EFFECT OF THE USE OF THE HUMAN AMNIOTIC MEMBRANE IN HEALING OF SURGICALLY INDUCED SKIN DEFECTS IN RABBITS (HISTOPATHOLOGICAL STUDY)

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ABSTRACT

INTRODUCTION: Soft tissue defects in Maxillofacial region remain significant health problems. The correct architecture and function of the vastly diverse tissues of this important anatomical region is mandatory. Amniotic membrane (AM) has been recently proposed as cost-effective alternative skin graft. It provides significant benefits by improving the process of wound healing and minimizing scar formation.

OBJECTIVES The aim of the present study was to assess clinically and histologically the effect of the use of the human amniotic membrane in healing of surgically induced skin defects in rabbits.

MATERIALS AND METHODS: This study was performed on 14 White New Zealand rabbits on which skin defects were created on both sides of their back. The right side was grafted with human amniotic membrane (AM) freshly obtained and cryopreserved (side A), and the left side was left to heal spontaneously by secondary intention (side B). The rabbits were divided later in to three groups according to intervals of evaluation and sacrification (1st week, 2nd and 3rd week postoperative). The wounds were examined for gross morphological evaluation, histological and immunohistochemical studies.

RESULTS Percentage of wound closure in AM grafted wounds was significantly higher than control wounds at 1st and 2nd week, but not significant at 3rd week healing wound ($P<0.061$). Histologically, the wounds of side (A) showed less inflammatory reaction and thicker newly formed epidermis layer. Collagen fibers were arranged in many directions and had higher density than those found in control wounds. Immunohistochemical evaluation showed higher expression of CD31 in side (A) than side (B) wounds indicating better angiogenesis in AM grafted wounds.

CONCLUSIONS: AM graft enhanced and speeded up the healing process and wound closure with less scar healthy tissue.

KEYWORDS: Amniotic membrane graft, skin defects, wound healing.

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INTRODUCTION

Soft tissue defects in Maxillofacial region remain significant health problems, this may be congenital (as in cleft lip and palate) or acquired. Acquired defects are usually more common which may result from trauma, surgery or infections. It is vital to seek new opportunities to optimize care for individuals suffering complex maxillofacial tissue loss (1 -3).

The correct architecture and function of the vastly diverse tissues of this important anatomical region are critical for life supportive processes such as breathing and eating. The face is also central to aesthetics, facial expression and social interaction (3). Maxillofacial tissue loss is commonly associated with significant scarring, disfigurement, and psychological sequelae as an inevitable consequence (4).

Management of disturbed wounds, large skin defects and areas where skin tension precludes wound closure is of high clinical importance. Healing in such wounds occurs through epithelization and contraction processes (second-intention healing) that may result in certain undesirable complications including keloid formations, poor final cosmetic appearance or the formation of a fragile epithelial layer. Therefore, the treatment methods that enhance wound healing and minimize related complications are desirable (6, 7).

In this respect, many wound healing materials consisting of medications, chemical and physical agents, nutrients and biomolecules have been tried in various experimental and clinical settings on human and laboratory animals with different degrees of success (7). These materials have been either incorporated into various vehicles such as gels, creams, various types of membranes or sponges. An ideal wound healing material has long been described as bioinert,nontoxic and bio- compatible, but this traditional idea is questionable, because the materials that can promote inflammatory reactions were found to shorten the healing time in both partial and full thickness skin wounds (9, 10).

Amniotic membrane (AM) has been recently proposed as a cost-effective graft that can be applied clinically in different medical conditions such as treatment of tissue adhesions, skin burns, ocular surface reconstruction, wound healing and tissue engineering (11). As it originates from ectoderm, its features are similar to human skin and hence it is an alternative dressing for skin graft. It provides significant benefits by increasing patients' comfort through decreasing pain sensation, prevent dehydration, trauma, associated infection and improving the process of wound healing with less scar tissue (12).

In the present study, the effect of the human amniotic membrane in healing of surgically induced full thickness skin defects in rabbits was evaluated clinically and histologically.

MATERIALS AND METHODS

This study was conducted on 14 healthy White New Zealand Rabbits. Their weight ranged between (2.5 - 3) kilograms. They were locally bred at Animal House of Research Institute, Alexandria University, Egypt.

A total of 28 experimental defects were created, two defects on each side of the back of each animal (right and left):
Side A: full thickness skin defect was created in the right side of the dorsolateral surface of each rabbit which was grafted by amniotic membrane (AM) (study group).
Side B: full thickness skin defect in the left side of the dorsolateral surface of each animal which will be left non-grafted to heal by secondary intention (negative control group).
Animals were further divided into 3 groups (I, II and III) according to the day of sacrifice:
Group I after one week: 4 rabbits.
Group II after two weeks: 5 rabbits.
Group III after three weeks: 5 rabbits.

