SPRAYED CULTURED AUTOLOGOUS KERATINOCYTES IN THE TREATMENT OF SEVERE BURNS: A RETROSPECTIVE MATCHED COHORT STUDY

AÉROSOLISATION DE KÉRATINOCYTES AUTOLOGUES CULTIVÉS DANS LE TRAITEMENT DES BRÛLURES ÉTENDUES: UNE ÉTUDE COMPARATIVE RÉTROSPECTIVE

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SUMMARY. The standard treatment of burns is early excision followed by autologous skin grafting. The closure of extensive deep burns poses a considerable challenge. Cultured autologous keratinocytes have been used since 1981 in an effort to improve healing. However, the time required to culture the cells and the lack of a dermal component limit the expectations of outcome. Our aim was to compare the duration of hospital stay between patients who were treated with autologous skin grafts and cultured autologous keratinocytes and those who were treated with autologous skin grafting without cultured autologous keratinocytes. In this retrospective study all patients treated with cultured autologous keratinocytes between 2012 and 2015 were matched by size and depth of burn with patients not treated with cultured autologous keratinocytes. Multivariable regression was used to analyse associations between duration of hospital stay and treatment adjusted for age, mortality, size and depth of the burn. Then, we investigated the possibility of differentiation of human bone marrow stem cell line to keratinocyte-like cells as a future direction. The regression analysis showed a coefficient of 17.36 (95% CI -17.69 to 52.40), p= 0.32, for hospital stay in the treatment group, compared with the matched group. Our results showed no difference in the duration of hospital stay between the two treatments. Autologous stem cells should be considered as a future modality of burn management, although further studies are needed.

Keywords: burn, sprayed cultured autologous keratinocytes, stem cells, outcome, duration of hospital stay

RÉSUMÉ. Le traitement de référence des brûlures est l’excision- greffe précoce, qui est problématique en cas d’atteinte étendue. La culture de kératinocytes autologues est utilisée depuis 1981 dans le but de répondre à cette problématique mais se heurte au temps nécessaire à sa mise en œuvre, ainsi qu’à l’absence de feuillet dermique, génératrice de séquelles. Cette étude a comparé la durée de séjour des patients traité par excision- greffe et culture de kératinocytes à celle des patients traités de manière conventionnelle. Les patients hospitalisés entre 2012 et 2015 ont été comparés à des patients de même surface et profondeur traités conventionnellement, en utilisant une analyse multivariée ajustée sur l’âge, la mortalité, la surface et la profondeur de la brûlure. L’analyse n’est pas significative (coefficient 17.36 ; IC95 -17.69 à 52.40 ; p= 0.32). Il serait utile d’étudier l’utilisation des cellules souches médullaires, différenciées en kératinocytes, dans un protocole de culture.

Mots-clés : brûlure, aérosol de kératinocytes autologues de culture, cellules souches, évolution, durée de séjour

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Introduction

The gold standard for the treatment of deep burns is excision followed by coverage with an autograft. Although this method is quick in covering skin defects, it is limited by the availability of donor sites. Thus, the development of novel techniques to create bioengineered skin is highly needed in clinical practice.1,2

Since 1975, it has been possible to cultivate human keratinocytes in vitro using irradiated 3T3 murine fibroblasts as a feeder layer and a specific culture medium.3,4 In Linköping Burn Centre we have been using a chemically mediated cell culture technique that does not require feeder layers of murine fibroblasts.3-5

In an attempt to speed up the process and shorten the duration of stay in hospital, patients with middle-sized burns were treated with human keratinocytes in an addition to split thickness skin grafts.6 In a different approach, grafts were cultured in epidermal sheets with several layers of keratinocytes. However, they were slow to culture, fragile, had abnormal dermo-epidermal junctions, and were expensive to cultivate.7 To overcome some of the disadvantages with multilayer sheet grafts, the keratinocyte cell suspension was developed.8,9 This approach, developed in 1998, reduced the time required for culture and allowed an easier method for cell delivery to the patients. In addition, the use of the keratinocyte cell suspension has improved the properties of the dermo-epidermal junction when transplanted to athymic mice.10 The single cell suspension method was further developed by spraying the cells onto the wound using an aerosol, which resulted in quicker epithelialisation.5,11 The autologous keratinocyte culture technique was later combined with split thickness skin grafts or Meek micrografts, which resulted in a shorter healing time.20 The control group was treated with meshed autografts on deep burns. The same routine was used for the treatment

