Intraepidermal injection of autologous non-cultured epidermal cell suspension in lesions of stable vitiligo: A novel method of repigmenting vitiligo

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ABSTRACT

Background: Transplantation of autologous non-cultured epidermal cell suspension containing melanocytes (ANEM) is one of the well-known surgical options for repigmenting stable vitiligo lesions. The recipient site for transplantation has traditionally been prepared by dermabrasion, liquid N_2 , or laser resurfacing, which is costly, cumbersome and has risk of scarring. **Objectives:** The objectives of this study were to experiment a novel method of repigmenting stable vitiligo lesions by intraepidermal injection of ANEM in the vitiligo lesions. **Materials and Methods:** A total of 50 stable vitiligo lesions in 50 patients were included in the study. The prepared ANEM was inoculated intraepidermally in the lesions. Patients were given psoralens and solar ultraviolet A therapy in post-operative period and followed up 4 weekly for 24 weeks to see repigmentation. **Results:** At 24 weeks, pigmentation was seen in 31 (62%) lesions of 50 lesions. It was excellent in 6 (12%), good in 10 (20%), satisfactory in 8 (16%), and poor in 7 (14%) patients. Adverse events were mild and insignificant. **Conclusion:** Intraepidermal ANEM inoculation in stable vitiligo lesions is an effective, safe, and cheap dermatosurgical procedure.

KEY WORDS: Intralesional, Intraepidermal, Vitiligo, Melanocyte

INTRODUCTION

Vitiligo is an acquired disorder with total or partial loss of melanocytes from the epidermis.^[1] Its presence on exposed areas of body leads to social embarrassment, psychological disturbance, and cosmetic impairment in those affected.^[2]

There are various medical and surgical therapeutic regimens used for the treatment with certain limitations:^[3-5]

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- Long duration of therapy
- Contraindications to different treatments (like age, photosensitivity, and lactation in case of oral PUVA therapy)
- Relative resistance of some vitiligo types (acrofacial and segmental) to medical therapy
- Inability to follow-up of protocol by patients (due to geographical, professional, and psychosocial factors)
- Financial constraints
- Side effect profile of both medical and surgical interventions.

Vitiligo is being treated by dermatosurgical techniques nowadays. Transplantation of melanocytes is one of the proved dermatosurgical techniques for the treatment of stable vitiligo. Both cultured^[5] and non-cultured^[6] melanocytes have been used.

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Autologous non-cultured melanocyte transplantation is a cellular grafting procedure that replenishes the melanocytes in vitiliginous epidermis.^[7] However, the recipient site preparation either by dermabrasion, blister formation, or laser resurfacing is slightly cumbersome and the occlusion dressing needed after resurfacing is costly. The part transplanted has to be kept strictly immobile. Hence, we thought of simplifying the inoculation procedure at recipient site. In this study, we injected the autologous non-cultured epidermal cell suspension containing melanocytes (ANEM) to the stable vitiligo lesions intraepidermally and started psoralens and solar ultraviolet A (PUVASOL) therapy in the follow-up period. This method of lesional injection technique has never been tried before in such large number of patients with control group.

MATERIALS AND METHODS

After approval of the institutional ethics committee, 50 patients of stable vitiligo were recruited from the dermatology outpatient department of a tertiary care center. Twenty-one males and 29 females (sex ratio 1:1.38) in the age range of 10–54 years (mean age of 28.10±8.76 years) having stable vitiligo lesions for the duration ranging from 2 to 18 years (mean duration of 7.54±3.81 years) were selected for the treatment with transplantation of ANEM [Table 1]. Patients who were having no new lesions and existing lesions were not increasing or decreasing in size from at least last 1 year were classified as stable vitiligo.

Inclusion criteria were stable vitiligo lesions, without an associated disease and age above 10 years.

Exclusion criteria were age below 10 years, women with pregnancy or lactation, any concurrent illness and any oral medication in the past 4 weeks, history of photosensitivity and known hypersensitivity to psoralens, progressive or unstable vitiligo, and any history of hypertrophic scar or keloid formation. Those who refused to give consent were excluded from the study.

Among the patients, type of vitiligo was localized in 38 (76%) patients (focal type in 23 [54%] and segmental type in 15 [22%]) and generalized type in 12 (24%) patients (acrofacial

Table 1: Age and sex distribution of the patients

Age (years)	Patients				
	Male	Female	Total (%)		
10–20	5	8	13 (26)		
21–30	8	12	20 (40)		
31–40	6	6	12 (24)		
41–50	2	2	4 (8)		
Above 50	0	1	1 (2)		
Total	21	29	50 (100)		
Mean±SD	28.95±8.52	27.48 ± 9.02	28.10 ± 8.76		

type in 3 [6%] patients and vulgaris type in 9 [18%]). The sites selected for the treatment of depigmented skin lesions by transplantation of autologous non-cultured epidermal suspension containing melanocytes were on the face in 13 (26%), neck in 3 (6%), trunk in 12 (24%), upper limb in 7 (14%), and lower limb in 15 (30%) patients. Of 50 patients, 24 patients (12 focal + 12 generalized) were having more than one lesion. Hence, one lesion in these 24 patients served as control site and another lesion served as test site. Twenty-six patients had only a single lesion so it served as test site. Hence, there were 50 test sites and 24 control sites.

