



Effect of Sprayed Cultured Autologous Keratinocyte Suspension Used Alone and in Combination with Fibrin Glue to Closure of 3rd Degree Burn Wounds in Rat

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Abstract

Objective- Our point is to determine if the treatment of the 3rd degree burn wounds makes any difference if sprayed cultured autologous keratinocyte used alone or in combination with Fibrin glue.

Design- Animal experimental study.

Animals- 30 male Wistar rats.

Procedures- Thirty male Wistar rats were selected and their keratinocytes were isolated and cultured from small skin biopsy. Rats were divided randomly into 3 equal groups and three 3rd degree burn wounds with 1 cm diameter were created on their back. Wounds were treated with normal saline (control group), Autologous keratinocyte suspension (AKS) (test group1) and AKS + Fibrin (AKS+ F) (test group2) in each rat. The wounds were photographed on selected days (0, 3, 5, 7, 10 and 14) and the percentage of wounds contraction was calculated with image analyzer. Biopsy samples were taken as well for histological studies on days 3, 7 and 14.

Results- The results showed faster wound contraction for AKS and AKS+ F groups during 14 days period than control group ($P < 0.05$). Histological observations showed significant difference in inflammation and fibrotic tissue formation between groups but other parameters did not show any remarkable difference.

Conclusion and Clinical Relevance- Although it can be concluded that fibrin glue could prevent cells from dripping out of the wound and also speed up the wound contraction and extend the inflammation and fibrotic tissue formation, it did not have any effect on fastening the re-epithelialization and granulation tissue formation during 14 days.

Key words- 3rd degree burn, Fibrin glue, Autologous keratinocyte suspension.

Introduction

The skin is the largest organ of the human body, representing approximately one-tenth of the body mass, and is quiet necessary for our survival. This organ serves several important functions, including physical barrier to the external environment, thermal regulation, and retention of normal hydration. Thermal injuries, chronic ulcerations secondary to diabetes mellitus, venous stasis and trauma have been the main causes for loss of skin integrity and severe morbidity.^{1, 2}

When a large percentage of skin is lost, cultured autologous epithelial (CAE) sheets are routinely used to make autologous grafts, which can be lifesaving for patients with extensive burns.^{3,4} Autologous keratinocytes can be isolated and cultured into cohesive sheets of epithelium that can be transplanted onto large skin defects on the patient.⁵

Clonogenic keratinocytes, termed holoclones, can be isolated from skin and serially propagated in culture for over 140 doublings, and have been shown to be bona fide multipotent stem cells based on their ability to renew multiple lineages in the skin.^{6, 7} The main advantage of the cultured keratinocyte method in treatment of the cutaneous wounds is the capacity of this method to propagate as much keratinocyte or epidermal sheet to cover the whole body surface from a small skin biopsy within a short period of time (almost 4-5 weeks). This is very significant especially in the case of lack of donor sites in extensive burn cases. Nevertheless there are still some deficiencies in CAE method. It takes at least 3-5 weeks to culture appropriate amount of epidermis out of a donor skin sample, and the

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final cultured epidermal layer is so fragile and difficult to detach from the culture dish and also transport to the patient's wound area.

The difficulty of CAE application made scientist to develop another transplantation method which is the spray of autologous keratinocyte suspension (AKS) to the wound area.^{8,9}

In this case, one of the main problems is cell loss during the post transplantation period of wound healing because of weak adhesion of the cells to the wound bed and their wasting during bandage changes.

To solve this problem we used fibrin glue to improve the adhesion of the cultured keratinocyte to the wound bed.

Fibrin glue has been already used in burn surgeries and has various benefits like haemostatic and antibacterial effect.¹⁰ Keratinocytes can also remain viable as a suspension in commercially available fibrin glue for at least 5 days.¹¹ Fibrin glue is an excellent template for cellular migration which can be modified to create an optimum pore size for keratinocyte, endothelial and fibroblast proliferation.¹² Fibrin glue has also been demonstrated as a delivery system for both growth factors and genetically modified cells engineered for enhanced growth factor expression¹⁰.