Materials

Harvesting and preparation of the amniotic membrane

Fresh amniotic membrane was obtained from healthy seronegative for hepatitis B, C, AIDS antigens and syphilis mothers who underwent caesarian section. Mothers were informed before donation (informed consent). Serological tests were carried out by both enzyme linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) 10 days before elective surgery. Vaginal delivered placenta was not used as it contains vaginal flora and is disfigured by stretching during delivery. Premature ruptured membrane was discarded.

Under sterile aseptic conditions, the placenta was cleaned with balanced sterile saline solution to remove all blood clots. The chorio-amnion was stripped from the placenta and further separation of amnion from the chorion was done by blunt dissection. The separated membrane was washed again properly with sterile normal saline after being rinsed with 0.025% sodium hypochlorite solution where all blood, mucus and debris were completely removed (13)

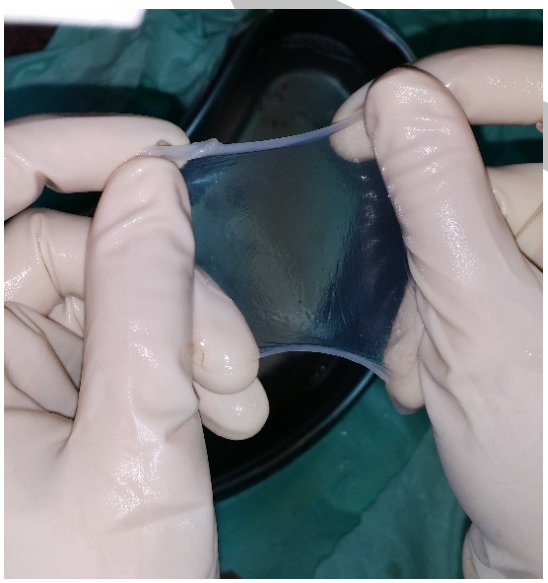


Figure (1): AM after being cleaned from blood and mucous and became ready for storage.

The membrane was cut into 5X5 cm sections and stored in a sterile bottle containing 85% glycerol (Pharma trade co, Egypt) at -80˚C. Immediately before its use, the membrane was kept in 480 ml saline containing 1,200,000 IU Penicillin (Pencitard, ACDIMA International Trading, NCPC North Best Co., Ltd) to 24 hours in refrigerator at 4˚C.

Animals

The rabbits were housed at room temperature under the same nutritional and environmental conditions and on the same balanced diet the whole period of the study. They were given 7 days acclimatization period before starting the experiment, and were treated in accordance with the international guidelines for the care and use of laboratory animals and the experiment protocol was approved by the Ethics Committee, Faculty of Dentistry, Alexandria University.

Methods

A-Preoperative care

Each animal received a dose of intramuscular antibiotics in the form of injectable amoxicillin (Flumox, Amoxicillin and flucloxacillin, EIPICO, Egypt) 15mg/kg body weight just before the operation.

B- Surgical procedures

1- Anesthesia

All operating procedures were performed under sterile conditions in an animal theatre. Each animal was generally anaesthetized via intramuscular injection of ketamine (Ketamin Alfasan 10%, Woerden, Holland) 50 mg/kg and xylazine (Xyla-Ject injectable solution, ADWIA Co., S.A.E., Egypt) muscle relaxant 5 mg/kg body weight.

2- Surgical operation

Hair shaving of the operation sites was performed and the surgical field was swabbed with 10% povidone iodine (Betadin antiseptic solution, The Nile Co., Egypt) before the operation. Two (2.5 x 2.5cm) full thickness critical size skin defects were created using blade no.15 in the dorsolateral cutaneous of the animals, one defect in each side after drawing by methylene blue stain on the sterile skin using a square template prepared from an X-ray film. The back of each rabbit was divided into two sides:

Side A: where full thickness skin defect at the right side of the back of each animal was created and grafted with amniotic membrane. The membrane was washed by sterile normal saline and spreaded over the surface of the defect then sutured in place using vicryl 5/0 suture material (Johnson & Johnson Int., C/O European Logistics Centre, Lenneke Marelaa 6, BE-1932 ST-Stevens- Woluwe, Belgium) with its epithelial side up and the mesenchymal surface in contact with the wound, to facilitate adherence of the membrane to the wound surface. To differentiate between the two surfaces we applied the tips of a blunt fine forceps to one surface of membrane and pinch lightly with the forceps and lift. A fine strand of “vitreous-like” substance can usually be drawn up from the mesenchymal but not the epithelial (basement membrane) side of the amniotic membrane. The membrane should spread without folds or voids beneath.