Detailed history which included name, age, sex, duration of lesion, size and number of vitiligo lesions, associated illness, any medication, and date of operation was recorded. Detailed general and systemic examination was done. A written and informed consent was taken from all patients. Clinical photographs were taken before and after therapy.

Donor Site

About 2% lignocaine local anesthesia (after antigen sensitivity test) was given under asepsis at the anterolateral aspect of the thigh with size about one-third to one-eighth of the recipient area. A split-thickness skin graft was taken using a shaving blade grasped in a straight artery forceps and transported to the laboratory in normal saline in a sterile container. The skin graft was transferred to trypsin-ethylenediaminetetraacetic acid (EDTA) solution (0.25% trypsin and 0.02% EDTA) in a Petri dish and incubated overnight for 18–24 h at 4°C under aseptic precautions. The donor site was dressed after applying topical fusidic acid ointment. Oral antibiotic (cefadroxil) and analgesic (ibuprofen) were given for 7 days.

Preparation of Non-cultured Melanocyte Suspension

After 18–24 h of incubation, the donor graft was taken out of trypsin-EDTA solution and put in Petri dish containing EDTA (1/5000 in saline) for 15 min at 37°C in incubator. Then, the graft was taken out of the EDTA solution and transferred in Petri dish containing phosphate-buffered saline to neutralize the trypsin. The epidermis was separated from dermis with tweezers and the basal layer of epidermis was rubbed gently with the help of blunt side of surgical blade to release cells from the basal layer of epidermis. Finally, the solution was pipetted out to obtain epidermal suspension. The solid waste of tissue was removed and the suspension thus obtained was centrifuged at 1000 rpm for 5 min. The supernatant was then discarded and the pellet containing melanocytes and keratinocytes was obtained. Pellets were mixed with normal saline to make a suspension, which was collected in sterile capped tube.

Recipient Site

The recipient area was prepared with the help of savlon, spirit and povidone-iodine. The ANEM was injected

intraepidermally in the lesions using tuberculin syringe with a 26 G needle. The inoculations were done at the level of basal layer (approximately at the depth of 0.5 mm–1.5 mm), depending on the site. About 0.1 ml of epidermal suspension was inoculated at each site to produce a wheal and several such injections were given to cover the whole of the test site at the approximate distance of 5 mm. Recipient site was kept open without any dressing.

The donor and recipient sites were examined for any untoward effects, and then, the patient was sent home with advice to continue oral antibiotic for 1 week. At the selected control site (C), direct intraepidermal inoculation of phosphate buffer saline solution was given to serve as control.

Follow-up

The patients were called after 1 week for follow-up and any untoward effects noted. The dressing at the donor site was removed. The patients were advised PUVASOL therapy thrice weekly using 8-methoxy psoralen at the dose of 0.6 mg/kg body weight/treatment day. Thereafter, every 4-week follow-up was done for next 20 weeks to note any pigmentation. The percentage repigmentation at 24 weeks post-surgery was assessed by two independent investigators with the help of baseline photographs and the lesser value was taken for the assessment of results. Concordance between the assessments by two investigators was good.

The response on test sites was divided into four categories which consisted of excellent response when the pigmentation produced was in approximately 75–100%, good in 50–75%, satisfactory in 25–50%, and poor when pigmented area was <25%. Compared with the surrounding skin, the color of repigmentation was graded as follows: Somewhat darker; somewhat lighter; and the same. The observations obtained were tabulated and statistical analysis was done. Adverse events were noted carefully.

Statistical Analysis

Statistical analysis was performed using the SPSS®21.0 for Windows®.

RESULTS

Appearance of pigmentation varied according to the duration in the follow-up period. No pigmentation was noted at 1 week; however, faint erythema was present at many of the recipient sites. Repigmentation was first noted at the 4 weeks in the follow-up period. Number of lesions showing repigmentation increased with follow-up. The patients showing onset of repigmentation were 6 at 4 weeks and 16, 8, and 1 at 8, 12, and 16 weeks, respectively. Hence, overall,

production of pigment was observed in 6, 22, 30, and 31 patients at 4, 8, 12, and 16 weeks of follow-up, respectively. No new site showed pigment production after 16 weeks. The areas of pigmentation increased in size during the follow-up. Pigment production was not observed in 19 patients till 6-month follow-up. In the control areas, which received only vehicle, no repigmentation was observed.

