Egypt. J. Rad. Sci. Applic. **Vol. 37**, No.1-2, pp.79-91 (2024)

Egyptian Journal of Radiation Sciences and Applications
http://ejrsa.journals.ekb.eg/

Preclinical Evaluation of Amniotic Stromal Layer as a Wound Dressing in Healing-Suppressed Rats by Gamma Irradiation

Nashwa K. Radwan[1], Waleed A. Nemr[2] and Salwa Farid Ahmed[1*]
[1]*Health Radiation Research Department, National Center for Radiation Research and Technology, Egyptian Atomic Energy Authority, Cairo, Egypt.*
[2]*Department of Radiation Microbiology, National Center for Radiation Research and Technology, Egyptian Atomic Energy Authority, Cairo, Egypt.*

WOUNDS with healing difficulties may lead to chronic ulcers. Gamma irradiation causes cellular damage that can delay tissue regeneration of wounds. Human amnion (HAM) is a promising wound graft because it contains many healing factors, such as growth factors and anti-inflammatory cytokines. This work aims to prove the therapeutic effect of amniotic stromal layer (ASL) grafts in wounded rats post-irradiation. The impact of two different preparations of ASL grafts (fresh versus cultivated) was compared by skin grafting of two groups of wounded rats; each rat has two equivalent areas of full-thickness wound (FTW). The healing was suppressed by exposing all rats to 6 Gy gamma-irradiation. The wound on the right side was treated by skin grafting with one preparation of ASL, whereas the left-sided wound was untreated as a control. The improvement of wounds was evaluated by clinical observation, histologic examinations, and immunostaining of the expressed tumor necrosis factor-alpha (TNF-α) and epidermal growth factor (EGF). Significant differences were observed among the different treatments concerning the contraction of wounds. Complete healing of wounds with the perfection of skin appendages was achieved only in the wounds treated with the cultivated ASL preparation within 11 days post-wounding. In general, ASL grafts reduced the degree of inflammation, accelerated the formation of granulation tissues, reduced the expressionof TNF-α and induced the expression of EGF in the treated wounds; compared to the untreated control.In conclusion, ASL is histologically compatible and promising for wound grafting and skin rejuvenation.

Keywords: Radiation, Amniotic membrane, Skin, Tumor necrosis factor, Epidermal growth factor.

Introduction

Stimulating wound healing requires many integrated and overlapping biological processes, including inflammation, angiogenesis, and cellular proliferation and differentiation. The inflammation stage starts in the first 2 days of injury. This stage triggers the proliferation of keratinocytes at the wound edges, fibroblasts in granulation tissue, and stem cells for tissue regeneration. Supplying wounds with blood (through angiogenesis) is necessary to feed cells and induce their differentiation into new tissues. Each of these biological processes requires essential healing factors to regenerate the damaged tissues, such as extracellular matrix (ECM) and growth-stimulating factors (Potekaev et al., 2021).

Any defect in one or more of these healing stages leads to an abnormal process of tissue repair, this means chronic, non-healing wounds which pose a difficult clinical practice challenge. A cause of the disruption of wound healing, particulary in the current times, is radiotherapy due to the widespread spread of cancer worldwide. Ionizing radiation impaired the various stages of the normal

*Corresponding author: E-mail:salwaa_fared@yahoo.com
Received 11 / 7 / 2024 ; Accepted 3 / 9 / 2024
DOI: 10.21608/EJRSA.2024.303532.1170

process of wound healing. Such impairment was mediated either directly due to cellular DNA strand breaks through ionization of electrons or indirectly by cellular damage resulting from different free radicals' generation (Devalia and Mansfield, 2008). Such impairment led to many pathological changes, including cellular depletion with suppression of proliferation and increased apoptosis, ECM alterations, and microvascular injury with associated local tissue hypoxia (Qu et al. 2003).

The amniotic membrane (AM) is the innermost placental layer facing the amniotic fluid and fetus. It composes a basement membrane lining up with epithelia, and the other side matches the chorion through ASL, which contains a meshwork of type III collagen linked with glycoproteins and proteoglycans. A previous research has shown that ASL suppressed the expression of pro-inflammatory cytokines, such as interleukins 1α and β) to help improve the pain and inflammation in wounds (Niknejad et al. 2008). During the inflammation stage following woundings, matrix metalloproteases (MMPs) are expressed by infiltrating polymorphonuclear cells and macrophages. Natural inhibitors of MMPs have been found in the AM (Hao et al. 2000 and Kim et al. 2000). In addition, large quantities of hyaluronic acid (HA, a high molecular weight glycosaminoglycan) exist in AM. Hyaluronic acid is important in wound healing because it acts as a ligand for CD44, which is expressed on inflammatory cells and plays an important role in the adhesion of inflammatory cells (including lymphocytes) to attach with AM stroma (Higa et al. 2005). Recently, the amniotic membrane as a biological stimulating tool for accelerating the healing of various types of wounds, especially chronic ones has been widely used. Its ease of availability, low cost, and superior healing properties make it the ideal choice in clinical use for treating different pathological conditions such as skin burns, chronic ulcers, wound healing and tissue engineering. It is considered a suitable alternative to skin graft due to its similarity to human skin. In addition to its healing capacity, it provides a set of beneficial features, including prevention of dehydration, good infection control, decreased pain sensation, diminished risk of trauma, perfect wound adherence and easy handling, thus promoting the healing process without massive scar formation (Salehi et al. 2015).

The ability of separated ASL to restore tissue-repairing mechanisms in healing-suppressed wounds after exposure to gamma irradiation has not been proved yet. Therefore, the current study aimed to treat these wounds using xenotrans plantation of two different ASL preparations (fresh versus cultivated) and to compare the healing progression with that of the untreated wounds through clinical and histological investigations.

Experimental

Ethical considerationand animal grouping

This experiment was conducted after receiving the approval of the Research Ethics Committee of the National Center for Radiation Research and Technology (REC-NCRRT), Egyptian Atomic Energy Authority (17A/22). All experiments were performed in accordance with relevant guidelines and regulations. The subjects of the experiment were 18 healthy male albino rats each weighing 200 ± 20 g. The housing conditions included gathering three rats per cage, adjusting the housing room with a 12-hour light/dark cycle, controlling the room temperature range within 20-24 ^{o}C, and preserving the availability of water and dry diets all the time. The rats were exposed to a whole body dose of 6 Gy of gamma radiation to suppress self-healing mechanisms. Then, they were randomly divided into two groups, each consisted of 9 rats. In the first group, rats were treated in the right-sided wound with gamma-sterilized, fresh-prepared amniotic stromal layer (FASL). Rats in the second group were treated in the right-sided wound with antibiotic-sterilized, fresh-prepared amniotic stromal layer (CASL). The left-sided wound in both groups acts as a control with no treatment.