Side B: where full thickness skin defect at the left side of the back of each animal was created and left to heal by secondary intention after placing 4 sutures at the border of the wound with vicryl 5/0 to anchor the wound to the underlying muscle fascia to prevent the wound from healing by contraction (negative control).

All wounds were covered with a sterile polyurethane (PU) film of breathable non-adherent pad to facilitate wound care without distortion of healing process.

C- Postoperative phase

Clinical follow up period

After the surgical procedure, each animal received the same of course antibiotics of amoxicillin 15mg/kg body weight intramuscular injection for five days every eight hours. Ketoprofen (Ketofan, AMRIA PHARM. IND., Alexandria – Egypt) 3mg/kg intramuscular injection every twelve hours was given as an analgesic and anti-inflammatory drug to the animals for three days post-operatively. The animals were transferred to an individual clean cage with secured dressing for follow up where they were examined regularly for general health condition, dressing slippage and other complications such as infection or dehiscence. Changing of the dressing started from third day post-operatively, and removed permanently on the 7th day. In between, the dressing was changed regularly and daily to allow wound inspection, assessment and cleaning. Cleaning of wounds was done by washing them by normal saline. All wounds were photographed regularly using a digital camera at (1st, 2nd and 3rd week). Measuring the surface area of the healing wounds using sterile metal ruler and the percentage of wound closure was calculated through the following formula (14) :

$$\text{Percent of wound closure (\%WC)} = \frac{\text{area on day(0)} - \text{area on day X}}{\text{area on day 0}} \times 100$$

X = day of sacrifice 0 = day of surgery

D- Histopathological technique

The skin around the wounds was dissected till the depth of the muscles with safety margins of 0.5 cm of healthy skin, and the specimens were rapidly fixed in 10% neutral buffered formalin for 6 hours. Then they were dehydrated and impregnated in paraffin wax for producing paraffin block for each sample. The paraffin block were sectioned at 5 µm thick sections and proceeded for Hematoxylin (Sigma Chemical Company, Saint Louis, USA (340374T)) and Eosin (BDH standard stain, England (48251)) stains used to study the histological changes. Other slides were stained with Masson's trichrome stain (Standard stain, Sigma Chemical Company, Saint Louis, USA) stain for distribution of collagen formation. The sections were examined under light microscope.

E- Immunohistochemical technique (15)

The Paraffin blocks were cut into 5µm-thick sections placed on positively charged glass slides. The slides were deparaffinized, dehydrated and underwent several histochemical treatments that took two days long. Finally, the slides were stained with diaminobenzidine (DAB) chromogen to detect the reaction product (brown colored of epitope), stained with hematoxylin as nuclear counter stain and proceeded for examination under light microscope.

F- Image analyzer and Morphometric measurements

The images of each slide were captured with numerical aperture of a high resolution of (16-bit digital camera (1280X1024 pixel). Images were viewed and recorded using Olympus microscope – equipped with Spot digital camera in histochemistry and cell biology Depart. Medical research Institute, Alexandria University.

By using Image J software (Wayne Rasband, National Institutes of Health, USA) the following parameters were measured in all groups, epidermal thickness was measured in H & E stained sections, Integrated Optical Density (IOD) of collagen fibers in Masson's trichrome stained sections and the numbers of positive blood vessels and capillaries react for CD31 protein immunostaining.

Statistical analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. Student t-test was used to compare two groups for normally distributed quantitative variables. F-test (ANOVA) for normally distributed quantitative variables, to compare between more than two groups, and Post Hoc test (Tukey) (LSD) for pairwise comparisons. Paired t-test for normally distributed quantitative variables, to compare between two periods

RESULTS

I- Clinical results

The membrane showed an excellent adherence to the wound and was easy in manipulation and suturing.

The clinical manifestations were followed up, all wounds were clean and changing the dressing was done with no signs of infection till the day of sacrifice due to regular wound care.

At the 1st week postoperative, the control wounds (side B) showed mild bleeding, slight redness in color and the rabbits expressed abnormal movement and signs of discomfort when changing the dressing over wounds indicating that they were in pain. This reaction was less or completely absent at the AM grafted side (Figure 2 A & B).

2nd week postoperative, the AM grafted wound showed gradual degradation of the membrane from the periphery of the wound. All wounds that were grafted by AM had soft hydrated texture with thin soft delicate crust on top. The healing was gradual and regular from the periphery with preservation of the square shape of the wounds, while wounds of non-grafted control side started to lose their square shape, became irregular and topped by dry thick crusts (Figure 2 C & D).