The object of present study is to determine if the treatment of the 3rd degree burn makes any difference if sprayed cultured autologous keratinocyte used alone or in combination with Fibrin glue.

Materials and methods

Ethics approval for this experiment was obtained from Ferdowsi University of Mashhad Research and Ethic committee (approval code: FUM-3998).

Animals

A total of 30 male Wistar rats aging 6-8 weeks and weighting 250±20 gr were obtained from Laboratory Animal Research Center of Medical University of Mashhad, Faculty of pharmacy. Rats were allowed at least 2 weeks to adapt to the environment (25°C, 12-h light/dark cycle) and making sure about their health before the start of experiments. A commercial chow (Javaneh Khorasan, Mashhad, Iran) and tap water were available all the time.

Keratinocyte isolation and culture

Rat keratinocytes were isolated and cultivated according to the protocols already described by Freshney, Hager and Yano.¹³⁻¹⁵

Under general anesthesia through IP injection of 5 mg/kg Xylasin (Xylazine, 2%, Bayer, AG, Leverkusen) plus 60 mg/kg Ketamin (Ketamin HCl 50 mg/ml, Rotexmedica, Trittau, Germany) and followed by carefully shaving off the body hair of the area, a split

thickness skin of around 2×2 cm was harvested under sterile conditions from the Para-vertebral region of the rats, near the cervical area. The area was sutured by 4-0 nylon suture to prevent any infection; sutures were removed after 6 days from biopsy harvesting.

Skin biopsies were rinsed 3 times in cold phosphate-buffered saline without Ca²⁺ or Mg²⁺ (PBSA) (Dulbecco's PBS, PAA, Austria) with 2% antibiotic/antimycotic and immediately placed into transport medium (DMEM+10%FBS+2% antibiotic/antimycotic). In lab, subcutaneous tissue was eliminated with curved scissors and each biopsy was dissected into 1×0.5cm pieces with scalpels and rinsed two times in PBSA. Then the tissues were incubated on cold 0.25% trypsin (Gibco, Grand Island, NY, U.S.A.) at 4°C for 16–24 h. The epidermis was separated from the dermis using two fine curved forceps, and collected it in a 50-mL centrifuge tube containing 20 mL of complete culture medium followed by gently pipetting. The isolated epidermal cells were washed twice in complete culture medium by centrifugation at 500 g for 5 min. Finally the total number of cells and the viable cells were counted using Trypan blue staining.

The isolated rat keratinocytes were then seeded onto collagen coated flasks (25 cm²/ one flask per each rat) which had been pre-seeded with inactivated 3T3 cells (3T3 cell were inactivated by Mitomycin-C treatment [Kyowa, Tokyo, Japan]). The 3T3 cells were plated at the density of 1×10⁵ cell/cm² onto the flasks a day prior to the rat keratinocyte isolation and incubated at 10% CO₂ and 37°C. A seeding density of 3×10⁴ cell/cm² was used for keratinocytes, which produced an 80% confluent flask in 7–8 days. Our culture medium consisted of DMEM (Dulbecco's Modified Eagles Medium, High Glucose with L-Glutamine and Sodium Pyrovalate, PAA, Austria) supplemented with 10% FBS (Fetal Bovine Serum, Standard Quality, PAA, Austria), 10 ng/ml Epidermal Growth Factor (EGF, Collaborative Biomedical Products, Bedford, USA), 10⁻¹⁰ M Cholera toxin (CT, Sigma, USA), 1% Penicillin-Streptomycin (10,000 IU/ml Pen [Pen Potassium, Jaber-e-bne-Hayyan Co, Tehran, Iran] and 10,000 µg/ml Streptomycin [Rotexmedica, Trittau, Germany]), 5 µg/ml Insulin (Exir Co, Iran), 1.8×10⁻⁴ M Adenine (Himedia, India), 1.4 ng/ml Tri-Iodothyronine , sodium salt (Sigma, USA), 0.5 µl/ml L-ascorbic acid (Jaber-e-bne hayyan co., Iran) and 5 µg/ml Apo-Transferrin (Sigma, USA) and 0.5 mg/ml Hydrocortisone (Hydrocortisone Sod. Succ., Rotexmedica, Trittau, Germany).