Processing of human amniotic membrane

The initial steps for obtaining and transporting placental membranes were carried out by the amniotic tissue lab (NCRRT, Cairo, Egypt) in accordence with standard regulations set by the preventive medicine sector of the Egyptian Ministry of Health. Particularly, the collection and handling of placentas were performed after cesarean delivery from healthy mothers inside an operative room of Algalaa Maternal Hospital (Cairo, Egypt). Amniochorionic tissue was dissociated from the placenta and transferred into a sterilized jar containing 50 ml of Hanks' Balanced Salt Solution (HBSS, Lonza). The jars were then transported under cooling within 4 hours to the quarantine of the amniotic tissue lab for performing the subsequent procedures.

Under full aseptic conditions, 0.2 ml of saline medium was aspirated from each jar for virology testing of samples using ABON™ rapid tests (Abbott, USA), for detecting the hepatitis C virus (HCV), hepatitis B virus (HBV), and human immunodeficient virus 1 (HIV-1). Passed samples were considered as potential virus-free. Confirmatory diagnostic PCR testing was carried out to ensure the safety of such biological samples before processing them into the tissue culture lab. Briefly, 1 ml of aspirated saline solution from each sample underwent nucleic acid extraction using the PREP-NA-S DNA/RNA extraction kit (DNA-Technology LLC., Moscow). Then, the extracted sample was tested by the diagnostic multiplex PCR test published by Nemr and Radwan (2024). Accordingly, the virus-free amniochorionic samples were transferred from the quarantine to the tissue culture lab for further processing. HAM was separated from the amniochorionic tissue by blunt dissection and washed several times by sterile saline solution until full clearance from blood and debris (Nemr et al., 2016).

Preparation of ASL

Under aseptic conditions, the fresh amniotic stromal layer (FASL) was separated by scraping the stromal side of the washed HAM and collected into a sterile 50-ml tube. The cultivated ASL (CASL) was scrapped from 4 g. HAM after 2 days of cultivation in 10 ml Hank's Minimal Essential Medium (MEM). The culture medium was supplied with 1x disinfecting solution (100 U/ml Penicillin, 100 ug/ml Streptomycin, 100 ug/ml Gentamicin, 0.25 ug/ml Amphotericin B, and 0.02% EDTA). Ten HAM cultures were prepared from 10 HAM samples of different batches and cultivated separately into 9 cm-glass Petri dishes to verify the disinfection efficacy through sterility testing, as described below.

Bio-burden assay and sterility testing of ASL-finished products

The microbial bio-burden of five batches of FASL (sourced from different HAM samples) was assessed using the tissue bio-burden determination methodology (Kowalski et al. 2012). Briefly, 10 ml of sterile microbial dissociation solution (0.1% peptone, 0.1% polysorbate 80) was added to 10 g. of FASL. The sample was sonicated under cooling for 5 min and subsequently shaken for 30 min at room temperature. The total volume of the homogenized suspension was then determined and dispensed into 10 Petri dishes; each plate contained an equivalent volume to a weight of 1 g. FASL. Therefore, the estimation of microbial bio-burden will be relevant to a gram of FASL. The colony-forming units (CFUs) assay was carried out using pour plating with Trypton Soya Agar medium (Oxoid); 5/10 plates were incubated under aerobic conditions while the other 5 plates were incubated under anaerobic conditions (at 32 ^{o}C for 7 days). According to the microbial bio-burden of the FASL, gamma irradiation sterilization was carried out and validated using VD_{max}^{15} methodology (ISO 13004, 2022).

The sterility testing of FASL was carried out by adding 1 g. of irradiated FASL to 90 ml of thioglycolate medium (Oxoid). Thirty FASL samples were tested to determine the sterility assurance level of the irradiation process. On the other hand, the sterility testing of CASL products was carried out by adding 10 ml of the HAM-conditioned medium (cmHAM) to 90 ml of thioglycolate medium. The sterility of cultures was examined under incubation at both aerobic and anaerobic conditions (as above) for 7 days at 32 ^{o}C. Cultures that exhibited microbial growth were safely discarded with their original samples.

Induction of healing-suppressed FTW and ASL treatments

All rats were induced for healing suppression by exposion to gamma irradiation (^{137}Cs, Gamma cell 40 radiator facility, NCRRT) at a dose of 6 Gy (Fouad et al. 2023), At the time of the experiment, the dose rate was 0.37 Gy/min. Three days post-irradiation, rats were subjected to the wounding operation after being anesthetized with intraperitoneal injection of ketamine 10% and xylazine 2% (2:1) at a dose of 0.12 mL/100 g body weight (de VasconcelosCatão et al. 2015). The wounding operation was as follows: shaving the dorsum area and disinfecting it with 70% ethanol. Two circular areas of 1.5 cm in diameter (area ~1.77 cm^2) were demarcated on the right and left sides and excised using sterile scissors to create two FTW lesions in each rat, each resulted wound was cleaned by a piece of sterile cotton pad wetted previously with normal saline solution.

The right wound in the individuals in the 1st group was treated with topical application of 1 g. FASL, while the right wound in the 2nd group was treated with topical application of 1 g. CASL. The previously weighted FASL and CASL were applied using sterile tweezers.The left wound in both groups was left without any treatment. All wounds were covered with sterile surgical pads.

Then, each rat was housed separately until the time of sacrifice.

Clinical and histological examinations

Clinical examination was conducted on days 3, 5, 7, and 11 post-wounding. The antroposterior and lateral dimensions of each wound were determined. The Aperio's Image ScopeTMruler tool was used to create the linear dimensions in the photographs of the wounds, which were taken using a Canon digital camera (Hipp et al. 2011). The software used to determine the area was Image J (Chang et al. 2011). Then, the wound area and the percentage of the wound contraction were calculated during the experiment. For histological examination of wounds, 3 rats from each group were sacrificed on the day of examination by treating them with overdose ketamine on the 3rd, 7th, and 11th days post-wounding.

A circle of skin including the wound site and a rim of normal tissues was excised from each wound, fixed in 10% buffered formalin, and embedded in paraffin. Samples were sectioned (thickness ~5 um). Analogous sections were divided into 3 comparable sets. Sections of the first set were stained with hematoxylin and eosin for histological examination (Suvarna et al. 2018). The other two sets were evaluated for immunohistochemistry reactivity against tumor necrosis factor alpha (TNF-α) and epidermal growth factor (EGF) separately (Ramos-Vara, 2005). The area percentage of TNF-α and EGF expression was determined in 10 histological fields (×400) randomly captured in each slide using a digitized image analysis system with the software Leica Qwin 500.

Data auditing and statistical analysis

Data are reported as mean ± standard deviation (SD). A one-way analysis of variance was performed to compare group differences. Multiple range tests were used when differences among groups were significant. Statistical analysis was performed for each part of the table separately. The untreated rats and those treated with FASL were considered as a separate experiment from the untreated rats and those treated with CASL. Therefore each of them was statistically analyzed separately. Statistical analysis was performed using Statgraphics Centurion XVI software (Statpoint Technologies, Inc., 560 Broadview Ave. Warrenton, Virginia 20182). Practically, data with a *P* value smaller than 0.01 were considered highly significant.

