degrees of inflammatory exudate, epithelialization, fibroplasia and type of leukocytes infiltration. Results revealed marked reduction of the inflammatory phase during all periods of post treatment with amniotic membrane, the degree of epithelization and fibroplasia in addition to angiogenesis were enhanced in amniotic and pericardial dressed wounds during period of 14 and 21 days. The study concluded that no significant differences are between the pericardial membrane and of amniotic membrane in wound healing of skin.

Keywords: **Amniotic, Pericardial, Dressing, Wound healing**.

Introduction

Acute wounds mostly occur by trauma (burns, lacerations, or abrasions); these acute wounds can lead to complications such as poor healing and infection if not managed properly through removal of necrotic tissue, exploration for underlying injuries, control of bacterial burden and appropriate closure (1). Open wounds are particularly prone to infection, especially by bacteria, and also provide an entry point for systemic infections. Infected wounds heal less rapidly and also often result in the formation of unpleasant exudates and toxins that will be produced with concomitant killing of regenerating cells. Consequently, there is a need to stimulate healing and restore the normal functions of the affected part of the body to ease the discomfort and pain associated with wounds, preventing infection, and activating tissue repair processes. Antibacterial and healing compounds in a traditional remedy can induce this occurrence and may be beneficial in treating wounds (2). Amniotic membrane (AM), the inner layer of the fetal membranes has been investigated as an alternative biomaterial for various purposes in wound-healing; it was used as a wound dressing, with good qualities as compared with conventional dressings (3). Collagen-based matrices have been used as wound covers to treat chronic wounds and have been shown to improve and expedite healing; these matrices may consist of allogeneic, xenogeneic, or chemical constructs that act as a temporary scaffold for cell migration and activity that is eventually displaced by the host tissue (4 and 5). The objective of this study was to compare pericardial membrane and amniotic membrane in acute skin wound.

Materials and Methods

Bovine pericardium was obtained from the local abattoir, immediately after slaughtering. The pericardium was submerged in saline solution. The collected tissue was gently rinsed with saline to get rid of the adhered blood. Mechanical cleansing was performed manually to eliminate all undesired associated fat and connective tissues from the pericardium using dry gauze. The tissue was cut into (4×4cm) size pieces and decellularized in a mixture of acetic acid (0.1%) and ethanol (4%) for two hours and cleansed by phosphate buffered saline (PBS) and by deionized water

for 15 min. (6 and 7). The prepared cellular tissue matrices were stored at (4°C) in PBS containing 1% gentamycin (8). The amniotic membrane was obtained from pregnant cow after parturition, rinsed three times in sterile physiological saline solution in order to dislodge the clots, the membrane is agitated thoroughly during rinsing and occasionally gauze is used to remove the clots between rinses (9). The decellularization and storage was done as previously mentioned for pericardium preparation.

Ten apparently healthy male of local breed rabbits, aged six months and weighing from (1.5 - 2) kg were used for the present study. The dorsal surface of thoracic regions was prepared aseptically for the wounding. Thirty minutes prior to wounding, Penicillin-Streptomycin was administered I.M., in a dose (20000 IU/kg) and (10 mg/kg B.W) respectively. The rabbits were premeditated by intramuscular injection of Acepromazine (0.05 mg/kg), local anesthesia was accomplished by Ketamine Hydrochloride (35 mg/kg) and xylazine (5 mg/kg), three square full-thickness skin wounds (3×3 cm) were created on the dorsal thoracic sides of each rabbit (two wounds opposed to each other and the other wound for about 5 cm below them). The animals were submitted to three equal groups, the control group (CG), the pericardium group (PG) and the amniotic membrane group (AG). All wounds were not sutured (opened wounds). The control wounds were dressed by gauze only immediately after wounding, while in second group, the wounds were dressed by using the pericardium membrane which prepared previously, and the third group, the created wound dressed using the amniotic membrane prepared as previously, then in both treated groups the wound sites were covered by sterile gauze and bandaging to secure the membranes in their position. The bandages have been changed gently after 48 hrs.

All experimental animals were clinically observed daily for any complications and the wound site photographed immediately after wounding and then on days (7, 14, 21 and 28) post wounding. The percentage of epithelialization, wound contraction and total wound healing were calculated for each wound using equation: [(A0 – At)/A0] × 100 = % of wound closure where A0 is the original wound area, and at is the area of wound at the time of biopsy (10).

The histopathological evaluations were performed at days (7, 14, 21 and 28) after wounding, for all groups. For biopsies collection full-thickness skin ($1cm^3$) were harvested and fixed in (10%) neutral buffer formalin solution. The tissue specimens were processed in a tissue processor for paraffin technique. Tissue sections were cut at 5-6 µm and stained with Hematoxylin and Eosin and Masson's trichrome stains (11). The histopathological analysis was performed by graded subjectively as mild, moderate and severe for inflammatory phase included (inflammatory exudate and infiltration of whatever mononuclear leukocytes or polymorphic nuclear leukocytes) and epidermal or dermal remodeling phase included; re-epithelization, fibroblast proliferation and collagen depositions in dermis (12). The assessment of epithelialization was dependent on the proliferation of basal layer during periods and keratinization of superficial layer of epithelium and epithelial heights.