Patients and methods

Study design

The design of this study was a retrospective cohort with matched groups. The inclusion criteria for the treatment group were patients admitted with burns and treated with cultured autologous keratinocytes at the Burn Centre in Linköping between 2012 and 2015. Decision to treat with cultured cells was left to the senior treating physician’s discretion, and for some patients where depth was in doubt keratinocyte cultures were initiated in case they were needed at a later stage of the treatment. Seventeen patients were listed for treatment with cultured autologous keratinocytes according to the records of the local tissue bank at the Laboratory for Regenerative Medicine at Linköping University Hospital. Seven of these patients were excluded: one patient’s cell culture failed to proliferate ex vivo, three patients did not receive the cells after being cultured as their wounds were healed, two patients died during treatment, and one patient was given cells as part of a late reconstructive operation for scarring on his neck. The included ten patients treated with cultured autologous keratinocytes were matched by age, size and depth of burn, by individual matching with a case-control rate of 1:4 (total of 35 control patients). Data were retrieved from the local database19 and hospital medical records after approval by the Regional Ethics Review Board.

Surgical treatment strategy

Surgical treatment included staged excisions using allografts, porcine xenografts or silver sulphadiazine dressings as temporary cover before final autografting with split thickness skin grafts.20 The control group was treated with meshed autografts on deep burns. The same routine was used for the treatment
group with the exception of the extra use of cultured autologous keratinocytes.

**Treatment with cultured autologous keratinocytes**

**Production of the suspension.** Full thickness skin biopsy specimens (2 x 2 cm up to 6 x 3 cm, based on availability) were harvested with a scalpel under sterile conditions as near as possible to the day of admission, predominantly from areas with the most hair follicles (groin, axilla and abdomen). Immediately after being cleaned with saline, they were stored in Roswell Park Memorial Institute transport medium and sent to the local tissue bank at the Laboratory for Regenerative Medicine at Linköping University Hospital for the cultivation of autologous keratinocytes.

**Isolation of keratinocytes from skin biopsies.** Skin biopsies were used within a maximum of 72 hours. The biopsies were washed with phosphate-buffered saline mixed with penicillin 100 μg/ml and streptomycin 100 μg/ml, and cut into small pieces (0.5 x 0.5 cm). These pieces were then incubated overnight in a protease solution (dispase 13.7 mg/ml of Dulbecco’s Modified Eagle Medium (DMEM)). Then, the digested skin pieces were washed again and the epidermis was separated and placed in trypsin 0.25% and Versene® 1:5000 EDTA. Keratinocytes were obtained by centrifugation and seeded into a serum-free keratinocyte medium (with L-glutamine) with 25 μg/ml of bovine pituitary extract and 1 ng/ml of epidermal growth factor, and then cultivated at 37°C in 5% CO₂ and humidified air.

The cell culture medium was changed every second day. The sterile environment was checked for microbial growth one week before the planned treatment and on the day of treatment. When the cells became 70%–75% confluent, they were sub-cultured by detaching them from the culture dish using trypsin 0.25% and Versene® 1:5000 EDTA and washed with a solution of DMEM, foetal calf serum and antibiotics. A maximum of three passages were allowed. On the day of transfer, the cells were detached, counted with trypan blue, and prepared as 1 x 10⁶ viable cells/1 ml of DMEM.

**Treatment.** After removal of the temporary cover, the recipient site was debrided with a water jet or a scalpel. The cell suspension was mixed with fibrin glue such as Artiss® or Tisseel® (Baxter Healthcare Corporation, Deerfield, USA) and sprayed on the freshly-debrided (not bleeding) surface of the wound either alone, with a Meek micrograft, or a widely-meshed autograft that would expand from 3:1 to 6:1. The autograft was harvested at 8-12/1000 inches using a pneumatic dermatome and meshed using a skin graft mesher (Zimmer®, Brennen Medical LLC, Minnesota, USA). The harvesting, preparation and application of the Meek® micrografts was done according to the manufacturers’ instructions. Cells were sprayed on the surface of the wound and then the micrograft was applied. The meshed autografts were applied in the reverse order; i.e. the autograft was applied first and fixed with staples or fibrin glue, and the cell suspension was then sprayed on top of the graft.