Results

Microbial load and sterility assurance of ASL

The microbial bioburden of the collected FASL samples, from fresh HAM, was 1.3 CFU/ 10 g. (on average). The sterilization dose of 15 KGy gamma irradiation of the FASL revealed sterility assurance level <10-6, as assessed by VDmax15methodology. On the other hand, the disinfection of HAM samples in the tissue culture system for producing CASL ensured that the combination of 0.02% EDTA, 100 ug/ ml Gentamicin, 100 U/ ml Penicillin, 100 ug/ml Streptomycin, and 0.25 ug/ml Amphotericin B reduced the possibility of culture infection to less than 0.4x10-2.

Production of ASL

Each 1 g. (wet weight) of freshly prepared HAM produced 0.37 g. (±0.082) of FASL. The average dry weight of the obtained FASL was 0.04 g. (water content ~89%), while 1 g. of wet scraped HAM produced an average of 0.44 g. dried HAM (water content ~66%). On the other hand, the wet weight of the obtained CASL was 0.64 g. (±0.11) from 1 g. of cultivated HAM with an average water content in CASL reached ~92%.

Experimental outcomes

Clinical indications

The clinical evaluation of the treated wounds reflected the superior effect of ASL in accelerating the healing progression with a good plastic appearance more than in the untreated wounds (Table 1). In details, the percentage of wound contraction in the 1st group was 1.94 times greater than that in the untreated wounds during the first 3 days of treatment. In the 2nd group, this percentage increased by 2.6 times in the CASL-treated wounds over the untreated wounds. Overall, both the anteroposterior and lateral lengths of the treated wounds decreased significantly more than those of the untreated wounds by the 7th day of treatment. Complete healing was achieved on the 7th day in the treated wounds, whilewhile the untreated wounds only showed partial healing on the 11th day.

The post-healing appearance of wounds, on the 11th day, indicated good rejuvenation of skin and the growth of hair in the CASL-treated wounds more than in FASL-treated wounds, and both FASL and CASL improved the post-healing appearance than the untreated wounds. The calculated percentage data for wound contraction in each rat group are shown in Table 1 with their statistical evaluations. Overall, no immunological rejection or adverse reaction signs have been observed in the treated wounds with ASL grafts.

TABLE 1. Statistical evaluation and the calculated average of antroposterior (AP) and lateral (Lat) wound length (cm), wound area (cm^2) and wound contraction % in rat individuals with untreated and FASL-treated wounds (A) and individuals with untreated and CASL-treated wounds (B).

A	AP wound length (cm)		Lat wound length (cm)		Area of wound (cm^2)		Wound contraction %	
	Untreated	FASL-treated	Untreated	FASL-treated	Untreated	FASL-treated	Untreated	FASL-treated
Day 0	1.50 ± 0.00 a	1.50 ± 0.00 a	1.50 ± 0.00 a	1.50 ± 0.00 a	1.77 ± 0.00 a	1.77 ± 0.00 a	0	0
Day 3	1.35 ± 0.06 b	1.13 ± 0.13 c	1.25 ± 0.06 b	1.03 ± 0.17 c	1.33 ± 0.08 b	0.92 ± 0.19 c	24.8	48.2
Day 5	1.08 ± 0.05 c	0.83 ± 0.17 d	1.03 ± 0.10 c	0.78 ± 0.13 d	0.87 ± 0.10 c	0.51 ± 0.15 d	50.9	71.1
Day 7	0.63 ± 0.09 e	0.10 ± 0.12 f	0.63 ± 0.13 e	0.13 ± 0.15 f	0.31 ± 0.06 d	0.02 ± 0.02 e	82.5	98.9
Day 11	0.00 ± 0.00 f	0.00 ± 0.00 f	0.01 ± 0.01 f	0.00 ± 0.00 f	0.00 ± 0.00 f	0.00 ± 0.00 f	100	100
p-value	0.000		0.000		0.000			
B	AP wound length (cm)		Lat wound length (cm)		Area of wound (cm^2)		Wound contraction %	
	Untreated	CASL-treated	Untreated	CASL-treated	Untreated	CASL-treated	Untreated	CASL-treated
Day 0	1.50 ± 0.00 a	1.50 ± 0.00 a	1.50 ± 0.00 a	1.50 ± 0.00 a	1.77 ± 0.00 a	1.77 ± 0.00 a	0	0
Day 3	1.45 ± 0.06 a	1.10 ± 0.26 b	1.23 ± 0.05 b	0.93 ± 0.32 c	1.41 ± 0.05 b	0.85 ± 0.47 c	20.2	51.9
Day 5	1.10 ± 0.12 b	0.85 ± 0.13 c	1.00 ± 0.08 c	0.65 ± 0.17 d	0.87 ± 0.11 c	0.45 ± 0.15 d	50.8	74.4
Day 7	0.65 ± 0.13 d	0.05 ± 0.06 e	0.60 ± 0.14 d	0.08 ± 0.09 e	0.32 ± 0.13 d	0.01 ± 0.01 e	82.1	99.6
Day 11	0.01 ± 0.01 e	0.00 ± 0.00 e	0.01 ± 0.01 e	0.00 ± 0.00 e	0.00 ± 0.00 e	0.00 ± 0.00 e	99.9	100
p-value	0.000		0.000		0.000			

Different letters denotes significant difference $p \leq 0.01$.

Histological indications

On the 3rd day post-wounding, the bed of untreated wounds were filled with granulation tissue infiltrated by polymorphonuclear leukocytes (PMNL) and covered with necrotic debris. Newly formed blood vessels were detected at this time without collagen formation. The cellular growth of epithelia in the granulation tissue increased on the 7th day post-wounding with a slight appearance of collagen fibers in the basal layer only of the wound and still infiltrated with PMNL. On the 11th day. the appearance of the epidermis with complete epithelization became obvious with an incomplete cover of the keratin layer and without the appearance of hair follicles. Then, the basement membrane was almost straight without interdigitation with the dermis. The underlying dermis exhibited parallel collagen fibers rich in fibroblasts. The newly formed skin lacks all its appendages (Fig. 1, A-C and 2, A-C).

The treated wounds, whether with FASL (Figure 1 D-F) or CASL (Fig. 2 D-F) showed PMNL-infiltrated granulation tissue covered with necrotic debris on the 3rd day. Notably, newly formed blood vessels appeared in CASL-treated wounds greater than those in the FASL-treated and untreated wounds. Parallel collagen fibers appeared clearly in the dermis, in the sections of the treated wounds on the 7th day. In comparison, the CASL-treated wounds exhibited an intact layer of epidermis (with completed epithelialization) covered with a thin layer of keratin, while the FASL-treated wounds showed a partial cover of keratin over the intact epidermis layer.

Furthermore, an initial interdigitation of the epidermis with the underlying dermis was detected only in the CASL-treated wounds at this time. The examination of the 11th-day sections revealed the superior histological outcomes of CALS over the other comparatives as detecting; a complete interdigitation between dermis and epidermis, restored complete layers of keratin and epidermis with all skin appendages (such as hair follicles and sebaceous glands). Furtheremore, organized collagen fibers running in different directions in dermis. In contrast, the FASL-treated wounds showed no interdigitation into the dermis and faint onset formation of skin appendages.

