Accepted Date : 18-Jan-2011
Article type : Original Article

Non-cultured extracted hair follicle outer root sheath cell suspension for transplantation in vitiligo

Running Title: ORS cell transplantation in vitiligo

Authors: [1]S. Mohanty, [1]A. Kumar, [2] J. Dhawan, [3]V. Sreenivas, [2]S. Gupta

Departments of [2]Dermatology and Venereology, [1]Stem Cell Facility, and Biostatistics, All India Institute of Medical Sciences, New Delhi, INDIA

[3]All India Institute of Medical Sciences, Biostatistics

Corresponding Author:

Dr. Somesh Gupta

Asst. Professor,

Department of Dermatology & Venereology,

All India Institute of Medical Sciences,

New Delhi-110029, INDIA

Email: someshgupta@hotmail.com

Phone: 0091-11-26546626 (Office), 0091-9868899120

Fax: 0091-11-26588641, 26588663

What's already known about this topic?

- Hair follicle is an important reservoir of melanocytes and replenishes melanocytes in depigmented epidermis
- Transplanted plucked hair follicles do not grow, but extracted follicles do. This suggests that not all stem cell pools are present in plucked follicles.

What does this study add?

- Preparation of outer root sheath cell suspension is technically less challenging
- Extracted hair follicular ORS cell suspension transplantation is effective in repigmentaion of vitiligo stable for a year or more

Abstract:

Background: Current non-cultured cell-based transplantation therapies for vitiligo largely involve shave skin biopsy for preparation of non-cultured melanocyte suspension. As overall proportion of melanocytes is low in the epidermis, these techniques require basal cell layer enrichment, which adds few additional steps. We tried follicular unit extraction (FUE) to harvest hair follicles as a source of melanocytes.

Objective: To evaluate the efficacy of a novel surgical method for vitiligo: non-cultured extracted hair follicular outer root sheath cell suspension (EHF ORS CS) transplantation.

Methods: Fourteen patients with vitiligo, stable for at least 3 months, were included in this prospective study. 15-25 hair follicles were extracted from occipital scalp using FUE method. Hair follicles were incubated with trypsin-EDTA solution at 37^{O}C for 90 minutes to separate ORS cells. The cell suspension was filtered through a 70µm cell strainer, then centrifuged for 5 minutes at 1000rpm to obtain a cell pellet. The pellet was resuspended and applied to the dermabraded recipient area and dressed.

Results: The mean±S.D. repigmentation was 65.7%±36.7%. Overall, 9 out of 14 patients achieved >75% repigmentation. Mean percentage repigmentation was significantly higher in patients with ≥1year stability than those with <1 year stability ($p=0.02$).

Conclusion: EHF ORS CS can be a useful simplified transplantation method for vitiligo. Transplantation procedure should be reserved for patients with vitiligo stable for at least one year. A larger study is needed for further evaluation.

Introduction

Vitiligo is a common skin pigmentary disorder resulting from the loss of melanin which causes depigmented skin. It has a huge negative psychological and social impact on the patient. A number of therapeutic options of repigmentation of vitiligo are available including corticosteroids, calcineurin derivatives, and phototherapy. Surgical treatment is indicated in stable disease not responding to medical treatment. There are various surgical modalities available for vitiligo, which are based on the idea of restoring melanocytes on recipient site. These can be tissue grafting such as suction blister epidermal grafting, thin and ultra-thin split thickness skin grafting, minigrafting (punch grafting), and follicular grafting, or cellular transplantation such as cultured pure melanocyte transplantation, Co-cultured melanocyte-keratinocyte suspension cell transplantation, cultured epidermis, and noncultured basal cell layer enriched epidermal cell suspension transplantation.[1] Each of these techniques has some advantages and some disadvantages and attempts are on to evolve a better technique. Treatment of vitiligo on hairy skin by the tedious procedure of hair follicle grafting has been carried out by few investigators. This is based on the fact that outer root sheath (ORS) of hair follicle is a rich source of melanocytes and melanocyte precursor cells. Traditionally, hair follicles for FG are obtained by elliptical scalp biopsy, and then single hair follicles are cut and transplanted to recipient site. In a preliminary study, Vanscheidt and Hunziker[2] have used single cell suspension of plucked hair follicle in the treatment of vitiligo with good results. We describe for the first time the use of single cell suspension of ORS of hair follicles harvested by the follicular unit extraction (FUE) method. This technique has been utilized in hair transplantation surgery and has an advantage of invisible or insignificant scarring. The cell suspension prepared from hair follicles obtained by FUE can also be a potential source of multipotent epidermal and mesenchymal stem cells.

