

SOME MODIFICATIONS IN TRANSPLANTATION OF AUTOLOGUS NON-CULTURED MELANOCYTES-KERATINOCYTES SUSPENSION IN TREATMENT OF SEGMENTAL AND FOCAL VITILIGO (EGYPTIAN EXPERIENCE IN ALEXANDRIA UNIVERSITY)

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Abstract

Introduction: Transplantation of Autologous non-cultured melanocytes suspension is a simple yet effective cell-based therapy for vitiligo.

Materials and Methods: 20 patients with stable vitiligo were subjected to epidermal cell suspension transplantation using Osslon's method with some new modifications.

Result: The repigmentation at 7 of the test sites (35.0%) was excellent. It was good for more than half of the test sites (55.0%). Fair repigmentation was encountered among only 2 (10.0%) of the tested sites. None of the tested sites showed poor repigmentation. On the other hand, none of the control sites showed excellent or even good repigmentation. However, repigmentation was fair for nearly 10% and it was poor for 90% of the control sites.

Conclusion: Autologous non cultured basal -enriched epidermal cell suspension transplantation is an effective, simple and safe method for treatment of stable vitiligo.

Key words: autologous; cellular transplantation; non cultured melanocytes; stable; vitiligo

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Introduction

Vitiligo is an acquired skin disorder caused by the disappearance of pigment cells from the epidermis that gives rise to well defined white patches which are often symmetrically distributed [1,2]. The lack of melanin pigment makes the lesional skin more sensitive to sunburn [3,4]. Vitiligo can be cosmetically disfiguring and it is a stigmatizing condition leading to serious psychological problems in daily life. Stable vitiligo and lesions with depigmented hairs, indicating the depletion of the melanocyte reservoir in the hair follicle, mostly fail to repigment by conservative therapies [5,6]. In these cases, surgical techniques may be indicated but are often time-consuming and lead to undesired effects [7]. For extensive lesions, cell-based techniques offer an alternative option, requiring only a small biopsy. However, the appropriate matrix for delivery and fixation of the cells is still an unsolved question [8].

Aim of the work:

To study the effect of non cultured melanocyte-keratinocyte cell suspension on stable vitiligo.

Material and Methods

Patients

- Twenty patients; 10 patients with focal vitiligo and 10 patients with segmental vitiligo.
- 7 male, 13 female.
- Aged between 13 and 33 years.
- 12 patients (60% of cases) are skin type IV and eight patients (40% of cases) skin type III.
- In every patient one area was taken as tested (treated area) and one area taken as control.

Selection criteria

- 1) Patients should have a realistic expectation.
- 2) Patients not respond adequately to medical treatment.
- 3) Patients should be stable for 24 months (no new lesions, no expansion of old ones).

Exclusion criteria

- 1) Involvement more than 30% body surface area.
- 2) Patients aged less than 12 years and patients receiving any concomitant medical treatment.

3) Patients who are positive to infectious disease HIV, HCV, HBV.

4) Patients who have a history of koebnerization, Keloid tendency and coagulative disorders.

Methods

All Patients were subjected to the following:

- Personal history.
- History of vitiligo, onset, precipitating factors, duration, course and koebner phenomenon.
- Family history of vitiligo or autoimmune diseases.
- Previous forms of therapy; local or systemic.
- Local examination of vitiligo site, distribution and number of lesions.
- Laboratory examination as CBC, bleeding time, coagulation time, HCV antibodies and HBV antigens.
- Photography.
- Approval by ethical committee was obtained, and a written consent was taken from each participant.

This procedure of transplantation of non-cultured melanocytes-keratinocytes cell suspension has been modified from that of Osslon and Juhlin [9]. CO₂ incubator was replaced with an ordinary incubator. Operation theater was modified by adding UV cabin, an incubator and a centrifuge to the existing equipment, so that cell separation could be performed there instead of in a separate laboratory. Single vitiliginous areas is dermabraded and taken as control and exposed to topical PUVA.

Donor site

A donor area of 1/10th of the recipient area was marked on lateral aspect of the gluteal region. The area was then anesthetized with 1% xylocaine. The skin was stretched and a very superficial sample was removed with a skin grafting knife. The superficial wound was then covered with a sterile vaslined gauze.

Procedure for cell separation

The following procedure was performed in UV cabin kept in our laboratory near the operation theater.

