Number of keratinocytes and melanocytes in cell suspensions obtained from partial-thickness epidermal cuts and suction blister roofs for vitiligo treatment: a prospective randomized comparative study

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Background
Treatment of vitiligo not responding to conventional therapies involves the replacement of the absent melanocytes by surgical maneuvers, including minigrafting and cultured and noncultured cell suspension grafting. Cell suspensions were prepared from epidermal cuts or suction blister roofs. Success of these grafts depends on the transplanted cells.
Objective
The aim was to compare the number of melanocytes and keratinocytes in suspensions prepared from suction blister roofs vs that of epidermal cuts obtained by Thiersch knife.
Patients and methods
The study was carried out on twenty patients with nonsegmental vitiligo attending the outpatient clinic of El-Minia University Hospital. They were randomly divided into two groups to be treated by autologous noncultured cellular grafting. One group was treated using cellular suspensions prepared from epidermal cuts and the other obtained by suction blister roofs. The mean number of keratinocytes and melanocytes was estimated using certain equations, and the presence of melanocytes was confirmed by special stains.
Results
Despite that the number of melanocytes was slightly less in group 2 compared with group 1, there was no statistically significant difference between total epidermal cells or melanocytes' numbers of both groups ($P>0.05$).
Conclusion
This is the first report to compare the number of epidermal cells and melanocytes in the suspensions obtained from partial-thickness epidermal cuts and suction blister roofs as a source of noncultured cell suspension for the treatment of vitiligo. Both groups were comparable in the melanocytes' numbers, so both sources could be used for noncultured cellular grafting in vitiligo.

Keywords:
melanocytes, noncultured cellular grafts, suction blister grafts, Thiersch grafts, vitiligo

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Introduction

Vitiligo is a disorder of pigmentation that affects 0.4–2% of the worldwide population, without sex or skin color predilection [1]. For years, treatment of vitiligo was thought to be unsatisfactory, and patients were advised to seek effective cosmetic camouflage for the lesions on the exposed skin [2]. Treatment modalities included potent topical steroids [3] and topical or systemic psoralens with ultraviolet light exposure [4,5]. Various grafting methods including tissue grafts and cellular grafts have been used with some success [6].

Noncultured epidermal suspension (NCES) transplantation is a well-established surgical treatment for stable vitiligo not responding to either medical treatment or phototherapy. This suspension is traditionally prepared from partial-thickness epidermal cuts taken by Thiersch knives or a dermatome. In 2014, three independent studies reported the successful use of the suction blister roof in the preparation of the NCES [7–9]. A density of 1000–2000 melanocytes/mm^2 has to be achieved before transplantation [10].

Aim

In this work, we compared the number of melanocytes and keratinocytes in noncultured cell suspensions prepared from suction blister roofs and that obtained by the traditional method, that is, using Thiersch knife.

Patients and methods

Twenty patients with localized lesions of stable nonsegmental vitiligo with skin phototype III and IV of both sexes were recruited from the Dermatology Outpatient Clinic of Minia University Hospital, El-Minia, Egypt, to be treated using noncultured cell suspension grafting. Our institution's Research Ethics Committee approved the study design, and a consent form was obtained from each patient. Details of the maneuver, and all possible outcomes and adverse effects were explained to all patients, and confidentiality was assured.

The patients were fully randomized into two groups using the random number allocation method. In group 1 (10 cases), the suspension was obtained from partial-thickness epidermal cuts, whereas in group 2 (10 cases), it was obtained from suction blister roofs.

In group 1, the donor skin was obtained from covered normally pigmented area, mainly the gluteal region. Local infiltration anesthesia using 2% lidocaine under aseptic precautions was done. The graft was then taken using a silver Thiersch knife (NIC Instruments Ltd, London, England) in an average size of 15 cm^2 and transported to the laboratory in a sterile container in normal saline. In group 2, suction blisters were obtained using the Chinese cups (size 2–8 cm diameter) to induce blisters by the method described by Awad [11]. Up to four blisters were prepared for each patient on the anteromedial aspect of the thigh and were harvested within 2–3 h. The roofs of the blisters were cut and transferred to the laboratory in a sterile container in normal saline. The size of the graft was designed to be in a donor-recipient ratio (D/R) of 1/3 in both groups.

Preparation of cell suspensions

In the laboratory, the skin grafts were cut into smaller pieces about 2×1 cm, which were then immersed in a mixture of 0.25% trypsin and 0.02% EDTA (Sigma Tec Pharmaceutical, Giza, Egypt) in a Petri dish. In cases of partial-thickness epidermal cuts, the dishes were incubated 1 h at 37°C provided that epidermal side facing upward and completely covered with the solution, whereas in cases of suction blister roofs, the incubation was done for half an hour.