Repigmentation at 24-week Post-surgery

Intention to treat analysis was done and the patients which did not come were thought to have no response.

At24weeks,31(62%)lesions repigmented [Figures 1 and 2]. Of 50 lesions, excellent, good, satisfactory, and poor pigmentation was observed in 6 (12%), 10 (20%), 8 (16%), and 7 (14%) lesions, respectively. Nineteen (38%) lesions showed no pigmentation even at 6 months [Table 2]. Face was the site to show maximum result with 9 of 13 lesions (69.2%) showing



Figure 1: Vitiligo: (a) Preoperative photograph of a lesion of stable vitiligo under right eye treated with autologous non-cultured epidermal cell suspension containing melanocytes suspension. (b) Postoperative photograph of the same lesion, 16 weeks after transplantation

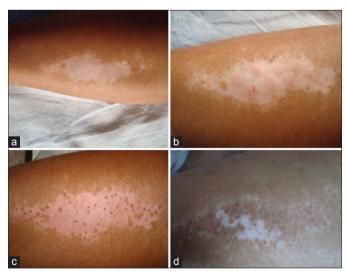


Figure 2: Vitiligo: (a) Preoperative photograph of a lesion of stable vitiligo on extensor surface of right forearm treated with autologous non-cultured epidermal cell suspension containing melanocytes suspension. (b) Intraoperative photograph of the same lesion (c) Postoperative photograph of the same lesion, 4 weeks after transplantation. (d) Postoperative photograph of the same lesion, 16 weeks after transplantation

pigmentation. Pigmentation appeared in decreasing order over face, upper limb, trunk, lower limb, and neck [Table 3].

In 94% of lesions, the color of the repigmented area was similar to the surrounding normal skin. In the rest, it was slightly darker than normal. The color tended to match with normal skin color overtime. Improvement in leukotrichia was observed in a few lesions [Figure 3].

We did not observe any correlations between start of repigmentation and duration of the disease, history of autoimmune disease, and familial history of vitiligo.



Figure 3: Vitiligo: (a) Preoperative photograph of a lesion of stable vitiligo on left hemiface treated with autologous non-cultured epidermal cell suspension containing melanocytes suspension. (b) Postoperative photograph of the same lesion, 16 weeks after transplantation. (c) Close up of the same lesion showing leukotrichia. (d) Improvement in leukotrichia seen at 24 weeks

Table 2: Appearance of pigmentation and duration in follow up

Duration in follow-up	Appearance of pigmentation			
period	Test site (%)	Control site		
1 week	0	0		
1 month	6 (12)	0		
2 months	22 (24)	0		
3 months	30 (60)	0		
4 months	31 (62)	0		
5 months	31 (62)	0		
6 months	31 (62)	0		

Adverse Events

Adverse events were mild. Mild bearable pain during the injection at recipient site was present, but none of the patients denied continuing with the procedure due to pain. Five patients had hyperpigmentation and one had scarring at the donor site. None of the patients reported any adverse effect such as secondary infection, crusting, discharge, and scarring at any of the recipient site. Mild faint erythema was noted in many patients, which was probably due to PUVASOL therapy. Color mismatch in the form of somewhat darker was a problem in two patients in immediate follow-up that gradually improved to give a similar color to surrounding skin at 24 weeks.

DISCUSSION

At 24 weeks, pigmentation was seen in 31 (62%) lesions of 50 lesions. It was excellent in 6 (12%), good in 10 (20%), satisfactory in 8 (16%), and poor in 7 (14%) patients. Adverse events were mild and insignificant.

Dermatosurgical techniques are grossly classified as tissue grafts and cellular grafts. Tissue grafts include punch grafts, blister method, and split skin thickness grafts and are limited by their inability to cover larger uneven areas. and hence, cellular grafts came into being. [5] Transplantation of melanocytes is one of the efficacious and relatively recent dermatosurgical technique for the treatment of stable vitiligo. Both cultured^[7] and non-cultured^[6] melanocytes have been used. Furthermore, the melanocytes have either been used in the pure form^[6] or along with other epidermal cells. [8,9] Culture of melanocytes is costly, time consuming and there are high chances of contamination during culture. Proliferating basal keratinocytes have been reported to produce melanogenic growth factors such as bFGF, mast cell growth factor, and endothelin-1.[10,11] ANEM is a simple and inexpensive method of cellular grafting, which is better than cultured melanocyte transplantation. [7] Repigmentation of vitiligo lesions using this method was first described by Gauthier and Surleve-Bazeille^[6] They achieved up to 70% pigmentation in 60% of the lesions. Since then, it has been used by a number of independent researchers with varying results.[12-21] More than 70% of pigmentation was obtained by van Geel et al.[13] with a modified procedure in which hyaluronic acid was added to the melanocyte suspension to