Separation of epidermis from dermis

The thin skin sample was transferred to a Petri dish containing approximately 4 ml of 0.2% trypsin solution. Care was taken to ensure that the sample was properly soaked in the solution. Finally, it was placed epidermis upwards. The sample in the petri dish was incubated for 50 min at 37 °C. After incubation, approximately 2 ml of trypsin inhibitor (Sigma, USA) was added to the petri dish to neutralize the action of the trypsin. The dermis was separated from the epidermis and was transferred to a test tube containing approximately 3 ml of DMEMF12 medium (Sigma, USA) and vortex mixed for 15 Sec.

Preparation of melanocyte cell pellet from epidermis

Epidermis in the petri dish was broken down into multiple small pieces. It was then washed with DMEMF12 medium and then transferred to a test tube containing the

same medium. Next it was vortex mixed for 15 sec. The test tube containing the epidermal pieces was then centrifuged for 6 min. The epidermal pieces were discarded and the cell pellet was suspended in DMEMF12 medium in a 1-ml syringe with detachable needle. The quantity of the suspension prepared was approximately 0.5 ml. Hyaluronic acid was added to increase viscosity. One drop of the cell suspension seen under light microscope (magnification 100x) had approximately 3-4 melanocytes.

Recipient site

Recipient site was marked out with a marker, cleaned with povidone iodine solution and 70% ethanol and then anesthetized with 1% xylocaine.

Transplantation

The recipient area was abraded down to the dermo-epidermal junction with a high speed dermabrader fitted with diamond fraise wheel. The ideal level was achieved when pinpoint bleeding spots appeared. Denuded area was covered with gauze pieces and moistened with normal saline until the cell suspension was applied. Cell suspension was applied evenly on the denuded area and covered with a vaslined gauze to help the transplanted cells to remain in place and to provide an optimal environment for cellular growth and vascularization. The patient was allowed to return home 4 hours after dressing. The patient was cautioned against any vigorous activities, which could displace the dressing. Absolute immobilization was however not necessary.

Postoperative care

Prophylactic antibiotics and analgesics are recommended for 1 week. Patients should be instructed not to move the transplanted area. The first 48 hours are crucial to ensure proper embedding of the melanocytes. The dressings are removed after a week. Then topical PUVA was done once weekly for the tested and control area for about six months.

Results

The main parameter to measure the efficacy of the intervention was the percentage of area of repigmentation in the test lesions relative to the baseline depigmented surface area. This percentage was subjectively assessed with area measurements on two week, one month, three months and 6 months after treatment (Tabl. I).

At the 4 observed time points two weeks, 1, 3 and 6 months there were statistical significant differences in repigmentation ($p < 0.05$) between actively treated lesions and conventionally (control) treated lesions. At two weeks, 1, 3 and 6 months, the mean percentages of the repigmented areas at the test site for the studied patients were 5.65 ± 3.17 , 18.25 ± 8.25 , 66.05 ± 13.45 , and 66.05 ± 13.45 % respectively). In control sites, at the 4 observed time points, the mean percentages of area of repigmented areas were 1.45 ± 1.57 , 4.70 ± 2.89 , 12.10 ± 6.49 , and 27.25 ± 10.05 % respectively.

Repigmentation in test and control sites was graded as excellent with 95% to 100% pigmentation, good with 65% to 94%, fair with 25% to 64%, and poor with 0% to 24% of the treated area (Fig. 1-3 a, b).

Time of outcome evaluation After treatment	Percentage of area of re-pigmentation in Test lesions (n = 20)	Percentage of area of re-pigmentation in Control lesions (n = 20)	Z of Mann-Whitney U Test (p value)
	Mean \pm SD	Mean \pm SD	
Two weeks	5.65 \pm 3.17	1.45 \pm 1.57	4.097 *
One month	18.25 \pm 8.25	4.70 \pm 2.89	4.718 *
Three months	66.05 \pm 13.45	12.10 \pm 6.49	5.413 *
Six months	66.05 \pm 13.45	27.25 \pm 10.05	5.413 *

Table I. Percentage (mean & standard deviation) of area of re-pigmentation in the test and control lesions of the studied cases with vitiligo (n = 20)

Significant at 0.05 level of significance



Figure 1a. Marking of the areas of vitiligo before treatment



Figure 1b. Same patient repigmentation of the tested area after 6 months (excellent result)



Figure 2a. The depigmented area before transplantation of melanocytes keratinocytes suspension



Figure 2b. Excellent result of the same patient after 6 months



Figure 3a. Patient before



Figure 3b. Same patient after 6 months (good result)

There was a statistical significant difference in the grades of repigmentation (Chi-square test = 33.067, $p=0.000$) between test lesions and control lesions. The repigmentation at 7 of the test sites (35.0%) was excellent. It was good for more than half of the test sites (55.0%). Fair repigmentation was encountered among only 2 (10.0%) of the tested sites. None of the tested sites showed poor repigmentation. On the other hand, none of the control sites showed excellent or even good repigmentation. However, repigmentation was fair for nearly 10% and it was poor for 90% of the control sites (Tabl. II).