Data are expressed as Mean±Standared Error (M±SE). Statistical analysis was carried out on the load bearing data using two-ways, Analysis of Variance (ANOVA) in addition to Least Significant Difference (LSD). p-value <0.05 was considered to indicate a statistical differences (13).

Results and Discussion

No complications such as infection, exudation or rejection were observed in all animals during the experimental period, and all animals showed normal appetite and normal physiological parameters.

The macroscopic finding in control group was that the wound area contracted progressively at the experimental periods, the calculation of wound closure was 10%, 25%, 75% at days 7, 14, 21 respectively, while in second group (PG) the wound contracted progressively with time and the closure percent was 20%, 50% and 100% at same periods, and the pericardial membrane adhered to the center of the wound, in the third group (AG) the amniotic membrane more firmly

adhered to the wound area and wound contraction and re-epithelization more rapidly and the closure percent was 35%, 50% and 100% at the same periods as shown in (Fig. 1).

Histopathological evaluation at 7 days in control: The sections showed very thick fibrin clot covering; the injured area with a thick layer of necrotic tissue those separated from underlying dermis showed sever hemorrhage, edema, and infiltration of inflammatory cells (Fig. 2- 4). These observation were recorded by (14 and 15) who notesd that at 7 days post injury in rabbits, the presence of inflammation was recorded during first three days in control rabbits.

Amniotic membrane group: The sections showed slightly thick layer of fibrin clot, the underneath dermis deposition of fibrin threads with mild infiltration of polymorphic nuclear leukocytes. The signs of regeneration was mild at the deepest part of dermis characterized by proliferation of fibroblast with marked angiogenesis (Fig. 5 and 6) (Table, 1); the present result revealed mild inflammatory reaction and angiogenesis during the 7th day, this agreed with (15 and 16) in amniotic treated rabbit, but the present study showed the infiltration of polymorphic nuclear leukocytes mainly neutrophils such records were reported by (14) in rabbits. The present result suggested that the reduce infiltration of leukocytes during 1st and 7th days was associated with antioxidant effect of amniotic membrane; consequently reduce chemotaxis factors were reduced (14 and 17) who estimated the free radicals and antioxidants in specimens of skin that dressed with amniotic membrane; also (18) referred to role of neutrophils in releasing chemokines and cytokines that induced oxidative stress; on the other hand (19) referred to the feature of amniotic membrane in induction of leukocytes apoptosis and consequently reducing acute inflammation.

On the other hand the result revealed mild proliferation of fibroblast not recorded by (14-16) and little inflammatory reaction. This suggested that due to presence of activities of antibacterial agent of amniotic membrane (20), this activity was due to certain agents such as lysozyme, transferrin, progesterone and immunoglobulin (21 and 22).

The present result suggested that the amniotic membrane had a good adaptation as dressing membrane for the injured skin characterized by no granulation, no edematous exudation and on other pathognomonic signs of inflammation. The fibrin deposition was the exudate in amniotic membrane similar result recorded by (16); also the activity of amniotic membrane in reducing wound exudation throughout it's highly adhesiveness activity to wound as mentioned by (23 and 24). At this period the epithelization was recorded by (14) at 7 days in rabbits.

Pericardium membrane: At the 7th days the section revealed thickest fibrin clot (Fig. 7) and (Table, 1). The dermis showed sever proliferation of fibroblast with marked hemorrhagic foci (Fig. 8). There was very thick inflammatory zone between the clot and dermis which mainly showed sever infiltration of polymorphic nuclear cells and fibrin deposits with moderate collagen production (Fig. 9) and (Table.1); the present finding revealed sever inflammatory phase in treatment with pericardial membrane that made such type of biological dressing more sensitive. The marked proliferation of fibroblasts was sever as compared with those in amniotic membrane.

At 14 days in control group: The sections revealed that, the epidermis showed poor epithelization with epithelial height (57.2 ±1.1µm) (Table, 2). The dermis showed cellular connective tissue characterized by marked angiogenesis, formation of new hair follicles and moderate infiltration of polymorphic nuclear leukocytes (Fig. 10 and 11) (Table, 1); the present result revealed delayed epithelization as compared with the result of **(**15**)** in control group, also the epithelization was advanced by (14) at 7 days post injury in rabbits.