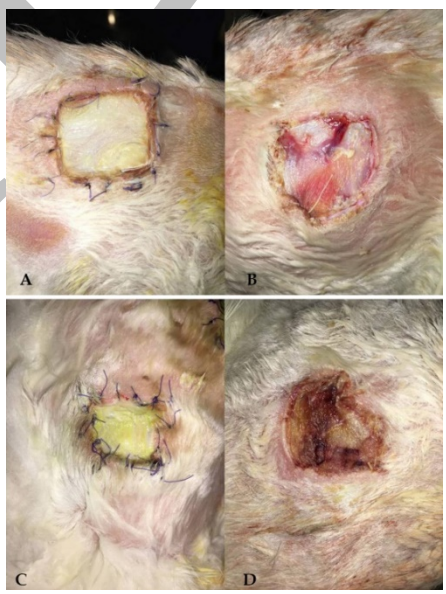


Figure (2): 1st week wounds, (A): AM Grafted wound, (B): None-grafted control wound. 2nd week wounds, (C): AM grafted wound, (D): None-grafted control wounds

At the 3rd week, the AM grafted wounds were still preserved their square shape and covered with thin delicate crust that was easily removed, while the non-grafted wounds showed irregular out line with thick dry crust on top that was difficult to remove without bleeding. None of the wounds healed completely (Figure 3).

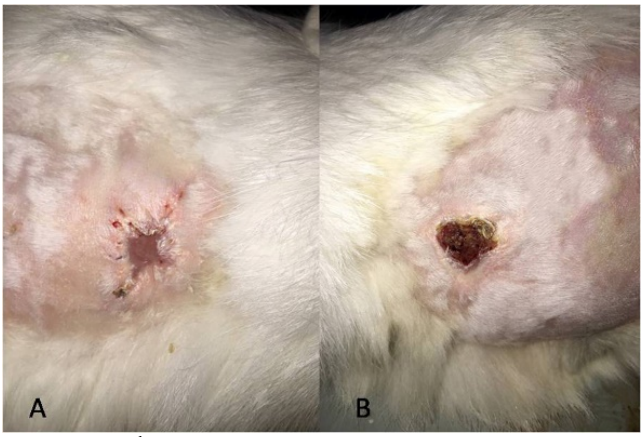


Figure (3): 3rd week wounds, (A): AM Grafted wound, (B): None-grafted control wound

Measurements of wound closure

Throughout the follow up period, the induced wounds of both sides (A & B) healed after surgery to variable extents. Comparing the wound closure between both sides, it was found that percentage of wound closure in AM grafted wounds was significantly higher than control wounds at 1st and 2nd week (P< 0.001/ P< 0.025 respectively), but not significant at 3rd week healing wounds (P< 0.061) (Table 1).

Table 1: Comparison between the studied groups according to percent of wound closure.

Percent of wound closure	AM grafted wounds	Control non-grafted wounds	t	p
Group I (1st week)				
Min. – Max.	26.40 – 32.80	16.70 – 23.22		
Mean ± SD.	29.31 ± 2.68	19.51 ± 2.82	15.609*	0.001*
Median	29.02	19.06		
Group II (2nd week)				
Min. – Max.	46.91 – 57.12	46.22 – 55.10		
Mean ± SD.	52.21 ± 4.38	50.86 ± 3.63	3.480*	0.025*
Median	52.33	51.42		
Group III (3rd week)				
Min. – Max.	83.23 – 88.12	34.65 – 73.90		
Mean ± SD.	86.22 ± 1.96	65.24 ± 17.12	2.580	0.061
Median	87.0	72.84		

t, p: t and p values for **Paired t-test** for comparing between AM and control grafted wounds in each group

*: Statistically significant at $p \leq 0.05$

II-Histopathological findings

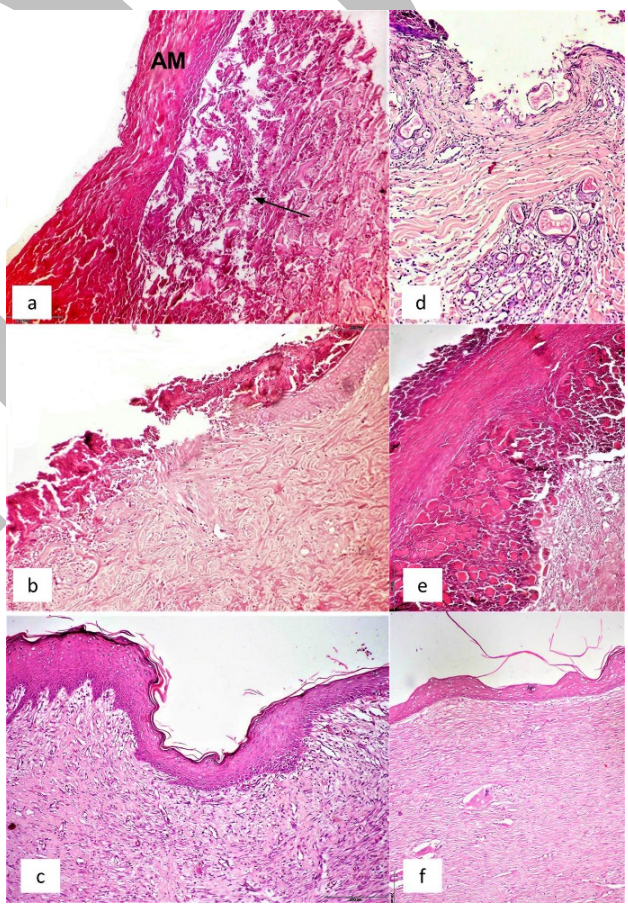
1-Histopathological changes (H&E)