Patients and Methods

Hair follicles were obtained from the subjects after taking their written informed consent as recommended by the Institutional Ethics Committee and Institutional Committee for Stem Cell Research and Therapy Guidelines of All India Institute of Medical Sciences (AIIMS), New Delhi, India. The study was approved by both these committees.

Patients coming to the Dermatology OPD for the surgical treatment of stable vitiligo were offered this therapy and those who agreed were enrolled for this prospective study. The stability was defined as no appearance of new lesions and no progression of existing lesions for past three months or more. Patients with vitiligo patches on scalp were excluded. None of the patients had clinical evidence of any other autoimmune disease.

Hair samples were taken from occipital area of scalp and only anagen hair were extracted. In pigmented populations, it is not very difficult to recognize anagen hair clinically. An anagen hair is an active, growing hair. From the surface, anagen hairs tend to be stronger in hair shaft tensile strength and more pigmented, that is, these hairs have more melanin. All subjects had undergone the FUE method for hair follicle tissue harvest. Samples were sent to the stem cell laboratory for processing within 15-20 min of harvesting of the tissue.

Follicular unit extraction:

Hair were trimmed to a length of approximately 2 mm. Field block anaesthesia was given with 2% lignocaine, which was infiltrated in the skin encircling the area chosen for FUE. To obtain follicular units, 1-mm punch was rotated till mid-dermis in the direction of hair follicle. Care was taken not to go up to subcutaneous space to avoid transaction of the hair follicle. Then follicular unit was pulled out gently using hair follicle holding forceps by holding the skin surrounding the hair shaft(s). Transacted hair follicles were discarded. Depending on the area to be transplanted, approximately 15-25 pigmented follicles were extracted per subject and collected in collection media, containing Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, St Louis, MO, U.S.A.), pH=7.2 supplemented with Penicillin, streptomycin and Amphotericin-B (Gibco BRL, Gaithersburg, MD, U.S.A.). The procedure of FUE took approximately 25-30 minutes.

Preparation of single cell suspension:

The extracted hair follicles were transported to the laboratory under sterile conditions and washed three times with phosphate buffered saline containing the antibiotics and antimycotics (Gibco BRL, Gaithersburg, MD, U.S.A.). The follicles were then incubated with 0.25% trypsin-0.05% EDTA (Gibco BRL, Gaithersburg, MD, U.S.A.) at 37 ºC for 90 minutes to prepare the single cell suspension. Cells started loosening up within 15-20 minutes (Fig. 1). After every 30 minutes the hair follicles were placed in a new tube of trypsin EDTA and the reaction in the previous tube was terminated

by adding the trypsin inhibitor (Sigma-Aldrich). This was done to prevent digestion of separated cells by trypsin. After cell separation only thin keratinous shafts of the hair were left (Fig. 2a,b), which were discarded. The cell suspensions of all the three tubes were added in a single tube and then filtered through a 70µm cell strainer (Becton Dickinson, Sunnyvale, CA, U.S.A.) to prepare a single cell suspension. Finally, the cell suspension was centrifuged for 5 minutes at 1000rpm to obtain a cell pellet, which was re-suspended in a small amount of DMEM and transported to the operation theatre for transplantation. The whole procedure of preparation of cell suspension took approximately 2-3 hours.