The culture medium was changed every other day. When 80% confluence has been reached, the flasks were passaged by dispersing the keratinocytes with 0.25% trypsin /0.02% EDTA (Gibco BRL, USA). These were then subcultured to collagen coated flasks, pre-treated with inactivated 3T3 cells. These secondary cultures reached 80% confluence in 4–5 days (Fig.1).

Preparation of the fibrin glue

Fibrin glue was kindly provided by Dr Darioush Hamidi (Department of biochemistry, Medical university of

Mashhad) based on routine protocols of Fibrin glue preparation.

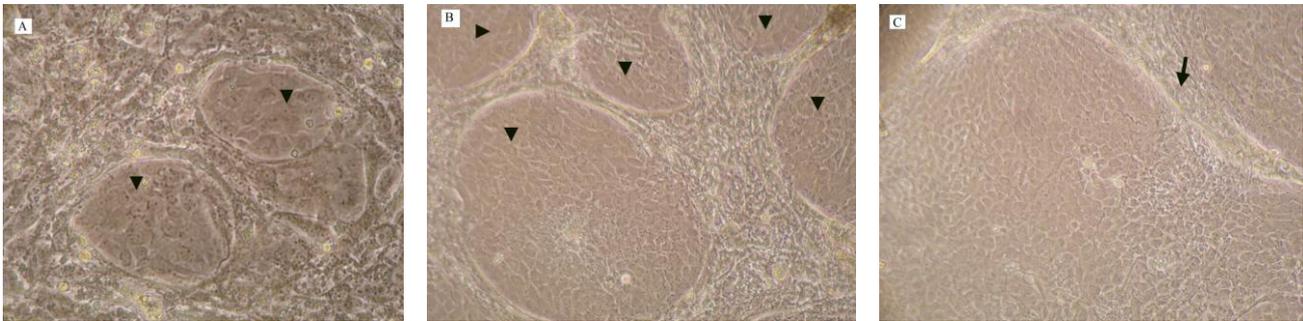


Figure 1. Primary keratinocyte culture 3 days after seeding, cultured keratinocyte colonies (arrows), (100X magnification) (A), Primary keratinocyte culture 6 days after seeding, increasing the amount and the size of the colonies are significant (100X magnification) (B), primary keratinocyte culture, 8 days after seeding, keratinocyte cell covered almost 80% of the flask surface and just thin lines of 3T3cell could be observed (arrow) (100X magnification)(C).

Formation of the burn wounds

Each rat was anesthetized by an IP injection of Ketamine and Xylazine (as described above). The dorsal thoracic area was shaved and scrubbed using 70% ethanol. After a deep general anesthesia had been reached, 3 round wounds, approximately 1cm in diameter was performed using a 100W electric soldering iron, heated to the point of redness (about 800° C) for 5 seconds.¹⁶ There was 1 cm distance between wounds. After 2 h, damaged tissues were removed from the fascia and the epidermal, dermal and hypodermal layers were removed down to the panniculus carnosus muscle layer, creating a 3rd degree burns wounds.

Experiment design and cell sprays

80% confluent autologous keratinocytes (second passage) were suspended in concentration of 1×10^6 cell/ml in DMEM. Each rat had 3 burn wound area included: Control which was sprayed by 1 ml of normal saline, Test1 sprayed by Autologous keratinocyte at the density of 5×10^5 cell/cm² (AKS) and Test 2 sprayed by Autologous keratinocyte at the density of 5×10^5 cell/cm² in combination with equal volume of fibrin glue (AKS+ F), at the last second before cell spraying, 20µl/ml thrombin was added to the AKS+ F suspension.