A-Side A (AM grafted wounds)

By the end of the 1st week, the grafted wounds were infiltrated by inflammatory cells that were entrapped in the dermis. Edema was found near the wound surface and the underlying stroma underwent degeneration. At the 2nd week, gradual degradation of the membrane was started and replaced by creeping of newly formed epithelium. The inflammatory cells numbers and tissue edema decreased when compared with the first week. At the end of the 3rd week, the wound became mature and covered by well-formed keratinized epidermis rested on basement membrane with dermal papillae (rete pegs) that lock the epidermis with the underlying connective tissue (Figure 4 a, b & c).

B-Side B (non-grafted wounds)

The non-grafted wounds paraffin sections photomicrograph showed severe inflammatory reaction at the wound surface and tissue stroma, the inflammatory cells condensed at the wound surface forming marked thick layer. Colonies of phagocytic cells and thick fibrotic blood vessels were also found in wound sections. At the 2nd week, the control wounds were covered by keratinocytes which were migrated toward wound surface forming thick crust and epithelial layer at wounds' peripheries. The inflammatory cells infiltration and wound edema were decreased. Phogocytic cells and fibrotic blood vessels were completely absent. There were newly formed small blood vessels and hemorrhage capillaries. After three weeks, the healing wounds formed thin layer of keratinocytes with incompletely formed epidermis. The edema and the number of inflammatory cells decreased, while new small blood vessels in the underlying dermis were formed (Figure 4 d, e & f).



Figure(4): Paraffin sections photomicrograph of skin wounds at 1st, 2nd and 3rd weeks. (a, b & c) grafted wounds. (d, e & f) non-grafted wounds (H & E stain Bar=200µm).

2-Histomorphology of collagen fibers

A- Side A (AM grafted wounds)

At 1st week, AM grafted wounds section photomicrograph showed dark and thick wavy collagen bundles, they were almost parallel to wound surface. After the 2nd week, The Collagen bundles became moderate blue stain and thinner. They were separated from epithelial part and condensed in the stroma in different directions. At the end of 3rd week, collagen bundles became mature with similar arrangement and distribution found in normal skin (Figure 5 a, b & c).

B. Side B (Non-grafted wounds)

At the 1st week of non-grafted wounds the collagen bundles appeared dark blue and thick bundles. They were arranged in different directions, condensed and migrated to wound surface. At the 2nd week the collagen bundles became thinner, condensed, straight and arranged in one direction parallel to each other up to the epithelial layer of the wound surface. After the 3rd week slight recovery occurred. Thus, collagen bundles were still preserving their arrangement and were marked with blue stain (Figure 5 d, e & f).

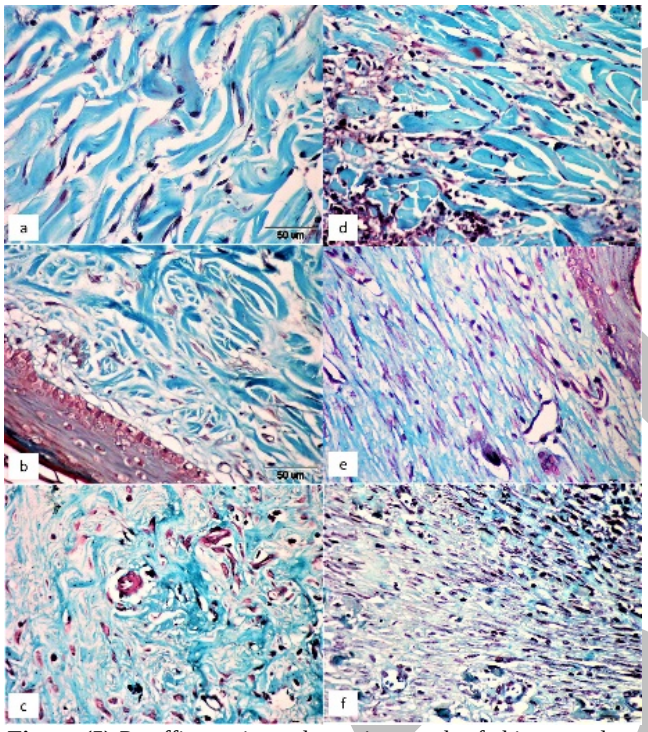


Figure (5): Paraffin sections photomicrograph of skin wounds at 1st, 2nd and 3rd weeks. (a, b & c) grafted wounds. (d, e & f) non-grafted wounds (Masson's Trichrome stain Bar=50µm).