While at the 14th days, in Amniotic membrane and pericardium membranes dressing, the epidermis revealed well epithelization characterized by mitotic figures at the stratum basalis cells layer and the epithelial height was significantly increased with dressing by the amniotic membrane (88.5±2.4µm); while with pericardial membranes the epithelial height was (77.8±1.7µm) (Table, 2) on the other hand the

epithelium showed keratinization. The dermis showed sever proliferation of fibroblasts organized in parallel bundles with collagen deposition and marked angiogenesis with mild infiltration of polymorphic nuclear leukocytes (Fig. 12- 14) (Table, 1). The present result agreed with results of (14 and 16), on the other hand well epithelization was observed in present study not recorded by (16). The present results revealed epithelization with significant epithelial height related to contents of the amniotic membrane for certain factors those enhance epithelialization (20, 24 and 25), while (15) had recorded the epithelization at three days in rabbits treated with amniotic membrane; this suggested that this could be related to the site of application whatever in external wound or internal; this agreed with opinion of (15) who mentioned that amniotic membrane acted as barrier covering the wound surface and protecting the newly formed epithelium which would enhance the overall re-epithelialization process. Also other recodes mentioned certain matrix proteins promoting the migration, adhesion, and differentiation of epithelial cells (26-28) who referred to using the amniotic membrane act as moistures maintenance, promoting epithelialization and wound contraction. The present result revealed severs fibroplasia that compatible with result of (14).

The present study proved that bovine amniotic and pericardial membranes could be used only as dressing for rabbit skin injuries. They were not anastomosed with the underlying tissue and blood vessels; this prof was supported by (29). Also the present study revealed that the dressing by pericardial membrane was characterized by marked proliferation of fibroblasts so was as that in dressing by amniotic membrane; this suggested that the content of both membranes in connective tissue growth factor (CTGF) that played as fibrogenic cytokine as highly expressed in wound healing and fibrotic lesions (30). Also the re-epithelization by pericardial membrane was fast and prolonged (Table, 1) that suggested that the proliferation and adherent of mesothelium cells of the pericardial membrane into the wounded (31); consequently the closure of wound was the result of cells migration but, the process was accompanied by an increase in proliferation of cells distributed through the wound this in agreement with (32).

At 21 days in control group, the sections revealed formation of hair follicles with repithelization, the epithelial height was (74.5±3.1µm). The dermis showed slightly organized fibroplasia with mild infiltration of polymorphic nuclear leukocytes (Fig. 15 and 16).

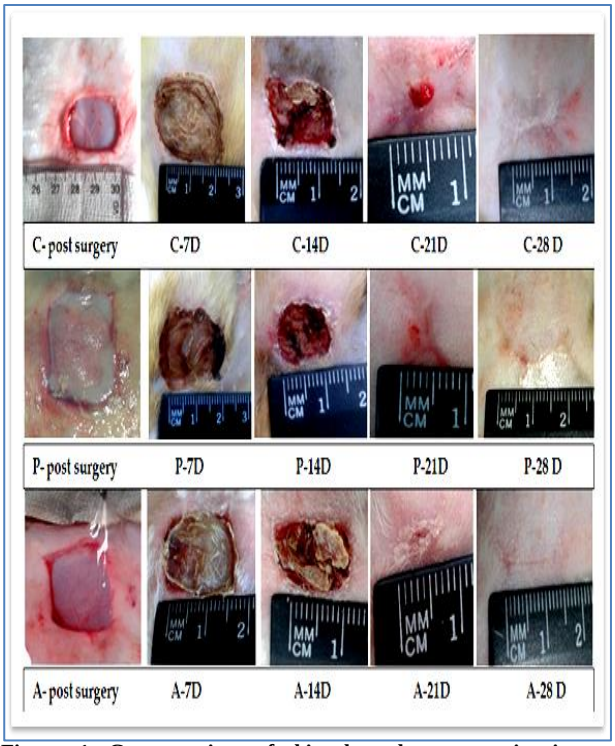
Amniotic membrane showed a continuation of moderated epithelization and the epithelial height was significantly increased (91.9±1.2 µm) (Table, 2). There were observed well organization of fibroblasts, collagen bundles deposits and angiogenesis with proliferation of hair follicle with no infiltration of leukocytes (Fig.17) and (Table, 1); the present result revealed no leukocytes infiltration during this period; this agreed with (16); the present result showed a well organization of fibroblasts incompatible with (16). On the other hand epithelization was still continuous in this period as that recorded by (16); the present study revealed a significant epithelial height (Table, 2).

Pericardial membrane showed advanced repithelization, and the epithelial height was insignificantly increased (79.9±3.1µm) (Table, 2). The inflammatory reaction was obscured with well-organized fibroblast and collagen bundles deposit (Fig. 18) (Table, 1); the present result revealed no inflammatory reaction during this period and a well organized fibrogenesis as those in amniotic membrane; the epithelization was more advanced showed thick layer of epithelium