Evaluation of the wounds contraction

Wounds were photographed using digital camera (Canon IXY32S, Japan) on days 0, 3, 5, 7, 10 and 14 post cell transplantation. The wounds areas were measured using image analysis software (Scion Image Software, USA). The percentage of wounds contraction was determined using the following formula:

$$1. N = (N_0 - N_x) / N_0 \times 100$$

N: % wound contraction at day x compared with day 0

N₀: wound size (mm²) at day 0

N_x: wound size (mm²) at day X

Wound biopsies and histochemical study

Biopsy samples were taken at days 3, 7 and 14 after wound formation (10 rats were sacrificed on a certain day). Rats were euthanized using CO₂ inhalation and whole wound biopsies with adjacent normal skin were collected and fixed in 10% buffered formalin, followed by tissue processing and embedding in paraffin, 5 µm sections were prepared and stained using H & E (Hematoxylin and Eosin) technique. Histological examination included of epithelialization, granulation tissue formation, inflammatory response and fibrous tissue formation were evaluated and graded based on the progression of the healing process (Table 1).

Statistical analysis

Repeated measures ANOVA followed by Bonferroni post hoc test were conducted to investigate the effects of treatments on wound contraction during the study period.

A non-parametric Kruskal-Wallis test at p<0.05 was used to investigate whether the histopathological indices of the three groups differed or not. Pairwise comparison performed by the Mann-Whitney U-test. Since this was multiple testing of the data the significance level was adjusted using the Bonferroni test. Three groups were compared and therefore the significance level became 0.05 divided by 3; (P<0.017). All statistical analysis performed using SPSS statistical software version 16 (SPSS Inc., Chicago, IL).

Table 1. Histopathological parameters scoring based on the progression of the healing process.

Grade	Histological Description			
	Granulation tissue formation	Inflammation	Fibrous tissue formation	Re-epithelialization
1	Not observed	Partly Acute	Not observed	Not observed
2	Primary granulation tissue has been formed	Very Acute	Primary fibrosis has been formed lightly at the wound bed	Partially observed at the wound bed
3	Granulation tissue completely formed	Moderate inflammation	Moderate fibrosis could be observed at the wound bed	Moderately covered the wound bed
4	Granulation tissue is regressing	Chronic inflammation	Completed at the wound bed	Completed at the wound bed
5	Completely regressed	Very mild inflammation	-	-

Results

All the rats survived the experiment and there were no obvious side-effects due to fibrin glue or cell spray usage.

30 rats were used for this study and a total of 90 wounds were created under the protocol which has been previously explained. Finally we had 30 wounds which treated with AKS, 30 with AKS+F and 30 control wound which received normal saline. Wounds were allocated to test groups in such a way that each group had equivalent numbers of wounds in similar positions on consecutive animals. The total number of cells delivered to each wound was 5×10^5 cell/cm² in both the AKS and AKS+F groups.

Gross observation of spray application

Better adherence and less dripping out of the cell suspension in AKS+F group in contrast with AKS group was observed.

Macroscopic results and evaluation of wound contraction

The average of wound contraction percentage in

wounds treated by AKS+F during the 3 (n=30), 7 (n=20) and 14 (n=10) days after wound treatment was greater than those treated by normal saline ($P < 0.05$). Also wound contraction percentage in wounds treated by AKS during the 7 and 14 days after wound formation was greater than normal saline ($P < 0.05$). Comparison of the average of wound contraction percentage in wounds treated by AKS+F and AKS didn't show any significant difference ($P < 0.05$) (Table 2, Fig. 2).

Microscopic results and histological observations

In the control wounds no epithelium was seen histologically at the center of wound bed, just thin Re-epithelialization around the border of the wound could be observed. But in test groups a thin epithelium was observed at the center of the wound area. The degree of Re-epithelization didn't show any significant difference between test groups ($p > 0.05$). Inflammatory response and fibrous tissue formation at days 3 of the study in rats treated with AKS+F were significantly greater than those treated with normal saline ($P < 0.05$, $P = 0.002$). None of the other histological parameters were significantly different between various groups in various days (Table 3, Fig. 3).

Table 2. Description of percentage of wound contraction at day 3, 5, 7, 10 and 14 in the studied groups.