Immunohistochemistry indications

Expression of TNF-α

On the 3rd day, the selective immunological staining of the expressed TNF-α was strongly exhibited in the granulation tissue of the untreated wounds, mild in FASL-treated wounds, and tiny in CASL-treated wounds. The expression level of TNF-α decreased in the sections of the 7th day; as moderate expression in the untreated wounds, negative in the FASL-treated CASL-treated wounds (Fig. 3). Statistically, TNF-α expression significantly decreased on the 7th day in untreated and FASL-treated wounds as compared to the 3rd day. Both FASL-treated and CASL-treated wounds exhibited significantly lower TNF-α expression compared to their untreated controls at the same time points (Fig. 4).

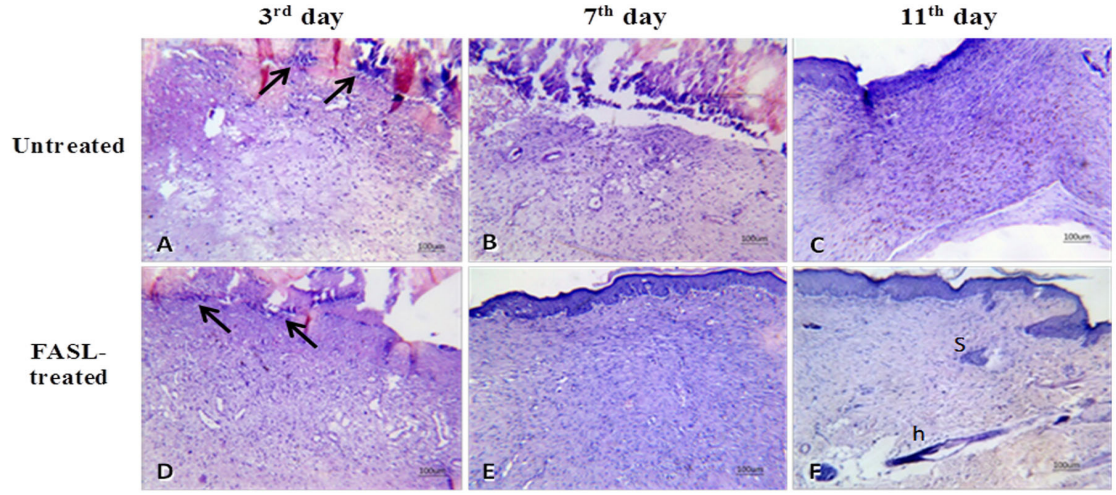


Fig. 1. Photomicrographs of H&E-stained sections of untreated and FASL-treated FTW. A. wound bed filled with granulation tissue infiltrated with PMNL (polymorph nuclear leukocytes). B. more cellular granulation tissue with newly formed blood vessels with a little appearance of collagen fibers in the basal layer,intact epithelium with no skin appendages and a deficient keratin layer with newly formed parallel collagen fibers. D. wound bed filled with granulation tissue infiltrated with PMNL (polymorph nuclear leukocytes). E. intact epithelium with no skin appendages and newly formed parallel collagen fibers. F. intact keratinized epidermis, dermis contained hair follicles and sebaceous glands.

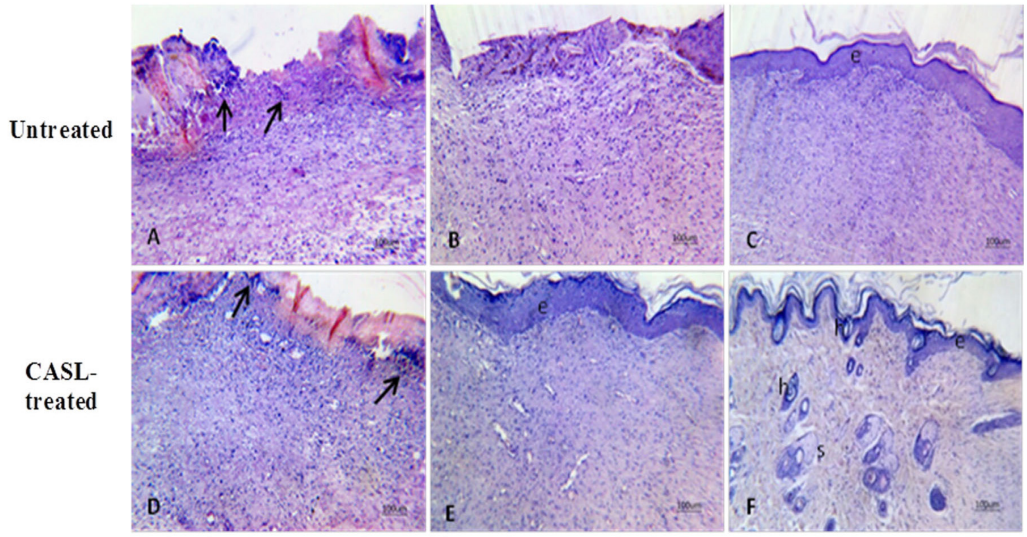


Fig. 2. Photomicrographs of H&E-stained sections of untreated and CASL-treated FTW. A. wound bed filled with granulation tissue infiltrated with inflammatory cells (arrows). B. more organized granulation tissue with migrating epithelial edges. C. intact epithelium (e) with no hair follicles, newly formed parallel collagen fibers. D. wound bed filled with cellular granulation tissue infiltrated with inflammatory cells (arrows). E. intact epithelium (e) with no hair follicles and newly formed parallel collagen fibers. F. intact epidermis (e) with many hair follicles (h), dermis contained hair follicles (h) and sebaceous glands (s).

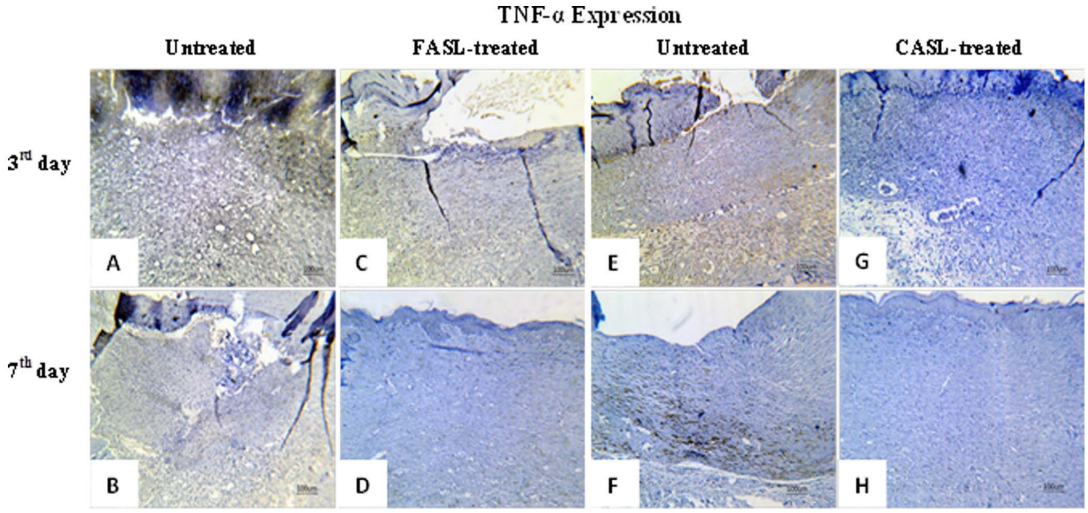


Fig. 3. Photomicrographs of immunostained sections against TNF-α in untreated, FASL-treated and CASL-treated FTW. The untreated wounds showed strong expression at the 3rd day (A and E) and decreased to be moderate at the 7th day (B and F). FASL-treated wounds exhibited mild expression at the 3rd day (C) with negative expression on the 7th day (D). While CASL-treated wounds revealed trace expression at the 3rd day (G) and negative expression at the 7th day (H).