III- Immunohistochemical expression of CD31 protein

A. Side A (AM grafted wounds)

At the 1st week, the AM grafted wound showed a brown stain of the CD 31 expression in the endothelial cell membrane and cytoplasm indicating the large numbers of newly formed blood vessels and capillaries. After the 2nd week, the expression of CD31 immunoreactivity stain decreased and the number of the capillaries as well. The large blood vessels were negative reaction (blue in color). At the end of the 3rd week the wounds showed weak expression of the CD31 immunoreaction and most of small blood vessels and capillaries were negative (blue in color) (Figure 6 a, b & c).

B. Side B (non-grafted wounds)

The non-grafted wounds microphotograph at 1st week showed a moderate brown immunostain of CD31 expression on endothelial cell membrane and cytoplasm. This moderate immunoreactions of the CD31 followed by the increased number of small blood vessels and capillaries. The immunoreactive of this side was decreased in compared with grafted side. At the 2nd week of non-grafted side, wound showed a decreased of the CD31 immunoreaction, a moderate immunostain was seen in a few endothelial cells of some capillaries. The CD31 expression was followed by decrease in blood vessel number where few of them became empty. Moderate reaction of immune-expression CD31 protein was also found. After the 3rd week of the non-grafted wounds showed a decreased expression of the CD31 immuno-stain in the endothelial cell of the small and large blood vessels as well as capillaries (Figure 6 d, e & f).

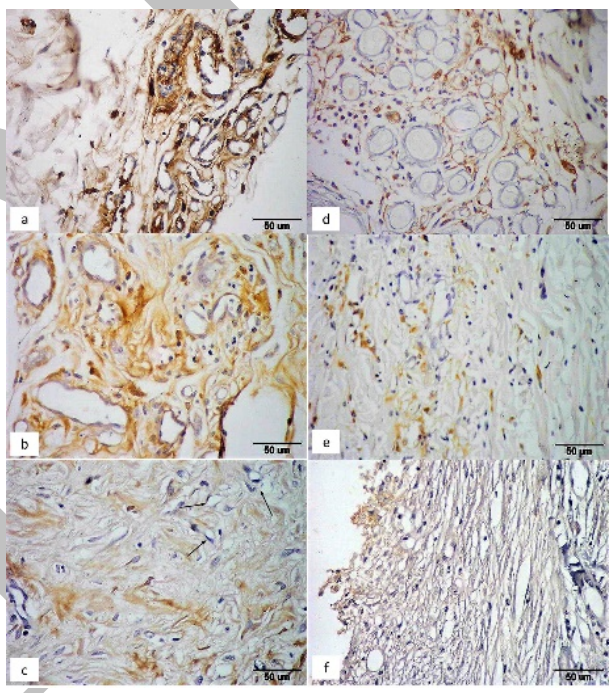


Figure (6): Photomicrograph of skin wounds at 1st, 2nd and 3rd weeks. (a, b & c) grafted wounds. (d, e & f) non-grafted wounds (CD31 & DAB-stain Bar=50µm).

IV- Image analysis findings

1. Thickness of epidermis

It was found that thickness of epithelial layer in AM grafted wounds (side A) was significantly higher than those non-grafted control wounds (side B) in last two weeks ($P<0.016$/ $P<0.001$ respectively).

2. Collagen bundles density

It was noticed that the density of collagen bundles in AM grafted wounds was darker than those of non-grafted for all groups ($P\leq0.002$/ $P\leq0.009$/ $P\leq0.006$) respectively. At the end of the experiment the collagen bundles of the AM grafted wounds showed no significant difference compared to normal skin bundles in density (mean value of normal skin collagen density by pixel= 36.92 ± 3.58) (Table 2).

Table 2: Comparison between the studied groups according to collagen density.