Day	N	Control		AKS		AKS+F	
		Mean	SEM*	Mean	SEM	Mean	SEM
3	30	9.87%	1.46	13.14%	1.38	17.28%	1.89
5	20	16.74%	1.41	20.06%	2.11	28.62%	2.64
7	20	27.84%	1.63	37.39%	2.42	47.15%	2.83
10	10	45.29%	3.98	60.56%	2.97	66.91%	2.73
14	10	77.8%	2.01	85.67%	0.88	88.41%	1.4

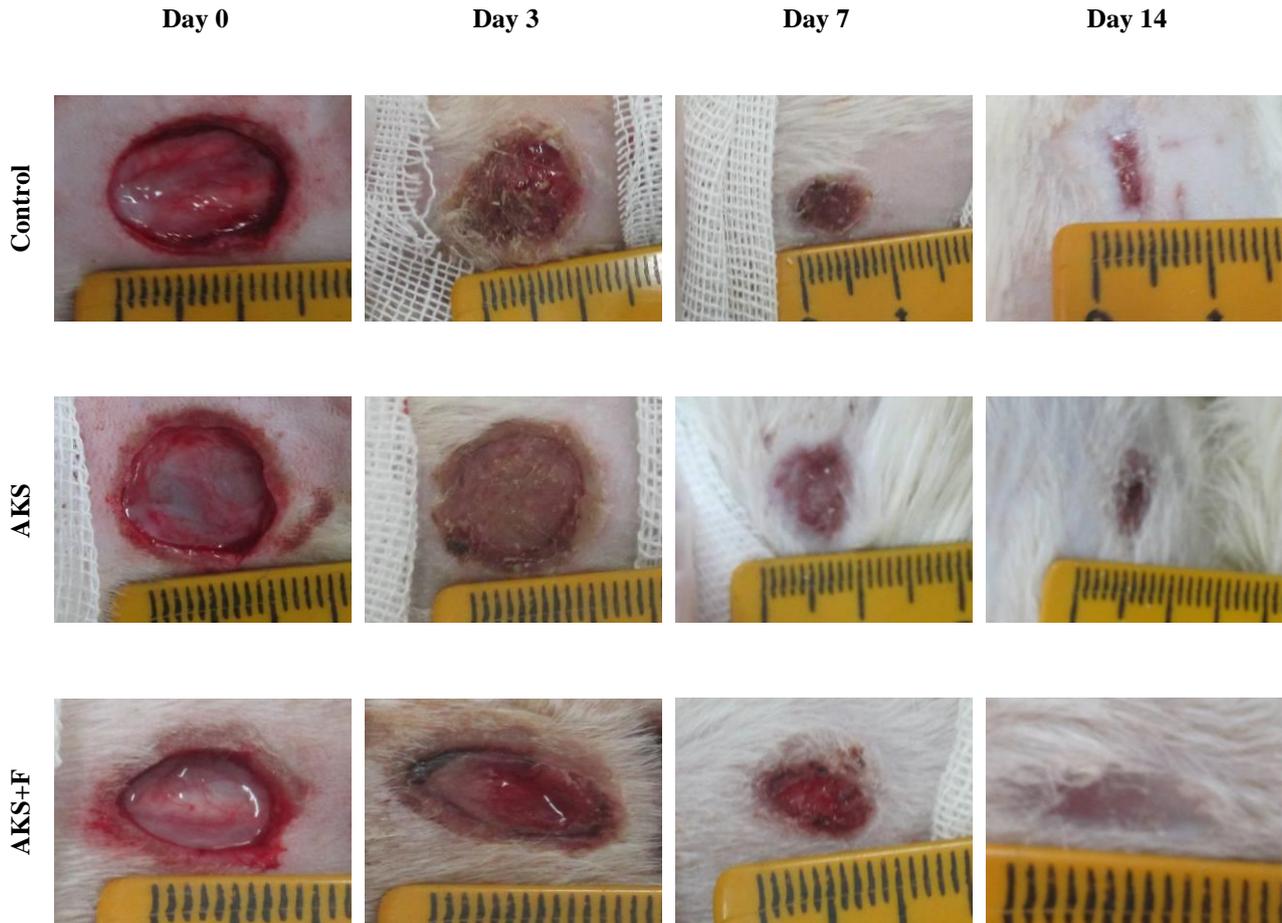


Figure 2. Photographs of macroscopic appearances of wound that were untreated (control), treated with autologous keratinocyte suspension (AKS), or treated with autologous keratinocyte suspension in combination with Fibrin glue (AKS+F) in various days.