After the incubation time, the trypsin and EDTA solution was discarded, and the tissues were washed three times with phosphate buffered saline.

For partial-thickness epidermal cuts, the epidermis was separated from the dermis with the help of blunt forceps within a petri dish containing Dulbecco-modified Eagle's medium (DMEM), whereas in cases of blister graft, the cells were directly removed gently from dermal side. The dermis was vortexed and mixed in a test tube containing 1 ml DMEM for ~1 min.

The dermal side of the epidermis was gently curetted using curved blunt forceps to release the epidermal cells into the dish containing DMEM. The solid tissue waste (mostly horny cell layer) was either removed or cut into minute pieces.

The obtained suspension was centrifuged for 5 min at 1000 rpm. The supernatant was then discarded, and the pellet, containing melanocytes and keratinocytes, was diluted with DMEM (1 : 10).

Counting of melanocytes in the suspension

The total cells in the epidermal suspension prepared for transplantation were counted using a hemocytometer. The diluted suspension with DMEM was mixed well, and then 10 µm was mounted onto a hemocytometer using a micropipette.

The viability of the cells was checked by the dye exclusion test using 0.4% trypan blue solution before loading into the hemocytometer chamber. The number of melanocytes transplanted per square millimetre was calculated by dividing the total number of melanocytes by the total area transplanted in square millimetres.

The cell concentration was calculated as follows:

Cell counts per milliliter=(total cell count in four squares/4×10^4)×dilution factor.

Percentage of viable cells=total viable cells/total cells (viable+dead)×100.

Total cells per sample=cell counts per milliliter ×original volume of fluid from which cell sample was removed.

Dilution factor=total volume (sample+diluting factor)/ sample volume.

Smears of the transplanted suspension were prepared and stained with hematoxylin/eosin and Papanicolaou

stains to count the average number of melanocytes in different fields. Melanocytes were confirmed by Fontana Masson and DOPA (3,4-dioxyphenyl alanin) stains as well as immunohistochemical staining using melanoma antigen recognized by T lymphocytes (MART-1) antibody (1 : 100; Neomarker, Fremont, California, USA). It recognizes a 18-kD molecular weight MART-1 antigen, which is present in melanosomes and endoplasmic reticulum of melanocytes [12].

The number of melanocytes in the stained smears was counted in each smear using ×400 magnification in 10 high-power fields, and the average percentage of melanocytes to the total epidermal cells was calculated.

The number of melanocytes was calculated by dividing the total epidermal cells by 36 (the normal melanocyte/keratinocyte ratio in the epidermal-melanin unit) [13].

Light microscope (Accu-Scope #3025 Five Headed 'A 3025–5'; Olympus, Tokyo, Japan) with a built-in camera (digital camera E-330 SLR; Olympus) was used to examine and photograph the stained smears.

Statistical analysis

Data were collected and tabulated using Microsoft Excel for windows. Statistical analysis was done using SPSS version 11 (Statistical Package for Social Sciences software, SPSS Inc., Chicago, Illinois, USA). The numerical data were expressed as mean±SD. Student's *t*-test was used to compare numerical values, whereas the χ^2 or Fisher's exact test was used for qualitative values. The values were expressed in terms of *P* value. One-way analysis of variance test was used for comparison between more than two quantitative variables. The level of significance for *P* value was determined as follows: *P* greater than 0.05, not significant; *P* less than or equal to 0.05, significant; and *P* less than 0.001, highly significant.

Results

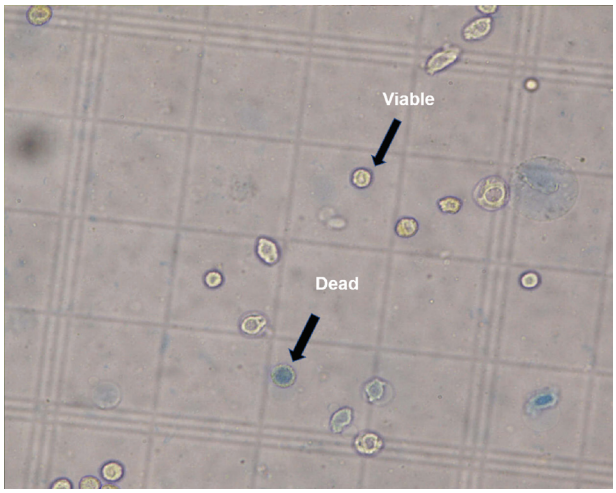
The age of the patients in group 1 ranged between 14 and 50 years, with a mean±SD of 36.8±15 years, and in group 2, it ranged between 12 and 40 years, with a mean±SD of 28.3±13.5 years, with no statistical difference between them. Moreover, both groups accidently included equal numbers of males and females, that is, each group included five males and five females.