Selected parameters of repigmentation of the treated area (Tabl. III)

In the majority of the responding lesions (85.0%), a diffuse repigmentation pattern was observed. This was rather than a typically follicular pattern in 3 patients (15.0%). In addition, colors of the treated areas in the majority of patients (90.0%) were the same as that of the surrounding normally pigmented skin. However, the color was somewhat darker (hyperpigmentation) in one patient and somewhat lighter in another patient (hypopigmentation). The time of initial repigmentation for the actively treated areas ranged between 2 and three weeks (a mean, 2.35 ± 0.49 weeks). The number of topical PUVA sessions required during the whole 6 months follow up period ranged from 20 to 42 sessions (a mean, 29.35 ± 7.17).

Grades of re-pigmentation of the treated area	Test lesions (n = 20)		Control lesions (n = 20)		Chi-square Test (p value)
	No.	%	No.	%	
Excellent	7	35.0	0	0.0	20.067 *(0.000)
Good	11	55.0	0	0.0	
Fair	2	10.0	2	10	
Poor	0	0.0	17	90	

Table II. Grades of repigmentation of the treated area in test lesions and control lesions of the studied cases with vitiligo
Significant at 0.05 level of significance

Parameters of re-pigmentation of the treated area		No. (n = 20)	%
Pattern of re-pigmentation after 1-3 months	Diffuse	17	85.0
	Perifollicular	3	15.0
Color matching	Somewhat darker	1	5.0
	Somewhat lighter	1	5.0
	The same	19	90.0
Time of initial re-pigmentation	Mean \pm SD (weeks)	2.35 ± 0.49	
	Minimum – Maximum (weeks)	2 – 3	
Topical PUVA sessions	Mean \pm SD (months)	29.35 ± 7.17	
	Minimum – Maximum (session)	20 - 42	

Table III. Distribution of the test lesions (n = 20) according to selected parameters of re-pigmentation of the treated area

Discussion

The age of the twenty studied patients ranged between 13 and 33 years with a mean age of 17.77 ± 6.98 years, that the highest proportion of the studied patients (60.0%) was in the age group less than 15 years and the lower proportion (40.0%) was in the age group of 15 to 33 years, this present study was revealed that the age group below 15 years is earlier in repigmentation and better in prognosis than older age group, this may be due to younger cell age that grows and multiplies rapidly, this is the same in comparison to the

study done by Czajkowski [10] who found that the time of proliferation of melanocytes in in-vitro culture conditions depends on the age of patient and, the younger the patient the faster the melanocytes proliferation. As regard to the skin type in this present study, twelve patients (60% of cases) are skin type IV and eight patients (40% of cases) skin type III and this work did not find any correlation between the skin type and the result of repigmentation, the same result was obtained by Gauthier and Surleve, [11] but different in comparison to Sobhy [12] who injected

the melanocytes suspension obtained from a graft from inner aspect of the thigh to the bullae introduced to vitiliginous area (group II patients) or pouring melanocytes suspension on dermabraded vitiliginous areas (group III patients) and found that skin type IV patients had better prognosis than the skin type III patients, Sobhy [12] found that the higher the skin type the Better the prognosis due to increase the density of melanocytes in donor areas and increasing melanocytic function and melanization of melanosomes in skin type IV patients, this difference from the present study may be due to the small case number in the present study and no patient with higher skin type in this study.

This present study did not find any significant correlation between the type of vitiligo and the response to this procedure, this is different in comparison to what found by Flabella [13] who found that the patients with focal vitiligo have a smaller success rate compared with the patients with segmental vitiligo, and this may be due to small cases number in the present study.

As regards the sex of the patients in this study there were 13 females and 7 males and this study found no correlation to this item and the result of repigmentation, this is the same as what found by the Gauthier result, [11] Sobhy [12] and flabella [13].