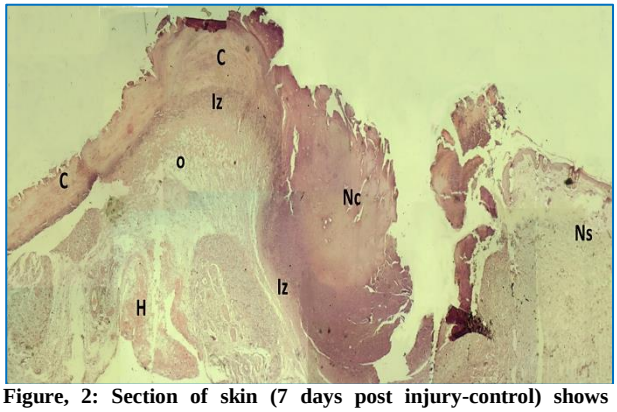
At 28 days in the control group, the sections revealed that the epithelia height was (95.9±1.5µm). Formation of sebaceous gland and fibrogenesis within the dermis with no polymorphic nuclear leukocytes, the absence of leukocytes agreed with the result of (16). In Amniotic membrane the results showed complete epithelization with significant epithelial height of (108.3±0.4µm) (Table, 2). Formation of sebaceous glands and mild proliferation of fibroblasts and collagen production with no infiltration of leukocytes (Fig. 19 and 20), (Table, 1), the absent of leukocytes agreed with result of (16); the present result showed mild fibrogenesis as

incompatible with (16). While in Pericardial membrane the sections showed significant epithelial height (105.1±.9µm) (Table, 2), with the formation of sebaceous glands and hair follicles with mild proliferation of fibroblast and little leukocytes (Fig. 21 and 22) and (Table, 1).

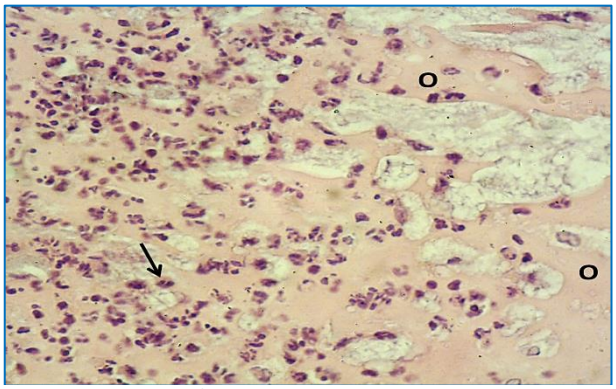
In Conclusions, the uses of bovine amniotic and pericardial membranes for rabbit skin injuries have no significant differences events. The amniotic and pericardial membranes have beneficial effect in promoting skin healing injuries in rabbits.



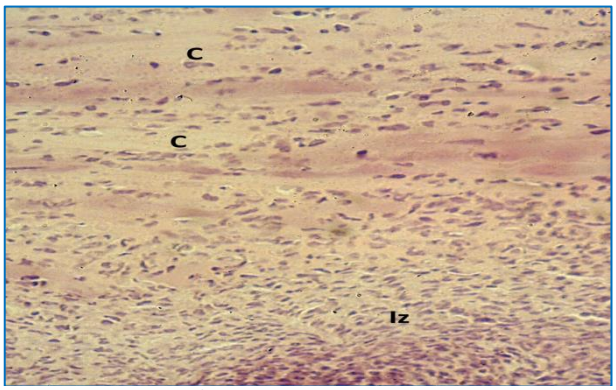
Figure, 1: Gross sections of skin showed macroscopic picture macroscopic picture of the of three groups at the different periods, C (control), P(pericardium), A(Amniotic, D(day).



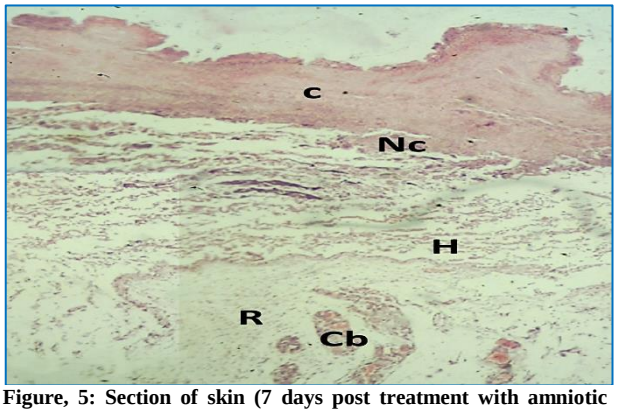
Figure, 2: Section of skin (7 days post injury-control) shows nflammatory zone (Iz), hemorrhage (H), fibrin clot (C), edema (O) and areas of normal skin (Ns). H and E 40x.



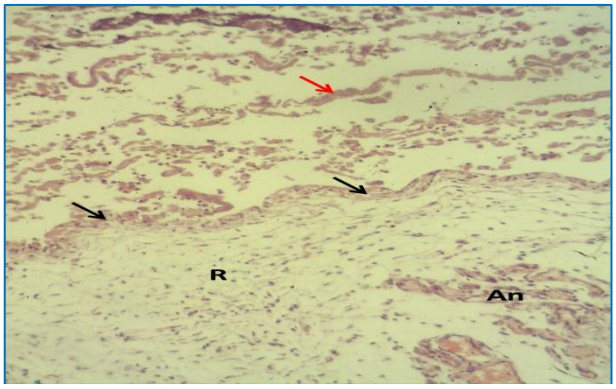
Figure, 3: Section of skin (7 days post injury-control) shows: Inflammatory cells (arrow), (H), edema. H and E stain. 400x.