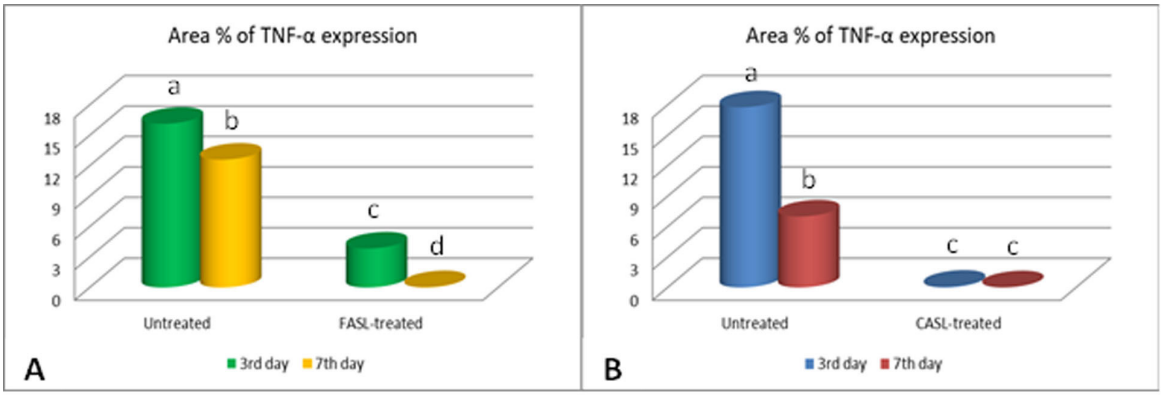
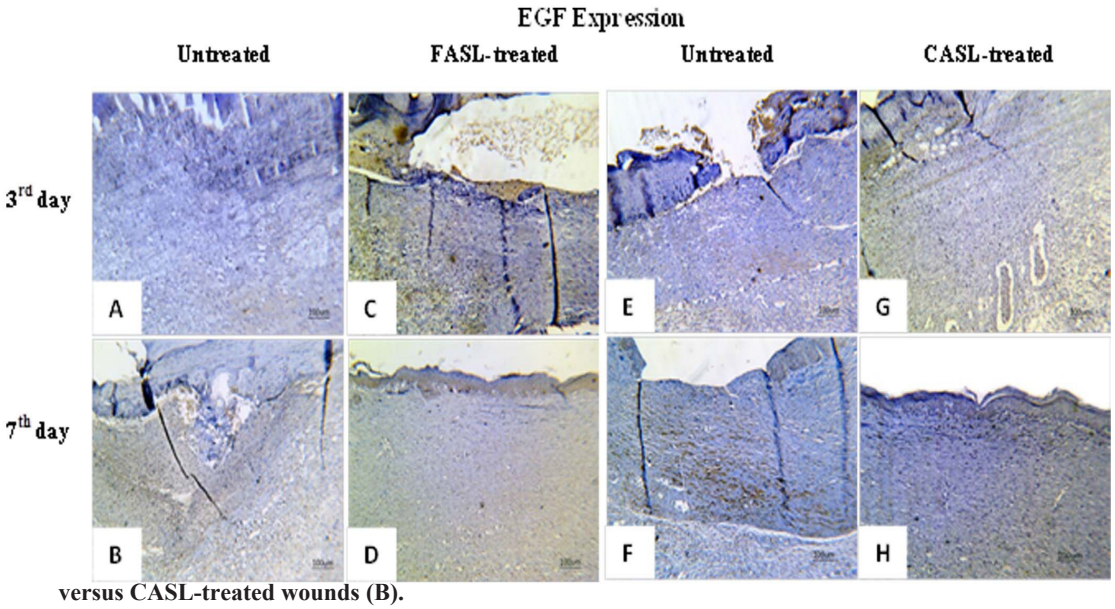


Fig. 4. Charts of image analysis for quantification of TNF-α immunostaining in the histology sections of FTW. The charts represent the average staining area percentage resulted on 3rd and 7th days post-wounding among individuals of each rat group, including the untreated versus FASL-treated wounds (A) and untreated

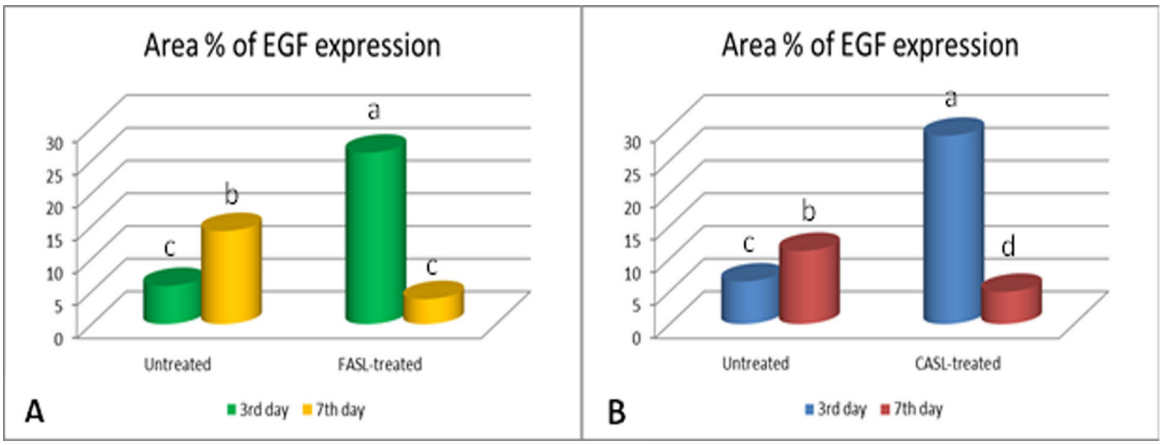
Expression of EGF

On the 3rd day post-wounding, the untreated wounds revealed mild expression of EGF, while this was expressed strongly in all treated wounds (whether with CASL or FASL). The EGF levels were observed in a mild-moderate expression on the 7th day in the untreated wounds, when they They declined in the treated wounds due to the completeness of healing (Figure 5). Statistically, the untreated wounds showed lower EGF expression on the 3rd day that which was significantly increased on the 7th day. On the contrary, FASL-treated and CASL-treated wounds revealed high EGF expression on the 3rd day that showed a significantly reduction reduced on the 7th day. Besides, the treated wounds either with FASL or CASL showed a significantly higher EGF expression on the 3rd day and a significantly lower expression on the 7th day compared to untreated controls at on the same dates (Figure 6). the 3rd day post-wounding, the untreated wounds revealed mild expression of EGF, while this was expressed strongly in all treated wounds (whether with CASL or FASL). The EGF levels were observed in a mild-moderate expression on the 7th day in the untreated wounds, when they declined in the treated wounds due to the completeness of healing (Figure 5). Statistically, the untreated wounds showed lower EGF expression on the 3rd day that was significantly increased on the 7th day. On the contrary, FASL-treated and CASL-treated wounds revealed high EGF expression on the 3rd day that showed a significant reduction on the 7th day. Besides, the treated wounds either with FASL or CASL showed a significant higher EGF expression on the 3rd day and a significant lower on the 7th day compared to untreated controls at the same dates (Figure 6).



versus CASL-treated wounds (B).