**Cultured autologous keratinocyte treatment “take” rate and scar formation.** Each area that had been sprayed with cells was photographed when the wound was first taken down (5 -11 days after the treatment) and 2, 3 and 4 weeks after treatment. The “take” rate was evaluated retrospectively by two independent, experienced surgeons by digital imaging. The scar was assessed three months after injury with the Vancouver scar scale (0 = normal and 13 (maximum total score) the worst scar possible).

**Statistical analysis**

STATA (STATA version 12.0, Stata Corp. LP College Station, TX, USA) was used for statistical analysis. As the data were skewed, non-parametric tests were used in the first part of the analysis. Descriptive data are given as median (10-90 centiles) unless otherwise stated. To detect any significant differences between the two groups we used the two sample Wilcoxon rank-sum test and chi squared test when appropriate. Probabilities of less than 0.05 were accepted as significant. A multivariable regression was used to analyse the significance of all the associations between duration of hospital stay and treatment groups (matched controls and treated group) adjusted for percentage total body surface area burned (TBSA%), deep burns (percentage body surface area), age, and mortality.
Results

Description of the treatment group and the matched control group

Cultured autologous keratinocytes were applied to 10 patients, 6 males and 4 females, with a median age of 40 years (17-61). All burns were thermal and the median burned body surface area was 49 (16-89) % of the total body surface area (TBSA). Median full thickness burn surface area was 11 (0-52) %, deep dermal burns 18 (1-80) % and superficial dermal burns 1% (0-51). Median duration of hospital stay was 63 days (47-168) or 1.6 (1.1- 2.6) days / TBSA %.

The control group included 35 patients. The two study groups were matched regarding age, sex, size and depth of burn (Table I).

Table I - Details of the patients by treatment group

<table>
<thead>
<tr>
<th>Description of the patients by treatment group</th>
<th>CAK</th>
<th>Control</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>10</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Sex, male</td>
<td>6 (60)</td>
<td>26 (74)</td>
<td>0.44</td>
</tr>
<tr>
<td>Age, years</td>
<td>40 (17-61)</td>
<td>45 (22-78)</td>
<td>0.40</td>
</tr>
<tr>
<td>TBSA%</td>
<td>49 (16-89)</td>
<td>39 (16-68)</td>
<td>0.46</td>
</tr>
<tr>
<td>Full thickness burns BSA%</td>
<td>11 (0-52)</td>
<td>17 (0-45)</td>
<td>0.48</td>
</tr>
<tr>
<td>Deep dermal burns BSA%</td>
<td>18 (1-80)</td>
<td>10 (2-30)</td>
<td>0.35</td>
</tr>
<tr>
<td>Superficial dermal burns BSA%</td>
<td>1 (0-51)</td>
<td>0.5 (0-22)</td>
<td>0.95</td>
</tr>
<tr>
<td>Hospital stay, days</td>
<td>63 (47-168)</td>
<td>58 (2-208)</td>
<td>0.24</td>
</tr>
<tr>
<td>Burn type, thermal</td>
<td>10 (100)</td>
<td>27 (77)</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Data are presented as median (10-90 centiles) or number of patients (%). TBSA% = percentage total body surface area burned. CAK = cultured autologous keratinocytes

Scar evaluations after injury

The scars on the areas treated with cultured autologous keratinocytes were assessed in 9 patients at three months after injury, as one did not attend follow up. The median Vancouver score was 8 (6-10). Scars were assessed as hyper-pigmented in eight patients and hypo pigmented in one. Vascularity was assessed as “red” (considerably increased) in five patients and in the other four varied from normal to “purple”. In six of the patients the scar was firm but pliable, while the remainder were recorded as yielding, banding or contracted. No scar was of normal height, but eight were assessed as < 2 mm and one between 2 and 5 mm (Table III).