Counting of epidermal cells in the transplanted suspension in both groups was done, and it was found that 4–10% of cells were dead with a range of 0.4×10^5–1×10^5 cells/ml and mean±SD of $0.7\times10^5\pm0.3\times10^5$ cells/ml (Fig. 1).

Total epidermal cells/ml in group 1 ranged from 6×10^5–12×10^5 cell/ml with a mean±SD $9.5\times10^5\pm2.3\times10^5$ cell/ml. In group 2, it ranged from 7×10^5–10×10^5 cell/ml with a mean±SD of $8.6\times10^5\pm1.1\times10^5$ cell/ml.

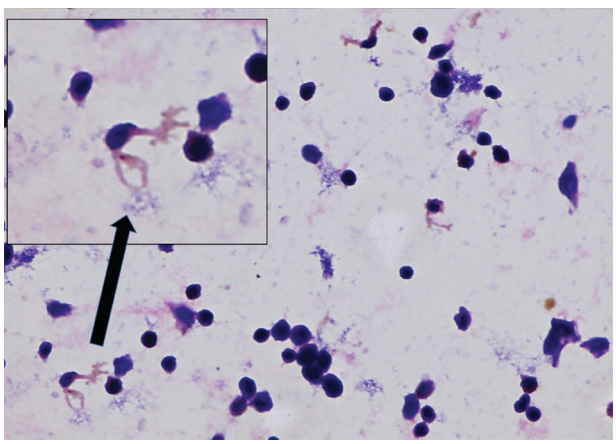
Hematoxylin/eosin-stained and Papanicolaou-stained smears of the suspension were done to count the average number of melanocytes in different fields (Figs. 2–3), and it was also confirmed by Fontana Masson (Fig. 4) and DOPA stains (Fig. 5), as well

Figure 1



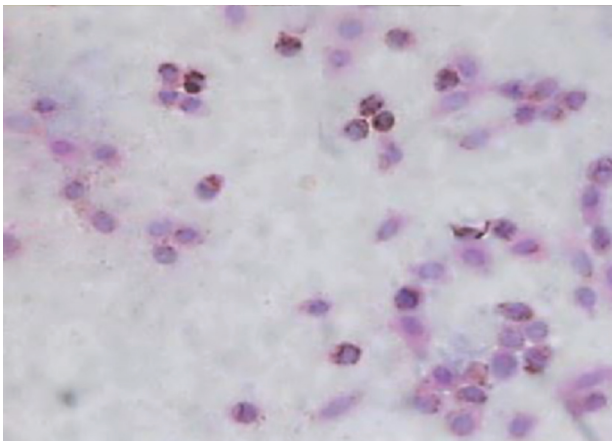
Epidermal suspension diluted 1 : 10 and stained with trypan blue in hemocytometer. Arrows point to the viable melanocytes (Shiny white) and dead melanocytes (in blue).

Figure 2



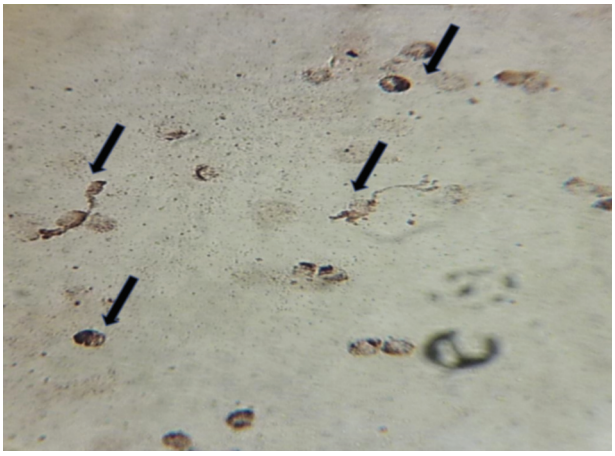
Epidermal suspension smears stained with hematoxylin/eosin (×400). The arrows show the melanocytes with their characteristic dendrites.

Figure 3



Epidermal suspension smears stained with Papinacolaou showing the melanocytes (×400).