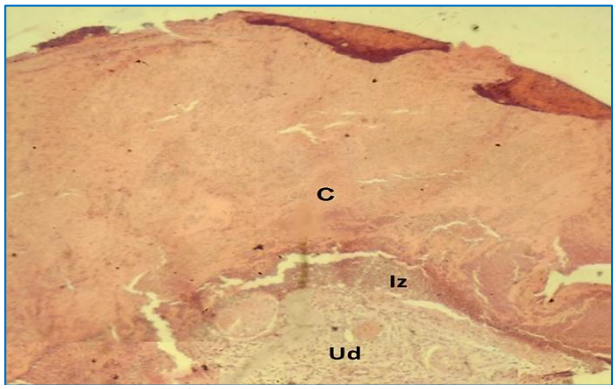
Figure, 4: Section of skin (7 days post injury-control) shows: Inflammatory zone (Iz) and clot fibrin. H and E stain. 400x.



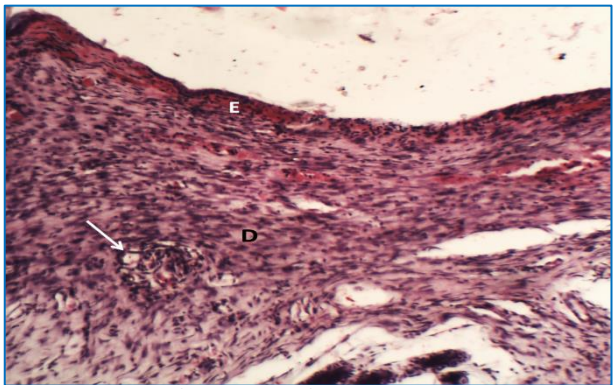
Figure, 5: Section of skin (7 days post treatment with amniotic membrane). Fibrin clot (C), necrotized cells (Nc), hemorrhage (H), regeneration (R), and blood vessels (Cb). Hand E, 40x.



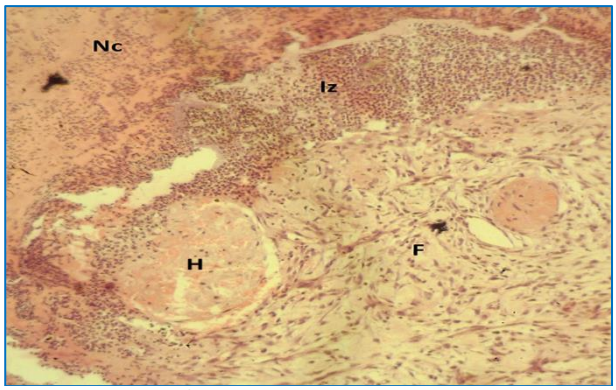
Figure, 6: Magnified section of skin (7 days post treatment with amniotic membrane) shows regeneration (R), newly formed blood vessels (An), line of thin inflammatory zone (black arrows) and fibrin thread (red arrow). H and E, 100x.



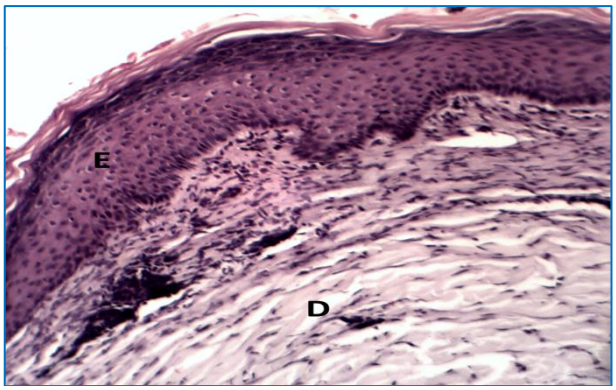
Figure, 7: Section of skin (7 days post treatment with pericardial craft) shows thick fibrin clot (C), inflammatory zone (Iz), and underlying dermis (Ud). H and E, 40x.



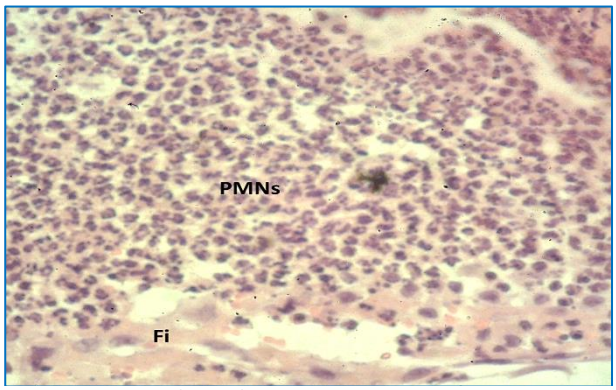
Figure, 11: Magnified section of skin (14 days -control) shows epidermis (E), dermis (D), and blood vessels (Arrows). H and E, 100x.