Fig. 5. Photomicrographs of immunostained sections against EGF in untreated, FASL-treated and CASL-treated FTW. The untreated wounds showed mild EGF expression at the 3rd day (A and E) and increased to be moderate on the 7th day (B and F). While the treatment with FASL or CASL revealed strong EGF expression on the 3rd day (C and G, respectively), and this expression became down-regulated on the 7th



day to be mild (D and H, respectively).

Fig. 6. Charts of image analysis for quantification of EGF immunostaining in the histology sections of FTW. The charts represent the average staining area percentage resulted on 3rd and 7th days post-wounding among individuals of each rat group, including the untreated versus FASL-treated wounds (A) and untreated

Discussion

For a long time, HAM has been used as a wound dressing due to its richness in healing factors such as growth factors, anti-inflammatory cytokines, and ECM (Lei et al. 2017). ASL is rich in HA that acts as a ligand for CD44 expressed on inflammatory cells to mediate cellular adhesion and migration (Misra et al. 2015). Hyaluronic acid products can improve wound healing by maintaining tissue hydration and supporting the ECM during re-epithelialization (Higa et al. 2005). In addition, HA has some biophysical properties that improve its therapeutic effect, such as viscoelasticity, water retention, and biocompatibility (Dovedytis et al. 2020). This causes the increment of the water content in CASL compared to FASL of in the current study.

HAM is considered an allograft in humans, some studies recommended using it in a cell-

free form to reduce its immunogenicity (Li et al. 2023). However, the decellularization procedure requires chemical or enzymatical treatment of HAM, followed by mechanical removal of cells. This increases the cost and may cause mechanical deterioration (Ashouri et al. 2022). Therefore, this study aimed to develope a simple method for producing a cellular derivative of HAM for wound grafting. The HAM stromal side contains an acellular spongy layer of type III collagen in a meshwork structure rich in proteoglycans and glycoproteins (Gupta et al. 2015), this led to the idea of extracting the HAM stromal layer to use as a wound graft. mechanically In this study, we applied this extraction method of ASL without the need for chemical or enzymatic treatment of HAM. However, the manual extraction procedure increases the possibility of microbial contamination due to the excessive handling. This requires a microbiological assessment to ensure safety during the production steps. Therefore, the microbial bioburden assay reflects the inclusion of HAM with microorganisms. This study tested two disinfection procedures to reduce the risk of infection: the first is by irradiating the finished product package and the second is by incubating HAM in a nutrition medium containing antibiotics. The impact of each disinfection procedure on the therapeutic effect of ASL was evaluated by the clinical application on wounded rats. Furthermore, wounded rats were subjected irradiation to cause healing complications (Gu et al. 1998 and Johnson et al. 2017) for simulating wounds with difficult healing.

The impact of ionizing radiation in suppressing wound healing was illustrated previously (Qu et al. 2003). Accumulation of a high amount of MMPs in the wounds after irradiation was detected, causing elongation of the inflammation phase and delaying wound healing. This leads to healing complications in more than 60% of cancer patients after the radiotherapy treatment, including desquamation, fibrosis, skin atrophy, rupturing of blood vessels, fistula formation, and epithelial ulceration. Many healing factors were affected in the irradiated wounds, such as growth factors, interferon-γ, TNF-α, and proinflammatory cytokines (Haubner et al. 2012). By the way, the results of the current study confirmed that irradiation delayed wound healing in untreated wounds and caused insufficient perfection of skin appendages.

According to related studies (Campelo et al., 2018 and Abdel Gawad et al., 2018), wound grafting with HAM induced faster healing compared to untreated wounds, with more profound formation of granulation tissue, collagen deposition, and angogenesis ending with complete healing with keratinized epithelium. These findings are consistent with the results of the current study concerning using ASL products in wound grafting. This proves that ASL as an acellular HAM derivative preserves the healing effect of HAM with reducing the risk of immunological rejection of native (contains cells) HAM grafts.

The results of this study indicate that wounds revealed infiltrating PMNL in the first 3 days with the incidence of necrosis and inflammatory reactions, referring to the expression of TNF-α. The same results were also reported by Campelo et al. (2018), who found that wounds treated with human amniotic membrane exhibited a mild inflammatory response with earlier formation of granulation tissue that showed intense angiogenesis and increased deposition of extracellular matrix that proceeded to well-vascularized granulation tissues and organized collagen deposition. Moreover, protected wounds showed earlier inception of repair compared to exposed ones. This is exactly what the membrane provides for the wounded tissues against pathogens in addition to preservation from local tissue dehydration (Duarte and Duval-Araujo, 2014). The expression level of TNF-α is reduced in treated wounds with ASL than in untreated wounds; this may be due to the ability of HAM to inhibit MMPs expressed by PMNL (Hao et al. 2000 and Kim et al. 2000) and subsequently cause improvement of inflammation. The anti-inflammatory effect of the collagenous stroma of the amniotic membrane could also be attributed to the presence of IL-1 receptor antagonists, IL-10 and hyaluronic acid (Hao et al. 2000 and Manuelpillai et al. 2011).

Wounds treated with ASL showed faster wound closure with complete epithelization and restoration of skin appendages, while the untreated group showed complete closure with thin epithelium lacking any appendages. Our results are consistent with those obtained by Sastri et al. (2022), who reported a significant reduction of wound area in amnion-treated wounds as compared to untreated control. The rate of epithelialization per day was faster in the amnion group than the control one. Additionally, the thickness of granulation tissue at wound edges was greater in the amnion group compared to the control (Kim et al. 2013).

The acceleration of wound healing was favorable in the treatment with CASL over the treatment with FASL. This reflects that sterilization with gamma irradiation reduces the quality of healing factors present in ASL. In harmony, Suroto and his colleagues (2021) found that the level of growth factors in HAM preparations was significantly lower after gamma irradiation. However, these irradiated preparations were still sufficient to promote wound healing. The improvement of skin appendages in the treated wounds indicated the induction of growth factors by ASL to stem cell differentiation. Therefore, the expression of EGF was observed in the CASL-treated wounds before the FASL-treated and untreated wounds. Koizumi et al. (2000)documented that the AM expresses mRNAs of 8 growth factors that remarkably influence epithelial regeneration. This effect happens when intimate contact with the stromal matrix occurs (Sant'Anna et al. 2017). The amniotic membrane possesses many beneficial biological properties that accelerate and augment the healing process, such as anti-bacterial, anti- inflammatory, analgesic and re-epithelializing effects (Tseng et al. 2004). In addition, it stimulates the production of cytokines and growth factors that modulate cell migration and proliferation (Frykberg and Banks,2015).