Discussion

Since 1998 which Fraulin et al.¹⁷ described their novel method of epithelial cell spray on wounds in pigs, many other trials have been organized to develop this technique for better healing, lowering the time of cell culturing and increasing the taking rate of transplanting. Spray of cultured autologous keratinocyte suspension which we have used in our study has also some disadvantages such as low capacity of adhesion to the wound bed. We tried to see if fibrin glue has any inhibitory effect on dripping the cells down. If yes, does it have any acceleratory effect on treatment of the 3rd degree burn in contrast with autologous keratinocytes which were sprayed alone to the wound bed.

Results showed that fibrin glue prevents cells from dripping out of the wound but it didn't have any effect on progressing of the Re-epithelialization in AKS+F group in contrast with AKS group and also we didn't observe any difference in wound contraction between these two groups. All of these results were similar to the Currie L. J. et al. study on pigs.¹⁰

Based on previous studies fibrin glue is an absolutely safe protein which has no inhibitory effect on both culturing and transporting of the keratinocytes, it is also a non-toxic protein to cultured autologous keratinocytes.^{18, 19} Despite all of these, there wasn't any remarkable difference between our test groups in epithelialization and granulation tissue formation, Currie et al results showed the same findings in pigs, they believe that fibrin glue might prevent the process of Neovascularization due to an inhibitory effect on serum imbibition which leads to less survive of the grafted cells. Also another explanation could be the sample size of our study which was 30 rats and we had 90 wounds in our study. If the number of samples is raised, results would be more significant in histological parameters.

Our result in fibrotic tissue formation and inflammatory response showed more reaction on day 3 in AKS+F group. Galletti et al studied on the effect of fibrin glue on treatment of open wounds and their experiment showed the same findings in inflammatory and fibrotic

tissue at day 5.²⁰ The difference between our study and theirs is the application of AKS in our trial.

We should pay attention to the point that inflammatory phase is a normal and necessary reaction following injury and in the first stage of healing. Therefore to find out whether the inflammatory response is part of the normal healing process or due to the effect of the material which we used is quiet difficult.^{21, 22}

Our result didn't show any significant difference in percentage of wound contraction between test groups, which was in agreement with Currie and Cohen's findings.^{8, 23}

Both of them reported that fibrin glue didn't have any acceleratory effect on wound contraction during their studies. Also Cohen et al. pointed that fibrin glue may have some effects in wound contraction in pigs during

first week of the treatment but after 4 weeks, there wasn't any significant difference between the pigs which were treated with cultured epithelial cells alone and the pigs which received both cells and fibrin glue as treatment.²³

It can be concluded that fibrin glue could act as an appropriate wound cell delivery medium and may prevent cell loss. It also can accelerate inflammatory response and fibrotic tissue formation but didn't have any promotive effect on re-epithelialization which is the most important aim of wound healing. However, application of higher cell concentrations or the use of greater sample size could be areas for further studies.

Table 3. Microscopic results and histological observations

	Day	Control			AKS			AKS+F		
		Median	Q ₁	Q ₃	Median	Q ₁	Q ₃	Median	Q ₁	Q ₃
Inflammation	3	2	1	2	2	2	2	2	2	2
	7	3	2.75	3	3	3	3	3	3	3
	14	4	4	4	4.5	4	5	4	3.25	4.75
Fibrotic tissue formation	3	1	1	1	1	1	2	1.75	2	2
	7	2	2	3	3	3	3	3	3	3
	14	4	3.25	4	4	4	4	4	4	4
Granulation tissue formation	3	2	2	2	2	2	2	2	2	2
	7	3	2	3	3	3	3	3	3	3
	14	4	4	4	4	4	4	4	4	4.75
Re-epithelialization	3	0	0	0	2	1	2	1.5	1	2
	7	0	0	0	2	2	2	2	2	2.25
	14	0	0	0	3	3	3	3	3	3