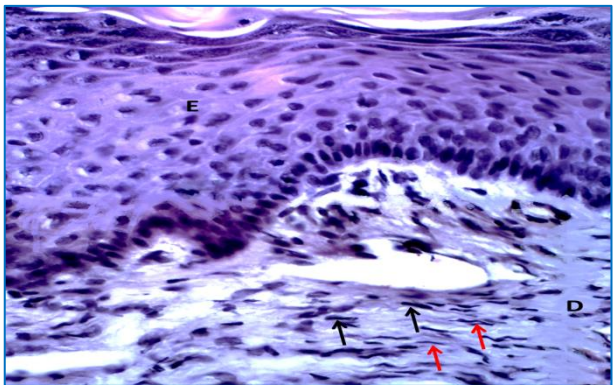
Figure, 8: Section of skin (7 days post treatment with pericardial craft) shows necrotized cells (Nc), inflammatory zone (Iz), fibrosis at the underlying dermis (F) and hemorrhage (H) H and E, 100x.



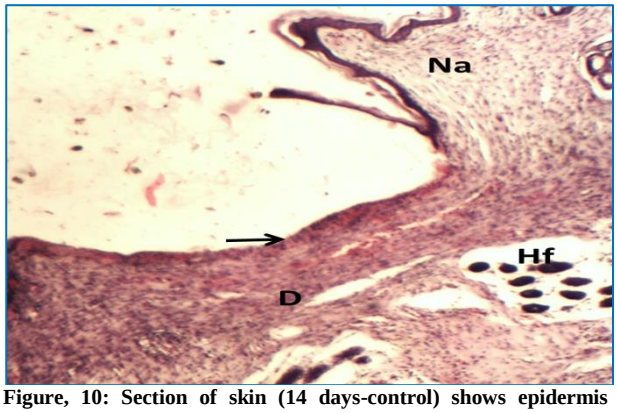
Figure, 12: Section of skin (14 days post treatment with amniotic membrane) shows epidermis (E) and dermis (D). H and E, 40 x.



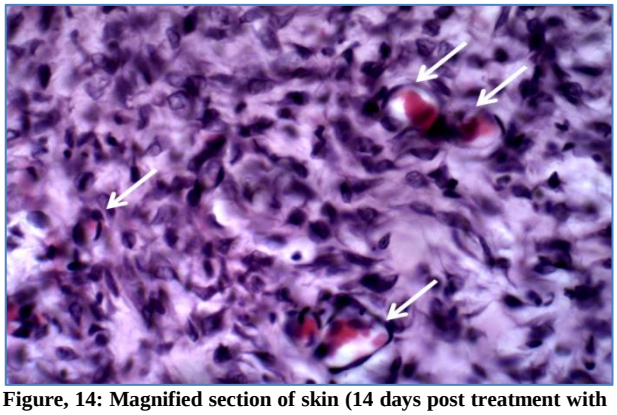
Figure, 9: magnified of skin (7 days post treatment with pericardial craft): Inflammatory zone composed of polymorphic nuclear cells (PMNs) and fibrin deposit (Fi). H and E stain, 400x.



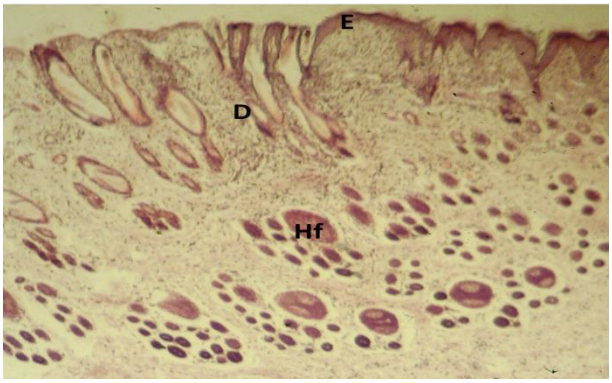
Figure, 13: Magnified section of skin (14 days post treatment with amniotic membrane) shows epidermis (E), dermis (D), and well organized fibroblast and collagen bundles (Black arrows) and fibrocytes (red arrows). H and E,400x.



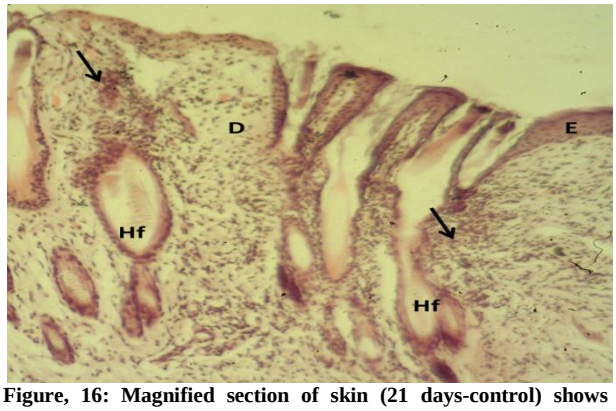
Figure, 10: Section of skin (14 days-control) shows epidermis (arrow), dermis (D), hair follicle (Hf), and normal area (Na) H and E, 40x.