Table 3: Extent of repigmentation on the test sites at 6 months

Location and number of sites	Excellent	Good	Satisfactory	Poor	Total pigmentation produced	No response
Face (<i>n</i> =13)	3 (23.07)	3 (23.07)	2 (15.38)	1 (7.69)	9 (69.2)	4 (30.8)
Neck (<i>n</i> =3)	0 (0)	0 (0)	1 (33.3)	1 (3.3)	2 (66.6)	1 (33.4)
Trunk (<i>n</i> =12)	1 (8.3)	2 (16.6)	3 (23.07)	1 (8.3)	7 (58.3)	5 (41.6)
Upper limb (<i>n</i> =7)	1 (14.3)	1 (14.3)	1 (14.3)	2 (28.6)	5 (71.4)	2 (28.6)
Lower limb (<i>n</i> =15)	1 (6.67)	4 (26.7)	1 (6.67)	2 (13.3)	8 (53.3)	7 (46.7)
Total (<i>n</i> =50)	6 (12)	10 (20)	8 (16)	7 (14)	31 (62)	19 (38)

increase its viscosity and prevent runoff of the suspension from the dermabraded recipient site. Holla et al. used chlorhexidine gauze smeared with epidermal suspension and using meshed collagen sheet or sterile gauze smeared with placental extract gel to achieve similar results.[19] The recipient site for the ANEM transplantation has traditionally been prepared by producing blister^[7] (either by liquid N2 or suction blister) or by dermabrasion or CO, laser resurfacing. Kauffman et al used Erbium-YAG laser,[22] Guerra et al. used diathermosurgery^[23] and ultrasonic abrasion has been utilized to dermabrade the recipient area.[24,25] The methods of recipient site preparation which are being used are time consuming, require special instruments, and need expertise. Moreover, when recipient site is prepared by dermabrasion or laser resurfacing, then we have to use occlusion dressing (e.g., collagen dressing), which is costly. Maximum movement restriction has to be done to keep the ANEM inside the recipient site, so the downtime is high. Lasers are not available in many centers and if available, its use increases total cost of therapy. Recipient site formation by producing cryoblister is quite painful, time consuming and can cause peripheral destruction of melanocytes. There are more chances of secondary infection and resulting in no pigmentation/dyspigmentation. Chances of scarring are high. Delayed healing of the transplanted sites and increased chances of infection in blister inoculative technique can be minimized by spreading the melanocytes over dermabraded skin with high success rate of repigmentation, but this is associated with a risk of runoff or drying up and dislodgement of epidermal suspension. These disadvantages of blister inoculation over dermabraded skin can be reduced by putting proper occlusive dressing at the transplanted sites.[13,19,21] Hence, we thought of simplifying the inoculation procedure at recipient site by directly injecting ANEM intraepidermally, approximately at basal cell layer. In this method, the epidermis itself acts as the occlusion dressing. The results obtained by us show that the comparable repigmentation can be obtained by the intraepidermal transplantation of ANEM. The produced pigmentation was stable with excellent color match. It is less time consuming, cheap and needs less expertise. A similar study was conducted in Iran by Khodadai et al. at the same time on 10 patients which also showed similar results.^[26] However, the small sample size and no control group were its limiting factor.

The large study population as well as a 6-month follow-up helped us support the claim of our study's efficacy better. The ease of inoculation at recipient site coupled with minimal side effects gives our technique its added advantage. The study did have its few limitations. The original method used by Gauthier and Surleve-Bazeille was used which takes approximately 24 h for trypsinization while today procedures have developed in which it takes less than an hour.^[23] The authors thought that since one aspect of the procedure was being modified by intraepidermal injections, the initial technique should not be modified to avoid skewing of results.

The viability of ANEM cells *in vivo* post-injection could not be assessed and authors are conducting further studies to corroborate it. An independent recent study has shown that epidermal suspension can survive up to 7 days in *in vitro* and further *in vivo* studies are being planned.^[27] The spontaneous repigmentation beyond 6 months of follow-up was not studied by us. Gan *et al.* showed significant maintenance of repigmentation at 12 months and 60 months follow-up.^[28]

CONCLUSION

Our study is a novel approach which makes the process of ANEM simpler, easier, and less cumbersome for both the patient and the dermatosurgeon. In comparison to the conventional modes of recipient site preparation, our method wins on the basis of its ease, simplicity, safety, and comparable efficacy.

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