* Interquartile Range

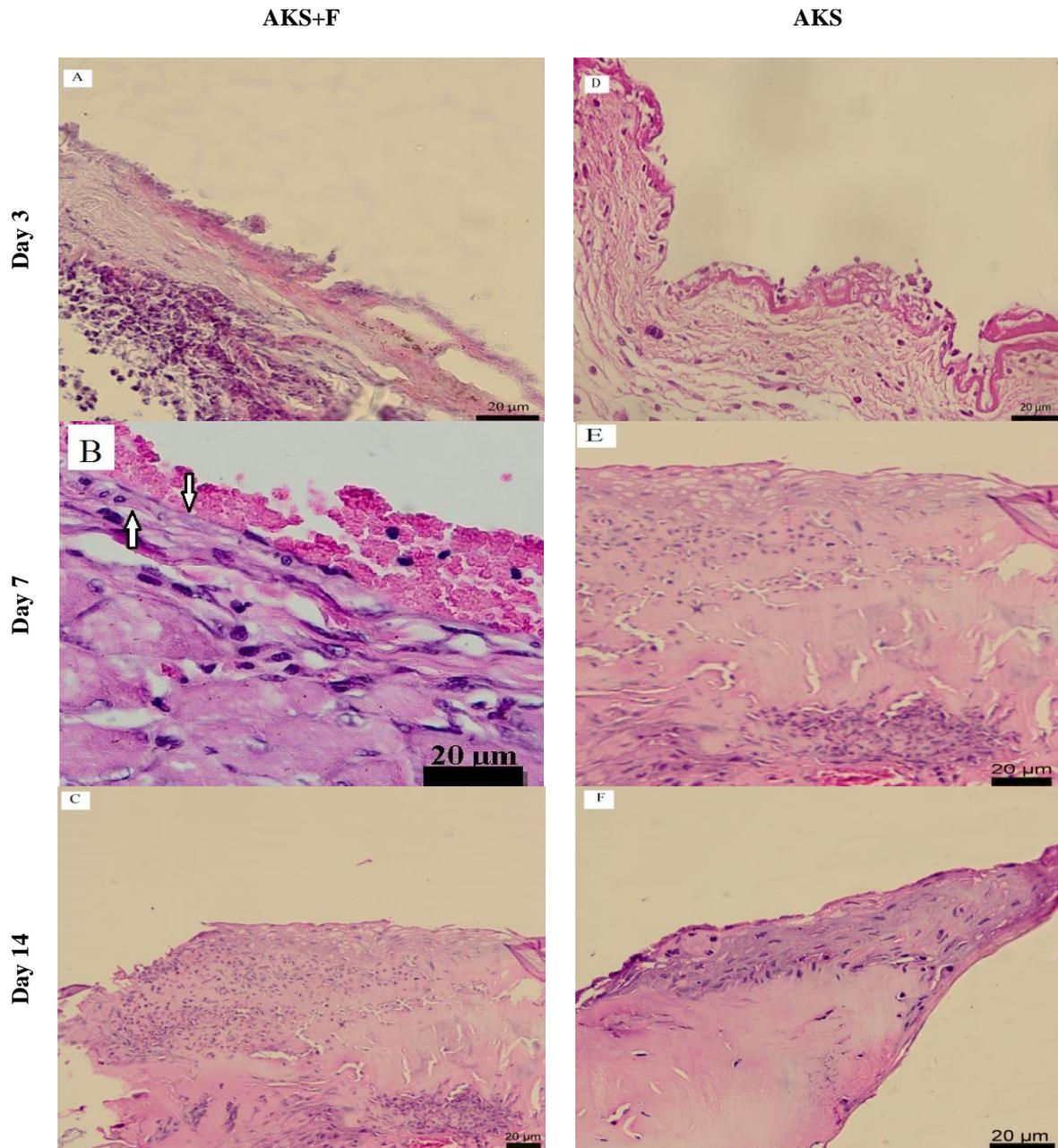


Figure 3. AKS+F group: day 3: a thin eosinophilic layer of fibrin could be observed over the wound bed (A), day 7: a thin layer of keratinocyte could be observed over the wound area (arrows) and fibrin glue is going to be regressed (B), day 14: multiple layers (4-6 layers) of keratinocyte covered the wound area (C). AKS group: day 3: there wasn't any significant keratinocyte layer, just diffused cell could be observed in the area (D), day 7: a thin 2 layers of keratinocyte was observed (E), day 14: multilayer of keratinocytes are stabilized (F).