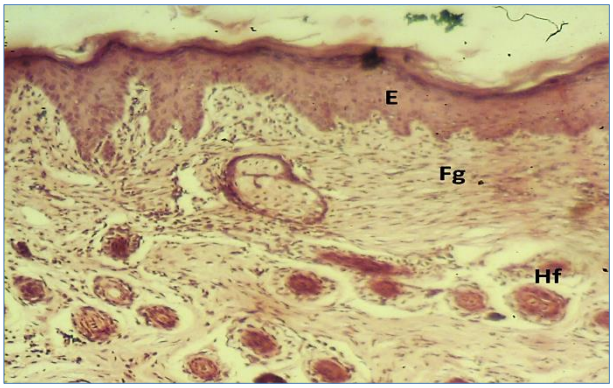
Figure, 14: Magnified section of skin (14 days post treatment with amniotic membrane) shows: marked angiogenesis within well-organized collagen bundles (Arrows), H and E, 400x.



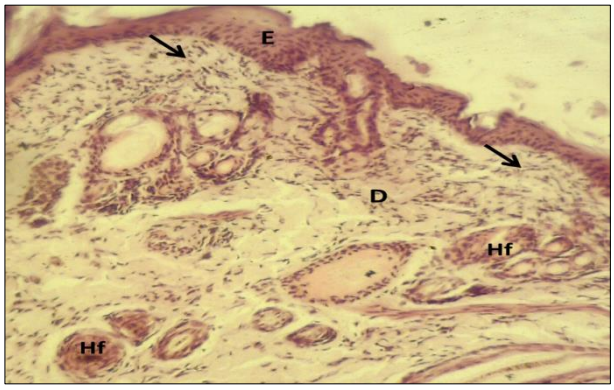
Figure, 15: Section of skin (21 days - control) shows epidermis (E) and dermis (D). H and E, 40 x.



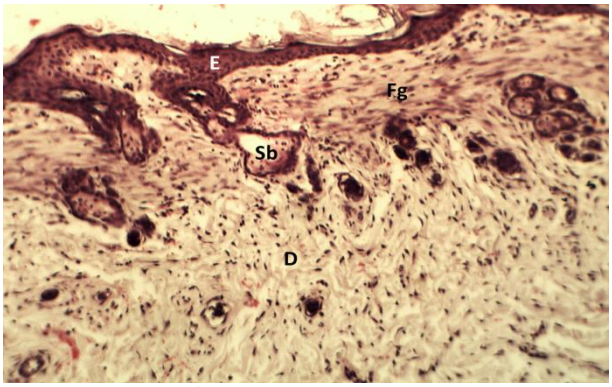
Figure, 16: Magnified section of skin (21 days-control) shows epidermis (E) and dermis (D), hair follicle (Hf) and aggregation of inflammatory cells (arrow) H and E, 100 x.



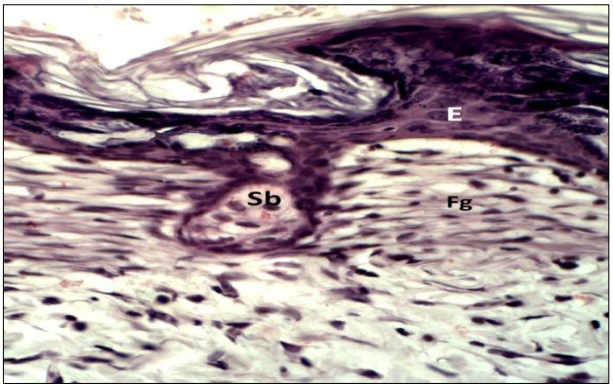
Figure, 17: Section of skin (21 days post treatment with amniotic membrane) shows: epithelium (E) organized fibroblast (Fg) and hair follicle (Hf). H and E, 100x.



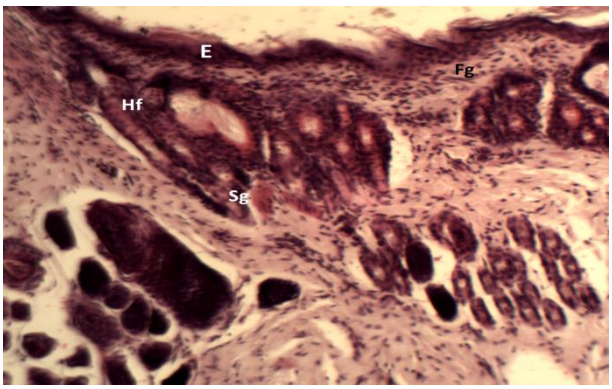
Figure, 18: section of skin (21 days post treatment with pericardial membrane) shows epithelization (E) dermis (D) and hair follicle (Hf) and organized fibroblasts (arrows) H and E, 100x.