Collagen Density	AM grafted wounds	Control non-grafted wounds	t	p
Group I (n= 5)				
Min. – Max.	81.81 – 90.46	65.22 – 77.82	7.526*	0.002*
Mean ± SD.	87.28 ± 3.59	71.17 ± 5.63		
Median	89.20	72.85		
Group II (n= 5)				
Min. – Max.	44.49 – 61.14	28.0 – 39.43	4.680*	0.009*
Mean ± SD.	54.17 ± 7.04	33.37 ± 4.77		
Median	54.81	34.96		
Group III (n= 5)				
Min. – Max.	36.01 – 43.93	19.47 – 29.49	5.249*	0.006*
Mean ± SD.	38.75 ± 3.22	24.16 ± 4.50		
Median	37.54	23.04		

3. Angiogenesis

All wounds of both sides (A & B) showed an increase in numbers of the newly formed small vessels in 1st, 2nd and 3rd week. The mean values of numbers of new vessels on grafted wounds side (A) (45.60 ± 6.66, 28.60 ± 2.07 and 17.60 ± 4.28 blood vessel) respectively, were higher than non-grafted wounds side (B) (34.80 ± 3.27, 23.20 ± 2.28 and 8.80 ± 3.19 blood vessel) respectively. There was significant correlation in the 1st and 2nd week between grafted and non-grafted wounds, but no significant difference between them in 3rd week (P=0.057) (Table 3).

Table 3: Comparison between the studied groups according to number of new blood vessels.

Number of new blood vessels	AM grafted wounds	Control non-grafted wounds	t	p
Group I (1st week)				
Min. – Max.	39.0 – 54.0	30.0 – 39.0	2.842*	0.047*
Mean ± SD.	45.60 ± 6.66	34.80 ± 3.27		
Median	44.0	35.0		
Group II (2nd week)				
Min. – Max.	26.0 – 31.0	20.0 – 26.0	3.592*	0.023*
Mean ± SD.	28.60 ± 2.07	23.20 ± 2.28		
Median	29.0	24.0		
Group III (3rd week)				
Min. – Max.	11.0 – 22.0	5.0 – 13.0	2.648	0.057
Mean ± SD.	17.60 ± 4.28	8.80 ± 3.19		
Median	19.0	8.0		

t, p: t and p values for **Paired t-test** for comparing between A and B in each group
*: Statistically significant at $p \leq 0.05$

DISCUSSION

Since the face is the physical foundation of attractiveness and identity, soft tissue defects in the oral and maxillofacial region result from either congenital (such as cleft lip and palate) or acquired causes (such as trauma, surgery, or infections) may negatively affect the perception of facial beauty, resulting in significant psychological morbidity and inevitable functional problems which make the person becomes socially and emotionally withdrawn (5, 16).

Dealing with large skin defects and the areas where skin tension retards wound closure is very challenging and of high clinical and cosmetic importance. Those wounds heals by secondary intention that results in undesirable complications such as scar formation and weak epithelial layer, that's why treatment methods that enhance wound healing and produce satisfactory results are desirable (15).

Because suitable autografts for transplantation are very rarely present and the demand for allograft skin exceeds the supply, not to mention the high cost of tissue engineered skin substitutes, it has been a significant need to use the amniotic membrane as a biological dermal substitute that has low antigenicity, good adherence to the wound surface, potential antimicrobial activity, beside its abundant availability (12).

In the present study amniotic membrane was chosen to fill surgically induced critical size full thickness skin defect (2.5 X 2.5 cm) in the dorsolateral surface of rabbits. There was ease in manipulation and suturing of the AM, this means there is no need for special skills compared with other skin substitute and grafts. It was found that the membrane has remained in place up to 10 days with no signs of any rejection due to its low immunogenicity and started gradually to dissolve and taken by the wound tissues. This means it could be placed in one stage with no need for farther removal. The biodegradability of AM advantage is absent in Integra skin substitute that needs a two-steps operation, being expensive, and accumulation of exudate underneath it that may lead to infection (16).

The slight signs of inflammation and absence of septic wounds and bleeding at AM covered wounds confirm the anti-inflammatory anti-microbial properties of the membrane. Its antimicrobial effect gives the surgeon the tool to use it in potentially septic traumatic wounds as well as wound dressing for infected wounds (17). On contrast, there is always a risk of infection when using Biobrane composite allograft and some studies have reported cases of toxic shock syndrome due to accumulation of exudate underneath it (16). The presence of bactricidin, beta-lysin, lysozyme, transferrin and 7-S immunoglobulins in the amniotic fluid explains the anti-microbial impact of AM as well as the mechanical action of being a barrier over the wound preventing it from being contaminated (18).

The clinical manifestations of the present study revealed the absence of abnormal movements and signs of discomfort expressed by rabbits while changing the dressing at the AM grafted sites, this confirms the pain reduction property of the amniotic membrane. Many studies revealed a rapid pain relief when using AM over burns (10), chronic ulcers (19), symptomatic bullous keratopathy and ocular Band keratopathy (20). Although one may attribute AM's effect in relieving pain to its anti-inflammatory anti-bacterial action and its adherence to wound covering the nerve endings, we suspect that such a rapid action in pain relief may due unknown anti-pain action that deserves further investigation.