In the present study, it was found that the site of the lesions did not significantly affect the result but the mean percentage of repigmentation of the treated area was greater in patients with skin lesions located in the face ($89.67 \pm 11.76\%$) compared to those of the patients with skin lesions located in the neck ($87.75 \pm 13.22\%$), and those located in the back ($86.65 \pm 12.53\%$) and those lesions located in the chest ($83.50 \pm 14.48\%$), this is different from the result of Pandya [14] study who found that in the fifty-one sites in 27 patients were chosen for autologous melanocytes transplantation, the most common sites were the feet (45.1%), legs (29.4%), hands (9.8%), knees (3.9%) and the face (3.9%), Pandya [14] results were most favorable on the legs, feet, face and the forearms, and poor on the elbows and the acral areas of the hand. Pandya [14] emphasis that the location of the recipient site was the major determinant of the outcome; acral parts including the dorsal aspects of the hands and feet, and the skin over the joints were less responsive, as 2 patients each with lesions on the hands and feet, and 1 patient with lesions on the elbow had a poor response, Pandya [14] said also that the fingers, the knuckles and the elbows were the most difficult areas to repigment, in part because of the relative uncertainty in controlling the depth of dermabrasion of such heavily cornified areas and also because of the high mobility of the skin covering these joints and so Pandya found also positive correlation with light exposed areas, this difference from the present study may be due to small case number and no acral cases and no cases with joint affection in the present study, but in accordance to Mulkar [15] who found poor response in the sun exposed areas and said that the sun may be a traumatic factor, but Sobhy [12] found that the exposed areas had better prognosis, these differences between the multiple studies may be due to the different degree of sun radiation on the earth and individual variation in melanocytic response to ultraviolet stimulation.

In the present study the face and the neck are cosmetically accepted with good response and rare side effect.

As regard to the duration of stability of the disease in this study, the stability ranged between 2 and 4 years with a mean stability of 2.25 ± 0.55 years, it was found that the duration of stability was not statistically significant in correlation with percentage of repigmentation, this observation was opposite to Van Geel [16] who found positive correlation, this may be due to small difference in duration between the cases in this present study, and this observation is the same found by Sobhy [12] who found that the most important thing that affects the response to treatment is the stability of the disease (2 years or more) rather than the duration of the disease.

In the present study, in the majority of the responding lesions (85.0%), a diffuse repigmentation pattern was observed, but there were rather than a typically follicular pattern in 10.0% or repigmentation from the perilesional margins in only 5.0% this is the same observed by Mulkar study, [15] Sobhy [12] and this observation may be due to spreading of melanocytes rich suspension liquid to all of treated areas giving chance to diffuse spread of transplanted melanocytes and so diffuse repigmentation pattern.

In the present study the transplanted melanocytic cell count per mm^2 ranged between 210-300 cells per mm^2 donor skin graft which were enough to induce repigmentation and this was similar in accordance to GR Tegta et al study [17] that found that the optimal melanocytes count was 210-250 cells per mm^2 donor skin graft and so no great difference in range of these transplanted cells statistically to affect significantly degree of repigmentation.

This study revealed that the time of initial repigmentation for the actively treated areas ranged between one week and three weeks, this was the same in comparison to what is done by Sobhy [12] and Flabella [13].

Every patient was encouraged to topical PUVA irradiation to tested and control areas in a slowly increasing dose to increase the extension of repigmentation and in the tested areas there was progressive increase in the size of pigmented spots until coalescence of pigment has occurred, this combination between autologous non-cultured epidermal cellular suspension and topical PUVA reduce the time of complete repigmentation by increasing melanocytes proliferation, function and suppress autoimmunity to transplanted cells.

The lack of pigmentation at the periphery of the transplanted skin observed in two patients was not due to the technical procedure, but probably to residual activity of some melanocyte-destroying factors, [12] another explanation could be an inflow of keratinocytes from the pigmented border which does not allow the transplanted pigment cells to remain where they were seeded, this influx of keratinocytes at the margins appeared as a white rim which could be treated by increasing dermabrasion beyond lesion boundary during the operation session or performing another session of transplantation of frozen autologous melanocytes not used in first session, this observation was also noted by Osslon et al [9] who found that the white rim decreased more often in the patients treated with the epithelial sheets compared with patients treated with the cells free in suspension.

Conclusion

1. Autologous non-cultured melanocytes transplantation is a rapid method for treating non-progressive vitiligo.

1. This method is simpler than methods involving cell culturing, but is still a quite advanced and requires a laboratory setup.
2. Selection of patients is crucial to the success of the outcome.
3. Segmental vitiligo and focal vitiligo almost always respond with complete repigmentation.
4. Segmental vitiligo and focal vitiligo cases retain all the repigmentation as noted by this study in up to one year of follow-up.

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