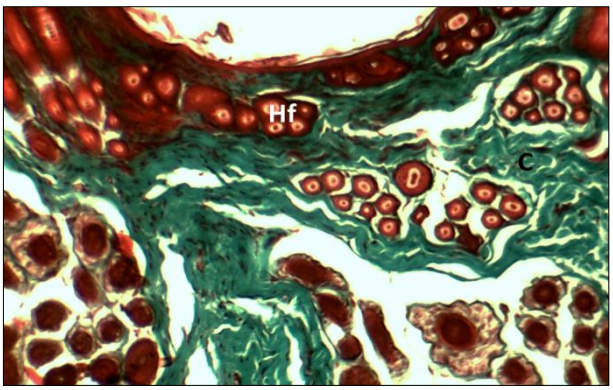
Figure, 19: Section of skin (28 days post treatment with amniotic membrane) shows complete epithelization (E) dermis (D) and sebaceous gland (Sb). H and E, 100 x.



Figure, 20: Magnified section of skin (28 days post treatment with amniotic membrane) shows complete epithelization (E) fibrogenesis (fg) and sebaceous gland (Sb). H and E, 400x.



Figure, 21: Section of skin (28 days post treatment with pericardial membrane) shows epidermis (E) much of hair follicles (Hf) sebaceous gland (Sb) and fibrogenesis (Fg) H and E, 100x.



Figure, 22: Section of skin (28 days post treatment with pericardial membrane) shows much of hair follicles (Hf) and collagen bundles within dermis. Masson's trichrome stain, 100x.

Table, 1: Shows the grades of histopathological evaluation of healing process in both amniotic and pericardial membranes.

	Fibrin deposited		Re-epithelization		Fibroblast proliferation		Mononuclear Cells		Polymorphneuclear Cells	
Period /day	Amniotic MM.	Pericardial mm.	Amniotic MM.	Pericardial mm.	Amniotic MM.	Pericardial mm.	Amniotic MM.	Pericardial mm.	Amniotic MM.	Pericardial mm.
7	moderate	sever	Nil	Nil	mild	sever	Nil	Nil	mild	sever
14	Nil	Nil	sever	Sever	sever	sever	mild	mild	Nil	Nil
21	Nil	Nil	moderate	Sever	moderate	moderate	Nil	Nil	Nil	mild
28	Nil	Nil	Nil	Nil	+	mild	Nil	Nil	Nil	mild

Table, 2: Shows the epithelial heights in control, amniotic and pericardial membrane groups during periods of experiment.

Period/ days	Control /µm	Amniotic membrane /µm	Pericardial membrane /µm
14 Days	57.2±1.1	*88.5±2.4	77.8±1.7
21 Days	74.5±3.1	*91.9±1.2	79.9±3.1
28 Days	95.9±1.5	*108.3±0.4	*105.1±0.9

*Significant at level of (P<0.05)

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تقييم مقارن لغشاء التامور وغشاء الامنيون البقري في شفاء جروح الجلد في الأرانب

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الخلاصة

هدفت الدراسة إلى تقييم مقارن لغشاء الأمنيون الجنيني وغشاءالتامورکنماذج لتضميد الجروح. استعملت في الدراسة عشرة أرانب من السلالة المحلية والتي قسمت على مجموعتين رئيستين: الأولى مجموعة التجربة (الغشاء الأمنيوني وغشاء التامور)، والمجموعة الثانية كانت مجموعة السيطرة، ووفقا لمدد الشفاء حيث قسمت كل مجموعة على أربع مجموعات فرعية (٧، ١٤، ٢١ و ٢٨) يوم بعد العملية. جُمعت عينات من الغشاء الامنيون من الأبقار الحوامل والتي اتمت مدد الحمل وأما غشاء التامور فقد جمع من الأبقار بعد جزرها مباشرة. اعتمدت الدراسة جرح الجلد لمواقع التطبيق وكانت الجروح الجلدية شاملة لكل سمك الجلد وبمساحة (٣×٣ سم) على الجانبين الصدري الظهري للجسم. اعتمد التقييم النسجي المرضي للدراسة درجات النضح الالتهابي، الاندمال للنسيج الظهاري، الاندمال للنسيج الليفي ونوع كريات الدم البيض المرتشحة. وكشفت النتائج انخفاض ملحوظ في الطور الالتهابي في جميع مدد ما بعد العلاج بغشاء الامنيون، تم تعزيز درجة الاندمال بتشكل النسيج الظهاري والليفي فضلاً عن الأوعية الدموية في الجروح المعالجة بغشاء الأمنيون والتامور خلال المدد ١٤ و٢١ يوم. وخلصت الدراسة إلى أن غشاء التامور يمتلك قابلية مشابهة لتلك التي في غشاء الأمنيون في تعزيز التئام الجروح الجلدية.

الكلمات المفتاحية: الأمنيون، التامور، تضميد، التئام الجروح.