Smears of a representative sample were prepared for cytological examination to ensure that the cells used for transplantation have normal morphology and for special staining with HMB45 antibody to see percentage of melanized cells. A histological examination (Haematoxylin and Eosin staining) of hair follicle was also done prior and after the preparation of cell suspension. The proportion of the CD200 positive cells was determined by a flowcytometer. The number of cells in the suspension was counted using haemocytometer. The viability of the cells suspension was checked by trypan blue dye exclusion method**.** For quality control, some of the cell samples were also tested for *Mycoplasma* contamination.

Transplantation of Single Cell Suspension

The recipient vitiligo patch was anaesthetized first using topical anaesthetic cream (EMLA) and then by infiltrating small amount of local anaesthetic (2% lignocaine). The recipient site was dermabraded superficially by motorised dermabrader fitted with diamond fraises. The depth of dermabrasion was determined by the bleeding pattern and dermabrasion was stopped once pinpoint bleeding points appeared. All precautions were taken to minimize any risk of transmission of infectious agents via aerosol produced by dermabrasion, such as all OT staff wearing mask and goggles during the procedure. Prepared hair follicle suspension was spread uniformly over the de-epidermised area with the help of a pipette. The suspension was then covered with a collagen dressing of fish origin (Neuskin-F$^{(R)}$). The dressing was removed on 8th days. After complete re-epithelisation (which took around 2-3 weeks), patients were put on oral methoxsalen followed by sun exposure (PUVAsol) or oral methoxsalen followed by ultraviolet A (PUVA).

The clinical outcome was documented monthly by standardized photographs for up to 6 months. The repigmentation was assessed subjectively by comparing pre-treatment and post-treatment pictures.

Statistical Methods:

The mean± standard deviation (S.D.) of area transplanted, number of cells transplanted, follow-up period, stability period, and repigmentation percentage were calculated. The average repigmentation between segmental and generalized (Acro-facial and vulgaris) was compared using Rank sum test. Average repigmentation was compared between patients with stability period <1 year and ≥1 year using Rank sum test.

Results

Demographic and disease characteristics:

Fourteen subjects were enrolled with 11 females and 3 males. Age range of the subjects was 17 years to 30 years with mean age±S.D. of 22.8±4.8 years. The average stability period was 43.6± 54.7 months (range 3-180 months). All but three patients had stability period of one year or more.

Laboratory Findings:

The average number of cells±S.D. transplanted was 36285±9659. The average proportion of viabile cells in the suspension was 91%. HMB45 antibody staining showed dark staining of almost all cells in suspension, suggesting that cells were heavily melanized (Fig. 3). The mean proportion±S.D. of CD200+ cells in the suspension was 11.4%±8.7%. Haematoxylin & Eosin staining of samples of hair follicles before trypsinization showed a classical hair follicle with all its components along with a small portion of surrounding dermis (Fig. 4a). However, post-trypsinization hair showed only central keratininzed shaft and few cells attached to it (Fig. 4b). This suggests that, with the method described most of the cells are separated from the hair to constitute cell suspension. The cell preparation was found to be negative for *Mycoplasma* contamination.

Clinical Outcome:

The mean±S.D. of area transplanted was 30.8± 26.5 cm^2. The post-procedure follow-up period ranged from 1 to 14 months (mean 6.9 months).

The mean percentage repigmentation±S.D. was 65.7%±36.7% (Fig. 5). The mean repigmentation±S.D. in segmental and bilateral disease were 86.7%±5.8 vs.

60%±39.7, respectively (p=0.8). However, mean percentage repigmentation was significantly less in patients with <1 year stability than in those with ≥1 year stability (18.3±16.1 vs. 78.6%±29.1, p=0.02). Details of patients and the outcome of procedure are given in Table 1.