Therefore, supplying such complicated wounds with inhibitors of MMPs, anti-inflammatory cytokines, and growth factors will improve wound healing. This explains why ASL decreases inflammation and accelerates the healing of wounds, with complete regeneration of skin appendages. Accordingly, ASL contains many healing factors that can improve complicated wounds and skin rejuvenation. These preclinical findings recommend further complementary studies to ensure the clinical feasibility of using ASL as a wound graft.

Conclusions

The *in vitro* cultivation of HAM in a nutrition medium with effective antibiotics ensured the elimination of microbial contamination, increased the production of ASL, and increased its healing effect. The clinical and histological results of the current study indicate that ASL products are histologically compatible for wound grafting. The sterilization by gamma irradiation of FASL resulted in a slight reduction in its therapeutic effect compared to the non-irradiated CASL. However, the approached ASL products in this study have good impacts on wound healing and skin rejuvenation, especially in patients who have had radiation therapy or whose wound healing is compromised. For these reasons, further medical research and complementary scientific studies on ASL products should be focused on.

Disclosure of interest

The authors declare that they have no competing interests.

References

1. Abdel Gawad, A. M., Shaaban, A. M. M., El Ashwah, A. A. and Yehia, M. A. (2018) Effect of the use of the human amniotic membrane in healing of surgically induced skin defects in rabbits (histopathological study). *Alex Dent J.,* **43**(2), 108-115.

2. Ashouri, S., Hosseini, S. A., Hoseini, S. J., Tara, F., Ebrahimzadeh-Bideskan, A., Webster, T. J. and Kargozar, S. (2022**)** Decellularization of human amniotic membrane using detergent-free methods: Possibilities in tissue engineering. *Tissue and Cell,* **76**, 101818.

3. Campelo, M. B., Santos, J. D., Maia Filho, A. L., Ferreira, D. C., Sant'Anna, L. B., Oliveira, R. A., Maia, L. F. and Arisawa, E. Â. (2018) Effects of the application of the amniotic membrane in the healing process of skin wounds in rats. *Actacirurgicabrasileira,* **33**(2), 144-55.

4. Chang, A.C., Dearman, B. and Greenwood, J.E. (2011)A comparison of wound area measurement techniques: visitrak versus photography. *Eplasty*, **11**, e18.

5. de Vasconcelos Catão, M. H. C., Nonaka, C. F. W., de Albuquerque Jr, R. L. C., Bento, P. M. B. and de Oliveira Costa, R. (2015) Effects of red laser, infrared, photodynamic therapy, and green LED on the healing process of third-degree burns: clinical and histological study in rats. *Lasers Med Sci.,* **30**(1),421-428.

6. Devalia, H. L. and Mansfield, L. (2008) Radiotherapy and wound healing. *Int Wound J.,* **5**(1),40-44.

7. Dovedytis, M., Liu, Z. J. and Bartlett, S. (2020) Hyaluronic acid and its biomedical applications: A review. *Engineered Regeneration,.*(1),102-113.

8. Duarte, I. G. L. and Duval-Araujo, I. (2014) Amniotic membrane as a biological dressing in infected wound healing in rabbits. *Acta Cir Bras.,* **29**(5),334–339.doi: 10.1590/ S0102-86502014000500008.

9. Fouad, A., Hegab, M. M., Ahmed S. F., Koraa, S. and El-Arab, A. E. (2023) Effect of Bone Marrow-Derived Mesenchymal Stem Cells and Nanoscaffold on Wound Healing in Irradiated Rats. *Perio J.,***7**(1), 1-11.

10. Frykberg, R. G. and Banks, J. (2015) Challenges in the treatment of chronic wounds. *Adv Wound Care,* **4**(9),560–582.

11. Gu, Q., Wang, D., Cui, C., Gao, Y., Xia, G. and Cui, X. (1998) Effects of radiation on wound healing. *J Environ Pathol Toxicol Oncol.,* **17**(2), 117-123.

12. Gupta, A., Kedige, S. D. and Jain, K. (2015) Amnion and chorion membranes: potential stem cell reservoir with wide applications in periodontics. *Int J Biomat.,* 274082.

13. Hao, Y., Ma, D. H., Hwang, D. G., Kim, W. S. and Zhang, F. (2000) Identification of antiangiogenic and antiinflammatory proteins in human amniotic membrane. *Cornea,* **19**(3),348-352.

14. Haubner, F., Ohmann, E., Pohl, F., Strutz, J. and Gassner, H. G. (2012) Wound healing after radiation therapy: review of the literature. *Radiat Oncol.,* (7), 162.

15. Higa, K., Shimmura, S., Shimazaki, J. and Tsubota, K. (2005) Hyaluronic acid-CD44 interaction mediates the adhesion of lymphocytes by amniotic membrane stroma. *Cornea*, **24**(2),206-212.

16. Hipp, J., Cheng, J., Daignault, S., Sica, J., Dugan, M.C., Lucas, D., Yagi, Y., Hewitt, S. and Balis, U.J. (2011) Automated area calculation of histopathologic features using SIVQ. *Anal. Cell.Pathol.*, **34**(5), 265-275.doi: 10.3233/ACP-2011-0025.

17. ISO 13004. (2022) Sterilization of health care products — Radiation — Substantiation of selected sterilization dose: Method VDmaxSD.https://www.iso.org/standard/82297.html.

18. Johnson, M. B., Pang, B., Gardner, D. J., Niknam-Benia, S., Soundarajan, V., Bramos, A., Perrault, D. P., Banks, K., Lee, G. K., Baker, R. Y. and Kim, G. H. (2017) Topical fibronectin improves wound healing of irradiated skin. *Sci. rep.,* **7**(1),3876.

19. Kim, H., Son, D., Choi, T. H., Jung, S., Kwon, S., Kim, J. and Han, K. (2013) Evaluation of an amniotic membrane-collagen dermal substitute in the management of full-thickness skin defects in a pig. *Arch. Plast. Surg.,* **40**(1), 11-18.

20. Kim, J. S., Kim, J. C., Na, B. K., Jeong, J. M. and Song, C. Y. (2000) Amniotic membrane patching promotes healing and inhibits proteinase activity on wound healing following acute corneal alkali burn. *Exp. Eye Res.,* **70**(3),329-337.

21. Koizumi, N., Inatomi, T., Sotozono, C., Fullwood, N. J., Quantock, A. J. and Kinoshita, S. (2000) Growth factor mRNA and protein in preserved human amniotic membrane. *Curr Eye Res.*, **20**(3), 173-177.

22. Kowalski, J. B., Merritt, K., Gocke, D. and Osborne, J. (2012) Assessment of bioburden on human and animal tissues: part 2—results of testing of human tissue and qualification of a composite sample for routine bioburden determination. *Cell Tissue Bank,* **13**(3),431-439.