Although healing with AM graft was found to be faster than control wounds in first 2 weeks of the present study, there was no significant difference in percentage of wound closure on day 21 between both groups (P=0.061). This is maybe due to repair with scar tissue in the non-grafted side, and healing with less scar tissue was undergone in the AM grafted site which may take more time. However, AM seemed to accelerate wound closure in this study and many studies supported this result. Hossam Elheneidy et al (14), used AM over chronic non healing leg ulcers and noticed complete healing of all ulcers with healthy granulation tissue with fast healing rate than those ulcers left uncovered and the patients expressed improvement in their pain level (14).

The histopathological findings obtained from this study of wounds in both groups confirmed the great value of the AM graft. It was found that, on day 7, the non-grafted wounds showed thick condensed inflammatory layer and obvious numbers of phagocytic cells. These phagocytic cells were completely absent in AM grafted wounds and only thin layer of scattered inflammatory cells. The anti-inflammatory impact of AM conducted by the inhibition of the pro-inflammatory cytokines such as interleukin (IL)-1a, IL-2, IL-8, IL- 10, IFN-c, basic fibroblast growth factor (bFGF), tumor necrosis factor b and platelet derived growth factors (PDGF). Moreover, the mechanical entrapment of inflammatory cells within AM storma ends up by their apoptosis (18).

In the present study, the thickness of the newly formed epithelial layer was increased in AM grafted wounds when compared to the non-grafted wounds. It has been demonstrated that the AM speed up the maturation of keratinocytes and epithelialization process. Beside acting as a scaffold for epithelial cell migration and support the basal epithelial cells, AM contains various growth factors that stimulate epithelialization, promote epithelial differentiation and prevent its apoptosis (20, 21). These findings evaluated by the significant difference in epithelial thickness between both grafted and non-grafted wound in last two weeks.

In normal skin, dermal fibroblasts produce elastin and triple helix proteins that form collagen fibers. These fibers contribute to the structure of the dermis and the elasticity of the skin. Scars occur mainly due to changes in quality and quantity of collagen and elastin bundles in the underlying dermal matrix. Scanning and transmission electron microscopy reveals an increase in thinned elastin fibers in skin scars that have different arrangement than those found in normal skin (23,24). The present work showed that by the end of the experiment the collagen bundles found in AM grafted wounds are nearly similar to that found in normal skin in histomorphological appearance and density. Image evaluation showed a high significant difference between grafted and non-grafted wound in collagen density where the density was higher in AM grafted wounds in all subgroups. This confirms that AM helps the wound to heal with less scar tissue that is almost similar to normal skin.

Sufficient blood supply through angiogenesis is crucial for the healing of a transplant (25). Studying the angiogenesis properties of the AM in this experiment, considering that the AM was placed with epithelial surface up and mesenchymal surface on contact with wound bed, revealed a significant increase in number of blood vessels and new capillaries when compared to non-grafted especially for the first two weeks of the experiment. The number of blood vessels started very high in the first week (45.60 ± 6.66) then gradually reduced in the second and third week. These results were obtained through the high expression of CD31 protein immune-stain in newly formed capillaries in the cytoplasm of endothelial cells on day 7 and the reduction of CD31 protein immune-expression on day 14 till it became weak by the end of the third week.

Hassan Niknejad et al (26), evaluated the angiogenic effects of epithelial and mesenchymal sides of the amnion implanted on dorsal skin of rats. It was found that amnion could induce surface dependent angiogenesis, in mesenchymal side up samples, decreases in number and length of vessel were observed. By contrast, in epithelial side up group the number and length of vessel were increased. The pro and anti-angiogenic activities of the AM can be utilized according to the medical conditions (26).

Ghasem Yazdanpanah et al (27), emphasized the previous study and proved that cryopreservation of AM did not change its angiogenic properties that found in fresh one.

Since impaired angiogenesis and collagen synthesis retard healing process and increase the risk of break down (28), and so the pathological scars are caused mainly due to increased or prolonged dermal inflammation, treatment modalities against scars focus on inhibiting the inflammation during healing process, increase vascularity and the endothelial cells differentiation (29). The amniotic membrane in this study showed a great value in accelerating wound healing with healthy tissue that had less inflammatory reaction, excellent collagen quality and good blood supply.

CONCLUSION

Amniotic membrane has many biological properties that make it one of the most promising inexpensive allografts for wound coverage. Beside accelerating the wound closure, it helps the wound to heal with less scar high quality tissue.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ACKNOWLEDGEMENT

We greatly appreciated Dr. Ahmed Zaky our veterinary doctor at Animal House (Medical Research Institute, Alexandria) for his help and Dr. Heba M. Fetiha at Histochemistry and Cell Biology department (Medical Research Institute, Alexandria) for her excellent immunohistochemical laboratory work to support this article.

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