Discussion

Hair follicle is an important reservoir of melanocytes and their precursor cells. Melanocyte-lineage antigens plus c-Kit (the receptor for Stem Cell Factor) stained cells are localized in the outer layer of the outer root sheath of the infundibulum and mid-follicle and the matrix of the hair bulb.[3] This reservoir of melanocytes and melanocytes stem cells is important in the treatment of vitiligo as the initial repigmentation in vitiligo patches often occurs around the hair follicles[4] and vitiligo patches on skin lacking hair follicles, such as palms and eyelids, are often resistant to medical therapies.

There are other populations of cells which might constitute the hair follicular cell suspension.[5] These include basal cells high in α6-integrin/keratin 14 (K14) expression, suprabasal cells low in α6-integrin/K14 expression; the hair germ cells expressing Lgr5, P-cadherin, and S100A4, the bulge cells expressing CD34 and CD200, while a more distal population expressing MTS24.[5]The perifollicular connective tissue sheath and the papilla is a potential source for mesenchymal stem cells in the cell suspension obtained from extracted hair follicles.[6]

Vanscheidt *et al.,*[2] in a small case series, have used single cell suspension of 'plucked' hair follicles in the treatment of vitiligo. They found almost complete (>90%) repigmentation in 3 of 5 patients with vitiligo, around 50% repigmentation in one patient and less than 10% repigmentation in one patient .Their technique is simple, non-invasive and allows easy, immediate and repeated application. However the cell yield is less in case of plucked hair follicles and optimization of cell harvest form the hair follicular unit needs to be standardized for optimum yield. We found that the cell suspension prepared from hair follicles obtained by FUE method contains more CD200+ cells (a marker for hair follicle bulge stem cells) as compared to plucked hair (unpublished observation). This is further supported by the observation that transplantation of plucked hair doesn't result in hair growth, however transplantation

of extracted follicular unit promptly accepted by the recipient site with resulting hair growth.[7,8] Hair follicle is a rich source of three different types of stem cells and, it appears that all of them are important in hair growth. These stem cells include melanocyte stem cells, keratinocyte stem cells, and mesenchymal stem cells.[9,10] Melanocyte stem cells in a melanocyte reservoir, located in the upper, "permanent," outer root sheath, have the capacity to migrate and enter vacant niches in epidermis. This phenomenon might be responsible for perifollicular pigmentation seen in vitiligo in response to phototherapy.

We have used ORS cell suspension of only anagen hair follicles obtained by FUE technique. Hair is actively pigmented only during the anagen phase and melanogenic activity of melanocytes is strictly coupled to anagen.[11] This method resulted in significant repigmentation in the majority of the patients. Apart from the technique used and expertise of the operating surgeon, the repigmentation after transplantation depends on several other factors: 1. disease type[12] (with better repigmentation in segmental than in generalized disease). 2. stability of disease[13] (repigmentation is better in stable disease than in active disease) 3. site of the vitiligo patch (skin over joints and acral parts is difficult to treat surgically due to mobility of the part). Three of our patients had less than one year of disease stability and none of them had >75% repigmentation while 9 out of 11 patients with at least one year stability had >75% repigmentation. Thus, the standard guidelines suggesting one year of disease inactivity as stability definition for the purpose of transplantation appear to be appropriate and patients with less than one year stability period should not be taken for transplantation.[14]

Significant repigmentation in the majority of our patients indicates that the procedure described by us works. It can be used to cover large depigmented areas. The procedure involves removal of only 15-25 follicular units, which provide 25000 to 50000 cells, sufficient to treat up to 25 cm^2. From the experience gained from epidermal cell suspension transplantation, the experts recommended the desired number of cells for repigmentation as 2000 cells/cm^2. This requirement is likely to be less with ORS cell suspension from FUE method, though only a comparative study can confirm this. In follicular melanin unit, there is one melanocyte for every five keratinocytes in the hair bulb,[15] which is much higher than epidermal melanin unit,

which has one melanocyte for every thirty-six keratinocytes. In comparison to epidermal melanocytes, anagen hair bulb melanogenic melanocytes are larger, more dendritic, with more extensive Golgi and rough endoplasmic Reticulum, and produce larger melanosomes.[15] Hair melanocytes have remarkable synthetic capacity and a relatively small number of melanocytes can potentially produce sufficient melanin to pigment up to 1.5 m of hair shaft.[16] Melanocyte stem cell has been recognized in the hair follicle but not in the epidermis. Melanocyte stem cells are less in number in epidermis in comparison to hair follicle. All these properties make hair a more attractive source of melanocytes than epidermis for cell based therapies in vitiligo.