23. Lei, J., Priddy, L. B., Lim, J. J., Massee, M. and Koob, T. J. (2017) Identification of extracellular matrix components and biological factors in micronized dehydrated human amnion/chorion membrane. *Adv Wound Care,* **6**(2),43-53.

24. Li, Y., An, S., Deng, C. and Xiao, S. (2023) Human acellular amniotic membrane as skin substitute and biological scaffold: a review of its preparation, preclinical research, and clinical application. *Pharmaceutics,* **15**(9),2249.

25. Manuelpillai, U., Moodley, Y., Borlongan, C. V. and Parolini, O. (2011) Amniotic membrane and amniotic cells: potential therapeutic tools to combat tissue inflammation and fibrosis? *Placenta,* **32**(Suppl4),S320-325.

26. Misra, S., Hascall, V. C., Markwald, R. R. and Ghatak, S. (2015) Interactions between hyaluronan and its receptors (CD44, RHAMM) regulate the activities of inflammation and cancer. *Front Immunol.* (6),201.

27. Nemr, W. A. and Nashwa, R. K. (2024) Development of a multiplex polymerase chain reaction assay for detection of hepatitis C virus, hepatitis B virus, and human immunodeficiency virus 1. *World J. Virol.,* **13**(1), 88164.

28. Nemr, W., Bashandy, A. S., Araby, E. and Khamiss, O. (2016) Biological Activity Alterations of Human Amniotic Membrane Pre and Post Irradiation Tissue Banking. *Pak. J. Biol. Sci.,* **19**(7),289-298.

29. Niknejad, H., Peirovi, H., Jorjani, M., Ahmadiani, A., Ghanavi, J. and Seifalian, A. M. (2008) Properties of the amniotic membrane for potential use in tissue engineering. *Eur. Cell Mater.,* **15**,88-99.

30. Potekaev, N. N., Borzykh, O. B., Medvedev, G. V., Pushkin, D. V., Petrova, M. M., Petrov, A. V., Dmitrenko, D. V., Karpova, E. I., Demina, O. M. and Shnayder, N. A. (2021) The role of extracellular matrix in skin wound healing. *J. Clinic. Med.,* **10**(24), 5947.

31. Qu, J., Cheng, T., Shi, C., Lin, Y., Yan, G. and Ran, X. (2003) Reduced presence of tissue-repairing cells in wounds combined with whole-body irradiation injury is associated with both suppression of proliferation and increased apoptosis. *Med. Sci. Monit.,* **9**(10),BR370-377.

32. Ramos-Vara, J. A. (2005) Technical aspects of immunohistochemistry. *Vet. Pathol.,* **42**(4),405-426.

33. Salehi, S. H., As'adi, K., Mousavi, S. J. and Shoar, S. (2015) Evaluation of Amniotic Membrane Effectiveness in Skin Graft Donor Site Dressing in Burn Patients *Indian J. Surg.,* **77**(Suppl2),427-431.

34. Sant'Anna, L. B., Brito, F. S., Barja, P. R. and Nicodemo, M. C. (2017) Long-term effects of human amniotic membrane in a rat model of biliary fibrosis.*Braz. J. Med. Biol. Res.,* **50**(7),e5692. doi: 10.1590/1414-431X20175692.

35. Sastri, N. C. A., Saputro, I. D. and Zarasade, L. (2022) A comparative study of full-thickness wound healing in rats using Nile tilapia skin and fresh human amnion. *Bali Med. J.,* **11**(3), 1945-1952.

36. Suroto, H., Aryawan, D. M. and Prakoeswa, C. A. (2021) The Influence of the preservation method and gamma irradiation sterilization on TGF-β and bFGF levels in freeze-dried amnion membrane (FD-AM) and amnion sponge. *Int. J. Biomat.,* 6685225.

37. Suvarna, K. S., Layton, C. and Bancroft, J. D. (2018) Bancroft's theory and practice of histological techniques E-Book. Elsevier health sciences; 8th ed.

38. Tseng, S. C. G., Espana, E. M., Kawakita, T., Di Pascuale, M. A., Li, W., He, H., Liu, T. S., Cho, T. H., Gao, Y. Y., Yeh, L. K. and Liu, C. Y. (2004) How does amniotic membrane work? *Ocul. Surf.,* **2**(3), 177–187. doi: 10.1016/S1542- 0124(12)70059-9.

التقييم قبل السريري لطبقة الستروما للنسيج الأمنيوسى كضمادة جروح في الجرذان المثبط التنامها بواسطة أشعة جاما

[1]نشوة كمال رضوان،[2]وليد عبد الجابر نمر، *[1]سلوى فريد أحمد

[1]قسم البحوث الصحية الإشعاعية. المركز القومى لبحوث وتكنولوجيا الإشعاع. هيئة الطاقة الذريةالمصرية, القاهرة, مصر.

[2]قسم الميكروبيولوجيا الإشعاعية. المركز القومى لبحوث وتكنولوجيا الإشعاع. هيئة الطاقة الذريةالمصرية, القاهرة, مصر.

الغشاء الأمنيوسي هو بديل للجلد وله العديد من المزايا مما يجعله مناسبًا لعلاج العديد من الجروح المقاومة للعلاج مثل جروح ما بعد التشعيع. تهدف الدراسة إلى تقييم فعالية الطبقة اللحمية التي تحيط بالجنين سواء كانت طازجة أو مزروعة لعلاج جرح الجلد الكامل السماكة في الجرذان المشعة بأشعة جاما. تكونت التجربة من مجموعتين من الفئران المشعة بأشعة جاما. تعرضت الجرذان لجرحين جلديين بسمك كامل. تم علاج الجرح الأيمن إما باستخدام الطبقة اللحمية التي تحيط بالجنين طازجة او مزروعة بينما يعمل الجرح الأيسر كعنصر تحكم. تم تقييم الجرح سريريًا لمراقبة عملية الالتئام. تم صبغ عينة الجرح بالهيماتوكسيلين و الايوسين للفحص النسيجي كما تم صبغه بالاجسام المضادة لعامل نخر الورم ألفا وعامل نمو البشرة لتحديد مستوياتها في الجروح مع تحديد نسبة مساحة التعبير. أظهرت النتائج معدل أسرع في التئام الجروح في الجرذان المعالجة بالطبقة اللحمية التي تحيط بالجنين سواء كانت طازجة أو مزروعة مقارنة بالفئران غير المعالجة. من الناحية النسيجية ، أظهرت الجروح المعالجة بالطبقة اللحمية التي تحيط بالجنين درجة أقل من الالتهاب وتكوين نسيج حبيبي أسرع مع إغلاق ظهاري كامل في وقت سابق مقارنة بالجروح غير المعالجة. علاوة على ذلك ، أدى علاج الجروح باستخدام الطبقة اللحمية التي تحيط بالجنين سواء كانت طازجة أو مزروعة إلى تقليل تعبيرعامل النخر الفا وتحفيز تعبير عامل نمو البشرةمقارنة بالجروح غير المعالجة.في الختام، إن الطبقة اللحمية التي تحيط بالجنين متوافقة نسيجيا وواعدة لتطعيم الجروح وتجديد شباب الجلد.