At the 6 months’ follow up only three patients attended the scar evaluations. Scars were assessed as hyper-pigmented and the pliability as firm in these patients. Vascularity was assessed as pink in one case, red in one and purple in one. No scar was of normal height, but two were assessed as < 2 mm and one between 2 and 5 mm.

At the 12 months’ follow up the same three patients attended the scar evaluations. Scars remained hyper-pigmented. Vascularity was assessed as normal in one case, pink in one and red in one.
pliability, one was recorded as ropes, one as yielding and one as supple. Regarding height, two were assessed as $< 2$ mm and one between 2 and 5 mm.

**Table III** - Vancouver Scar Scale results three months after treatment with cultured autologous keratinocytes

<table>
<thead>
<tr>
<th>Pat No</th>
<th>Pigmentation</th>
<th>Vascularity</th>
<th>Pliability</th>
<th>Height</th>
<th>Total score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
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<td>2</td>
<td>5</td>
<td>1</td>
<td>10</td>
</tr>
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<td>9</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>Total score</td>
<td>2 (1-2)</td>
<td>2 (0-3)</td>
<td>3 (2-5)</td>
<td>1 (1-2)</td>
<td>8 (6-10)</td>
</tr>
</tbody>
</table>

One of the 10 patients did not attend for follow up at three months. Scale min-max: Pigmentation 0-2, Vascularity 0-3, Pliability 0-5, Height 0-3, Total score 0-13 (from normal to worst scar possible). Total data are presented as median (10-90 centiles).

**Duration of hospital stay, multivariable regression**

There was no difference in duration of hospital stay between the groups ($p=0.24$, **Table I**). The multivariable regression model showed that the only factor that affected duration of hospital stay was size of full thickness burns (**Table IV**).

**Table IV** - Multivariable regression for effect on duration of hospital stay

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>p value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex - male</td>
<td>-0.34</td>
<td>0.74</td>
</tr>
<tr>
<td>Age - years</td>
<td>0.03</td>
<td>0.95</td>
</tr>
<tr>
<td>TBSA%</td>
<td>0.86</td>
<td>0.13</td>
</tr>
<tr>
<td>Full thickness burns BSA%</td>
<td>2.14</td>
<td>0.002</td>
</tr>
<tr>
<td>Cultured autologous keratinocytes*</td>
<td>-0.66</td>
<td>0.97</td>
</tr>
</tbody>
</table>

TBSA% = percentage total body surface area burned. Model adjusted $R^2$ 0.42 $<0.001$.

*The matched group is reference

**iMSC differentiation into keratinocyte-like cells**

Cells cultured on glass had higher density than those cultured on the classical tissue culture plastic, in spite of the initial seeding density. In addition, glass enhanced the expression of cytokeratin 14 in all studied culture conditions. While dexamethasone and epidermal growth factor had higher cell density, retinoic acid enhanced the expression of the studied marker per cell (**Fig. I**).

**Fig. I** - Cytokeratin immunostaining for cytokeratin 14 in iMSC cultured on plastic (A-D) and glass (E-H) with different media additives. Cells cultured on glass showed higher density than those cultured on plastic with enhanced expression of the protein. All the images were photographed at 40X.

**Discussion**

**Main findings**

In this retrospective study we evaluated the outcome of treatment with cultured autologous keratinocytes in terms of shortening of the duration of hospital stay in the Burn Centre. We included all patients who had been treated with cultured autologous keratinocytes during a four-year period and compared them with a matched control group, but found no support for the idea that the treatment shortened the duration of hospital stay. In agreement with previous studies, no statistical difference was observed in the duration of hospital stay between patients who received cultured autologous keratinocytes and patients who had not received the treatment, following the adjustment for TBSA%.23-25 On the other hand, a retrospective study in 1996 compared 28 burn patients treated with cultured allografts, autografts or both. However, in the cultured graft group 23 out of the 28 patients were given only cultured allografts and no autografts. This fact would give this study a different perspective.26 In 2000 a retrospective study on 20 children who survived massive burns (full thickness, 90% BSA) investigated the use of autologous keratinocytes, cultured as sheets. The authors
reported longer hospital stay than the group who had not received the treatment (128 compared to 89 days).27