Acknowledgments

This study was funded by a research grant from both Ferdowsi University of Mashhad and Mashhad University of Medical Sciences. The authors would like to thank Dr. Adel Moallem for his kind supports and advises during cell culture procedure and Dr.

Mohammad Azizzadeh for statistical analysis. We are also grateful to Mr. Ghanbari and his colleagues (Ghaem hospital laboratory of histopathology) for the preparation and staining of histological sections and Mr. Akbari for kindly assistance in animal experiments.

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چکیده

اثراسپری سوسپانسیون کراتینوسیت خودی به همراه و بدون چسب فیبرین در التیام زخم سوختگی درجه ۳ در رت

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هدف - بررسی و مقایسه اثر بخشی اسپری سوسپانسیون کراتینوسیت های خودی به همراه و بدون چسب فیبرین در درمان سوختگی درجه ۳ در رت.

طرح مطالعه - مطالعه تجربی در حیوانات زنده

حیوانات - ۳۰ سر رت نر نژاد ویستار

روش کار - ۳۰ سر رت انتخاب شدند و پس از استحصال یک قطعه کوچک بیوپسی پوستی از هر یک، سلول های کراتینوسیت پوست جدا شده و کشت داده شدند. رت ها به سه گروه مساوی ده تایی تقسیم شدند و تحت بیهوشی عمومی تعداد ۳ زخم سوختگی درجه ۳ به قطر ۱ سانتی متر در پشت حیوانات ایجاد شد. هر کدام از زخم ها در هر حیوان توسط نرمال سالین (کنترل)، سوسپانسیون کراتینوسیت خودی (تست ۱) و ترکیب سوسپانسیون کراتینوسیت خودی و چسب فیبرین اسپری شدند. به منظور مطالعه ماکروسکوپی از زخم ها در روزهای ۰، ۳، ۵، ۷ و ۱۴ عکس برداری شد و میزان بسته شدن زخم ها پس از محاسبه مساحت آن ها توسط نرم افزار آنالیز تصاویر انجام گرفت. جهت مطالعه هیستولوژیک، هر یک از گروه های ده تایی از رت ها در روز های ۳، ۷ و ۱۴ کشته شدند و نمونه های پوستی آنها تهیه شد و پس از طی مراحل تهیه مقاطع بافتی و رنگ آمیزی هماتوکسیلین و انوزین به جهت بررسی میزان پیشرفت در ترمیم بافتی مورد مطالعه قرار گرفتند.

نتایج - انقباض زخم های درمان شده با اسپری کراتینوسیت های اتولوگ در هر دو گروه تست ۱ و ۲ به نسبت گروه کنترل به طور معنی داری بیشتر بود ($P < 0.05$). در مطالعه بافت شناسی هم میزان آماس و تشکیل بافت فیبروتیک بین گروهها متفاوت بود اما پارامتر های دیگر تفاوت معنی داری را نشان نمی دادند.

نتیجه گیری و کاربرد بالینی - میتوان اعلام کرد که هر چند چسب فیبرین در جلوگیری از ریزش سلول ها از سطح زخم نقش دارند و انقباض زخم را سرعت می بخشند اما در دوره ۱۴ روزه با وجود تحریک واکنش آماسی و تشکیل بافت فیبروزی، تأثیری بر پیشرفت اپیتیلیالیزاسیون و تشکیل بافت گرانولاسیون در کف زخم ندارد.

کلید واژگان - سوختگی درجه ۳، چسب فیبرین، سوسپانسیون کراتینوسیت خودی.