Figure 4

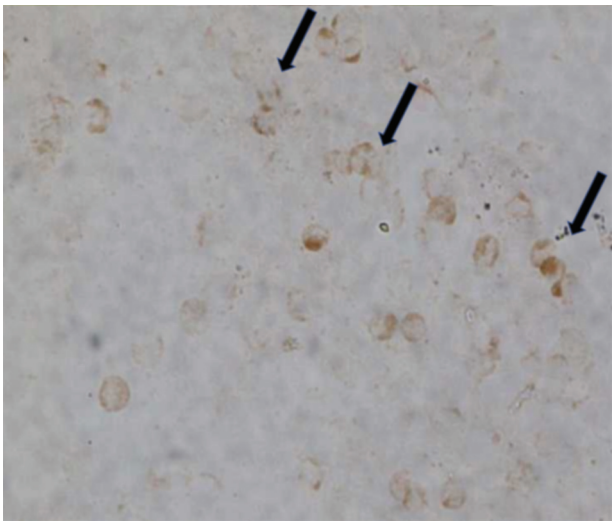


Fontana Masson-stained epidermal suspension smears confirming the presence of melanocytes (×400).

as MART-1 immunohistochemical staining (Fig. 6). The number of melanocytes per ml varied from 0.17–0.33×10^5 with a mean±SD of 0.3±0.1×10^5 in group 1 vs 0.19–0.28×10^5 with a mean±SD of 0.2 ±0.03×10^5 in group 2. There was no statistically significant difference between both groups regarding the total epidermal cells or melanocytes number (Table 1).

The mean±SD of melanocytes number/cm^2 donor area was 0.05±0.02×10^5 in group 1 compared with 0.04 ±0.01×10^5 in group 2, whereas that transplanted/cm^2 was 0.02±0.01×10^5 in group 1 compared with 0.018 ±0.004×10^5 in group 2. Despite the number of melanocytes being slightly less in group 2 compared with group 1, there was no statistically significant difference between both groups regarding the total

Figure 5



DOPA-stained epidermal suspension smears (×400). The arrows point at the positively stained melanocytes.

epidermal cells or melanocyte numbers of (P>0.05) (Table 1).

Discussion

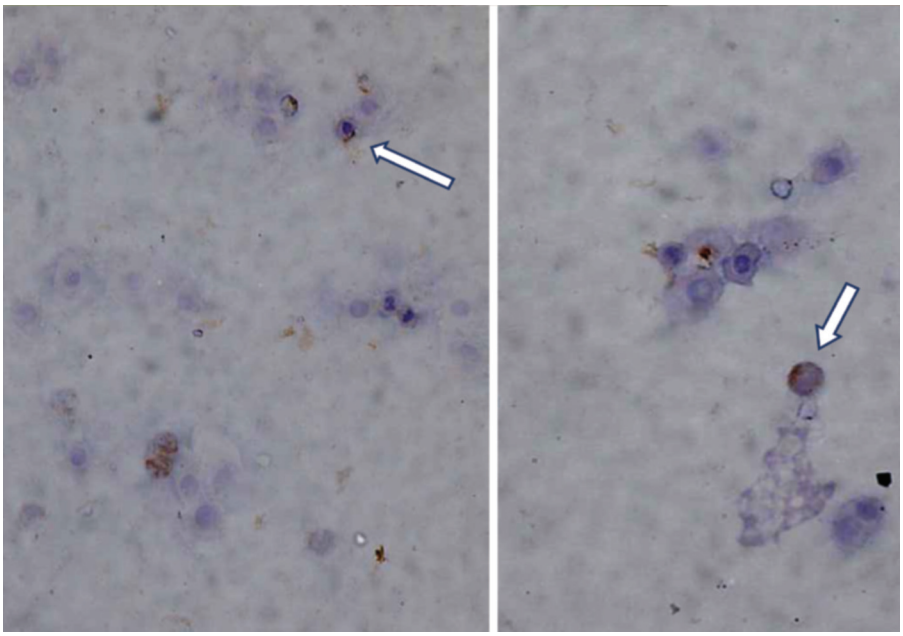
In our study, we compared the number of epidermal cells and melanocytes in the suspension obtained from partial-thickness epidermal cuts and suction blister roofs used for vitiligo treatment.

On revising the literature, it was reported that the gluteal region is the preferred site for Thiersch grafting [14] and the inner side of the thigh is the recommended site for harvesting suction blisters [15]; thus, in our study, we used these recommended sites to obtain the grafts.

The total epidermal cells and melanocytes' numbers within the suspension were slightly less in group 2 compared with group 1, but the difference was not statistically significant.