Other evaluated parameters showed no difference between the two study groups, which was not in agreement with previous studies. For example, more survivors in the group treated with cultured autologous keratinocytes were reported in an earlier paper.23 In a different approach, epithelial cells (keratinocytes and melanocytes) were placed on Matri-derm® (MedSkin Solutions, Billerbeck, Germany) together with split thickness skin grafts and compared to controls who received split thickness skin grafts alone. Faster epithelialisation was reported in the treatment group 5-7 days postoperatively. Because of the design of the study, intra-individual, no evaluations of hospital stay could be made.28

The median duration of hospital stay in our treatment group was 1.6 (1.1- 2.6) days / TBSA% which is similar to that of other burn centres that treat adult patients with moderate-sized burns who require operation.29 Among patients who have been treated with cultured autologous keratinocytes, hospital stay has been reported to be between 0.8-5 days/TBSA%, usually about 1.5 days/TBSA%, which is similar to our findings.6,7,14,23,24,26,27,30,31

"Take" rate and assessment of scar

Our “take” rate was lower than Gardien et al.28 and Yim et al.,32 similar to Braye et al.,6 and better than Lee et al.,15 who used the same techniques. Scarring was assessed after three months, when the median Vancouver score was 8, which is “severe” compared with others who have reported scores between 2.413 and 5.815 for burns treated with cultured autologous keratinocytes. The assessments were made later in these studies of more mature scars, which may explain why they had a better scoring than our patients,18,28,33 although Vancouver scores of 2 and 4 have been reported after three months’ follow up.13,32

A total Vancouver score of more than 7 has been suggested as a marker for hypertrophic scarring in burn patients,34 which would mean that five of our nine patients whose scars were evaluated had hypertrophic scarring after treatment with cultured autologous keratinocytes. Another study that used Vancouver scores to evaluate hypertrophic scarring suggested that scars that scored 1 or more on the “height” variable should be regarded as hypertrophic.35 This would give an incidence of 100% of hypertrophic scarring for our patients treated with cultured autologous keratinocytes. The reported incidence of hypertrophic scarring in burns, in general, ranges from 32% to 72%.36

Future expectations/plans

The method of using cultured keratinocytes has been clinically appreciated, as it is reported to reduce the need for available donor sites as well as being potentially lifesaving. Sprayed cells can be beneficial in accelerating the healing of donor sites of split thickness skin grafts, so that further grafts may be taken after less time. When it has been used for deep burns, several limitations were described, such as the time needed to culture cells, the fragility of the epidermal layer, the lack of dermis that results in the formation of blisters, prolonged hospital stay, severe scarring and related expenses.7,30,37

Seeding cultured cells over Matriderm® as a collagen carrier28 showed greater epithelialisation by days 5-7 and better scars on the side treated with cultured autologous keratinocytes than the non-treated side at three and twelve months. Although this could be a promising approach, there are some drawbacks with the study. The studied group had a relatively low TBSA% (mean 24%), which meant that the patients were “healthier” and had more available donor sites, the time for complete healing was not reported, and a mesh of 3:1 was used for all patients. The latter indicated that plenty of donor sites were available. Perhaps the most important limitation, though, was that the good results may have come from the use of Matriderm® and not from the cultured cells.

In addition, there are a number of issues that still need to be considered in a randomised, controlled study. The choice of outcome measures could include time to complete healing and the patient’s own assessment of the scar, but what should it be compared with: areas not sprayed, or areas on other patients? It is difficult to know if the scar is the result of the sprayed cells or the wide-meshed autograft used with them. There is also considerable variability among centres in the treatments used for exten-
sive burns, which makes multicentre studies difficult.

Alternatives to the classical cultured autologous keratinocytes, tissue-engineered products that combine cultured keratinocytes with other cell groups, might also be of use in the care of burns.\textsuperscript{17,27,37-39} Other possible methods to consider in the future are the use of non-cultured skin cells in mixed groups of cells,\textsuperscript{40} micro barriers, or scaffolds.\textsuperscript{41}

As an alternative and proof of concept, we investigated the possibility of the differentiation of a mesenchymal stem cell line into keratinocyte-like cell. Cytokeratin 14 is an important protein that is found mainly in the keratinocytes of the basal layer of epidermis, which is responsible for giving the rest of the epidermal cells. This protein is important for cell protection as well as their proliferative capacity.\textsuperscript{42} Short-term culture for 7 days was associated with the expression of this marker under different culturing conditions. We investigated the effect of glass as culture substrate on the differentiation effect and this inexpensive and available material was associated with enhanced proliferative and differentiation capacity of iMSC. The possible explanation is that the glass provided a surface for the adhesion and migration of iMSC during their culture and differentiation. Such an effect has been shown before in stem cell differentiation into the osteogenic lineage with various biomaterials.\textsuperscript{43} Such results would provide us with a new direction for cell-based therapy in our burn centre.

An innovative alternative

Human immortalized bone marrow stromal cells (iMSC) were purchased (Applied Biological Materials, BC Canada). The cells were cultured in keratinocyte differentiation media according to Depprich et al.\textsuperscript{44} for seven days, on two different culture surfaces, tissue culture plastic and glass. The latter can modulate cell adhesion, which is an essential step for keratinocyte differentiation.\textsuperscript{45} Different additives of the media were added to enhance the differentiation into the epidermal lineage, including dexamethasone, retinoic acid (RA) and epidermal growth factor (EGF). After one week, the media was removed and cells were prepared as described before.\textsuperscript{46} The cells were stained with anti-cytokeratin 14 antibody (Abcam, Cambridge, United Kingdom), according to the manufacturer protocol.

Limitations

The retrospective design of the study is a limitation. Patients treated with cultured autologous keratinocytes were those with large wounds that had not healed by the time the cell culture was ready. We compared them with a matched group of patients, which we considered as a suboptimal but reasonable control group. It is quite possible that hospital stay would be longer in a group treated with cultured autologous keratinocytes because the treatment had been consistently chosen for patients with complicated wounds. The fact that the size of the treated area was a fraction of the total area that required operation may also have affected the result in the sense that more than half of all the deep burns were treated in the same way in both groups.

One other important and perhaps influencing factor is the possible heterogeneity of the isolated cells. Melanocytes, fibroblasts, angioblasts as well as epidermal stem cells might have been included during the isolation process. In addition, keratinocytes can un-differentiate in the culture system into earlier precursors, which could be associated with increasing proliferative capacity.\textsuperscript{47,48}

Our culture time was somewhat longer than the 12-21 days that have been reported from similar studies.\textsuperscript{13,15,28,32} In theory, if the culture took one or two weeks less, it could have shortened the hospital stay as the cultured autologous keratinocytes would have been available earlier. It is also possible that the duration of healing would have been shorter if the treatment had been applied to wounds that were open for a shorter time. The size of the treated area was also a fraction of the total area that required operation, because deep burns were covered with split-thickness skin grafts on wounds that were ready whenever there were donor sites available. A shorter culture could also therefore have resulted in a larger proportion of areas of the total areas of deep burns being treated with cultured autologous keratinocytes, and subsequently the increased possibility that we could detect a clinically important difference between the two groups.

The one-centre approach with results generated
from a relatively small group is another limitation that makes it difficult to generalise the result. The limited indications of this kind of treatment, in combination with the extensive regulations and the cost factor, make its application limited and therefore few patients were included. Other studies investigating the same treatment also included a small number of patients. The use of hospital stay as an outcome measure can also be a limitation, as it is affected by many factors that are not directly related to the effect of the operation.

Based on the results, we now prefer to restrict the use of cultured autologous keratinocytes to major burns with extension >70%, and deep-full thickness burns. Other studies have described the use of cultured autologous keratinocytes in similar or more extensive burns. We will also start a new program for culturing patients’ autologous stem cells and their differentiation into keratinocyte-like cells. This step will need to follow the strict regulations of advanced therapeutic medical products in order to be applied clinically.

**Conclusion**

Our results show no difference in duration of hospital stay between the two treatments. Autologous stem cells should be considered as a future modality of burn management although further studies are needed.

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