In a study done in 1998, they compared melanocytes' number in biopsied skin with that of suction-blistered epidermis. They found that the average melanocytes' number in biopsied skin was around 1000, which was in the region of normal levels, whereas that of suction-blistered epidermis was less than 500. They suggested that some cells might have dropped to the dermal side and may not have been included in the blistered epidermis, resulting in fewer melanocytes in the epidermis. They suggested also that the surface area of the epidermis from the blister was wider than normal, without an

Figure 6



Melanoma antigen recognized by T lymphocytes-1 stained epidermal suspension smears (×400). Melanocytes are stainend positively (arrows).

Table 1 The total epidermal cells and number of melanocytes in the suspension in groups 1 and 2

Number of total epidermal cells	Group 1	Group 2	*P* value
Cells/ml			
Range	6–12×10^5	7–10×10^5	0.4
Mean±SD	9.5±2.3×10^5	8.6±1.1×10^5	
Cells/cm^2 donor			
Range	1.3–2.4×10^5	0.9–1.9×10^5	0.2
Mean±SD	1.7±0.6×10^5	1.4±0.4×10^5	
Transplanted cells/cm^2			
Range	0.4–1.6×10^5	0.4–0.8×10^5	0.2
Mean±SD	0.9±0.5×10^5	0.6±0.14×10^5	
Number of Melanocytes Cells/ml			
Range	0.17–033×10^5	0.19–0.28×10^5	0.3
Mean±SD	0.3±0.1×10^5	0.2±0.03×10^5	
Cells/cm^2 donor			
Range	0.02–0.07×10^5	0.03–0.05×10^5	0.2
Mean±SD	0.05±0.02×10^5	0.04±0.01×10^5	
Transplanted cells/cm^2			
Range	0.01–0.04×10^5	0.011–0.022×10^5	0.3
Mean±SD	0.02±0.01×10^5	0.018±0.004×10^5	

increase in the cell number, so fewer cells were left in the blistered epidermis [16]. These numbers are less than ours as we counted melanocytes in a melanocyte-enriched suspension/ml after discarding the stratum corneum and dispersing of the epidermal cells, whereas they counted it within the skin/m^2 with preserved architecture.

In another study, cells were counted manually by the hemocytometer in a suspension obtained from shave biopsy specimens of normally pigmented skin. The average cell yield (melanocytes and keratinocytes) ranged from 1–1.4×10^6 cell/cm^2. The number of melanocytes in 10 cm^2 donor skin was ~25–45×10^4 melanocyte. Each 1 ml of the suspension mixture contained ~25–40×10^3 melanocytes [17]. These results are comparable to ours.

In a study by Tegta *et al.* [18], the effect of diluting cell suspension on the outcome was assessed: in group A, where D/R is 1/3 and the density of melanocytes transplanted was approximately 231.60±27.03/mm^2, whereas in group B, where D/R is 1/5, the density of melanocytes transplanted was 154.90±27.65/mm^2

which affected the repigmentation outcome in patients, being better in group A. They suggested that the minimum number of melanocytes required to produce satisfactory repigmentation is probably in the range of 210–250/mm^2. This is consistent with the recommended desired number of cells for repigmentation as 2000 cells/cm^2 by Singh *et al.* [19].

Olsson and Juhlin [20] reported that the number of melanocytes in the epidermis from the buttock area is ~1900/mm^2. A 10-fold expansion of the donor area would thus give 190 melanocytes/mm^2 applied to the recipient area which seemed to be the lower limit to produce repigmentation, whereas Brysk *et al.* [21] found that ~1000–1500 cells/mm^2 of recipient area were required, and this is also supported by Pandya *et al.* [22].

The gluteal region as a donor graft site was reported to have a greater melanocyte density (1900±178 melanocytes/mm^2) than the thigh region, and they suggested that it might be preferred as a donor graft site (1700 ±139 melanocytes/mm^2) [23]. However, in our study, there was no statistically significant difference between the numbers of cells between both groups.

Conclusion

Our report is the first to compare the number of epidermal cells and melanocytes in the suspension obtained from partial-thickness epidermal cuts and suction blister roof as a source of non-cultured cell suspension for the treatment of vitiligo.

Both groups were comparable in the melanocyte numbers. Although the total epidermal cells and melanocytes numbers within the suspension were slightly less in group 2 compared with group 1, there was no statistically significant difference between both groups, so both sources, i.e., partial-thickness epidermal cuts or suction blister roofs, could be used for NCES grafting in vitiligo.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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