In contrast to the conventional epidermal cell suspension technique,[17] our technique is relatively simpler, as it does not require separation of epidermis from dermis, and manual breaking of epidermal samples into small pieces. Outer root sheath cells are easily separated when incubated with trypsin. Furthermore, the tiny scars of FUE on scalp are invisible and insignificant. The procedure of FUE involves removal of much less volume of tissue in comparison to epidermal shave biopsy. The healing time is much less as compared to conventional shave biopsy from buttock or thigh. We do not even dress donor site after FUE. The number of melanocytes is less in the epidermis, and most of them are located in the basal layers. Therefore, to enhance the proportion of Melanocyte in cell suspension, basal cell layer enrichment is necessary in the conventional method, which requires few additional steps in the cell suspension preparation. On the other hand, hair follicle has melanocytes in abundance and no enrichment is needed. This makes it a simpler procedure and potential method of choice for the treatment of large areas of stable vitiligo in a quick time. The cell suspensions used in this study are autologous and with minimum *in-vitro* manipulation. These cells are suspended in culture media without serum, so there are no safety concerns.

Patients with vitiligo have a tendency to develop canities at an early age. In addition, canities in any person develop after the age of 40-45. So there might be a concern that melanocytes of follicular origin might have a shorter life span. However, vitiligo patches repigmented by phototherapy induced peri-follicular repigmentation do not

show loss of pigmentation with ageing, so it is less likely that surgically transplanted melanocytes will have shorter life span.

This procedure can serve as a novel, minimally invasive, technically less challenging technique with good yield of melanocytes, melanocyte stem cells, and other stem cells (keratinocyte stem cells, and mesenchymal stem cells in the surrounding dermis). Stem cells may also help in rapid healing over recipient area by rapid reepithelialization. This method results in excellent pigmentation with no visible residual scarring. The limitations of this study are small sample size, too short follow-up period, and less than one year stability period in three patients.

References:

1. Bahadoran P, Ortonne J-P. Classification of surgical therapies for vitiligo. In Surgical Management of Vitiligo (Gupta S, Olsson M, Kanwar A, Ortonne J-P, Eds.). *Oxford: Blackwell Publishing, 2007, pp59-68.*
2. Vanscheidt W, Hunziker T. Repigmentation by outer-root-sheath-derived melanocytes: proof of concept in vitiligo and leucoderma. *Dermatology* 2009;**218**: 342-3.
3. Randall VA, Jenner TJ, Hibberts NA, De Oliveira IO, Vafaee T. Stem cell factor/c-Kit signalling in normal and androgenetic alopecia hair follicles. J Endocrinol 2008; 197:11-23.
4. Lan CC, Wu CS, Chen GS, Yu HS. FK506 (topical tacrolimus) and Enothothelin Combined Treatment Induces Mobility of Melanoblasts: New Insights of Follicular Vitiligo Repigmentation Induced by Topical Tacrolimus on Sun-Exposed Skin. Br J Dermatol 2010 Oct 29. doi: 10.1111/j.1365-2133.2010.10113.x. [Epub ahead of print]
5. Legué E, Sequeira I, Nicolas J-F. Hair follicle renewal: authentic morphogenesis that depends on a complex progression of stem cell lineages. Development 2010;137: 569-577.
6. Sellheyer K, Krahl D. Skin mesenchymal stem cells: prospects for clinical dermatology. J Am Acad Dermatol. 2010;63:859-65..
7. Gho CG, Braun JE, Tilli CM et al. Human follicular stem cells: their presence in plucked hair and follicular cell culture. *Br J Dermatol* 2004; **150**: 860-8.
8. Gho CG, Neumann HA. Hair transplantation of plucked hair biopsies. *Dermatol Surg* 2001; **27**: 913.
9. Nishimura EK, Jordan SA, Oshima H et al. Dominant role of the niche in melanocyte stem-cell fate determination. *Nature* 2002;**416**:854-60.
10. Nishimura EK, Granter SR, Fisher DE. Mechanisms of hair graying: incomplete melanocyte stem cell maintenance in the niche. *Science* 2005;**307**:720-4.
11. Botchkareva N, Khlgatian M, Longley B et al. Scf/c-Kit signaling is required for cyclic regeneration of the hair pigmentation unit. *FASEB J* 2001;**615**:645-8.
12. Gupta S, Kumar B. Epidermal grafting in vitiligo: influence of age, site of lesion, and type of disease on outcome. *J Am Acad Dermatol* 2003;**49**:99-104.
13. Gupta S. Stability in vitiligo: why such a hullabaloo? *J Cutan Aesthet Surg* 2009;**2**:41-2.
14. Parsad D, Gupta S. Standard guidelines of care for vitiligo surgery. *Indian J Dermatol Venereol Leprol* 2008; **74 Suppl**: S37-45.
15. Tobin DJ, Paus R. Graying: gerontobiology of the hair follicle pigmentary unit. *Exp Gerontol* 2001;**36**:29-54.
16. Slominski A, Wortsman J, Plonka PM et al. Hair follicle pigmentation. *J Invest Dermatol* 2005;**124**:13-21.
17. Paul M. Autologous non-cultured basal cell enriched epidermal cell suspension transplantation: A new perspective on the treatment of vitiligo. J Cutan Aesth Surg 2011 (In Press).

Legends for Illustrations:

Fig. 1. Outer root sheath cells (ORS) in early stage of separation from the hair shaft (HS) (X200).

Fig. 2 (a). Follicular units before trypsinization. (b) Follicular units after trypsinization- only hair shafts are left.

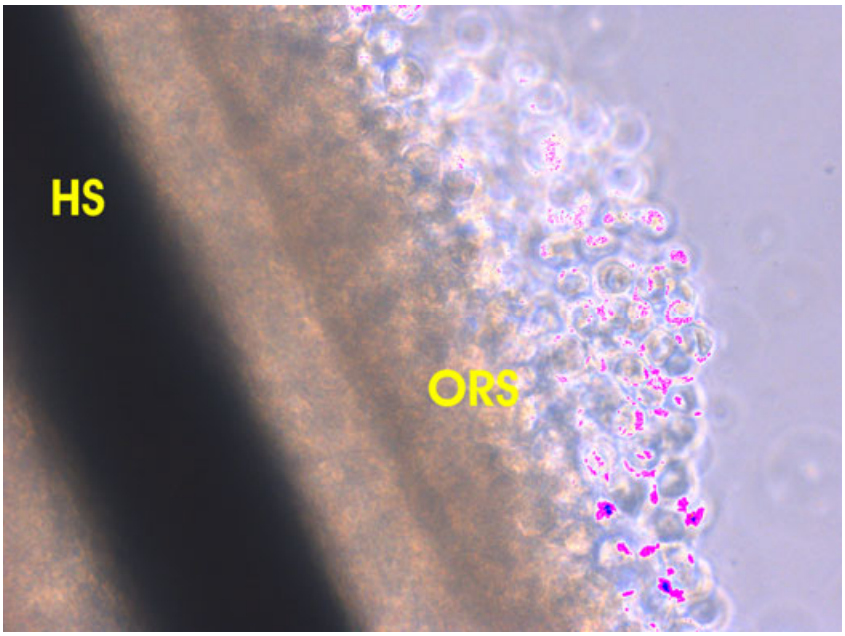
Fig. 3. HMB45 staining of smear of cells from cell suspension showing staining (>) of almost all cells (X400).

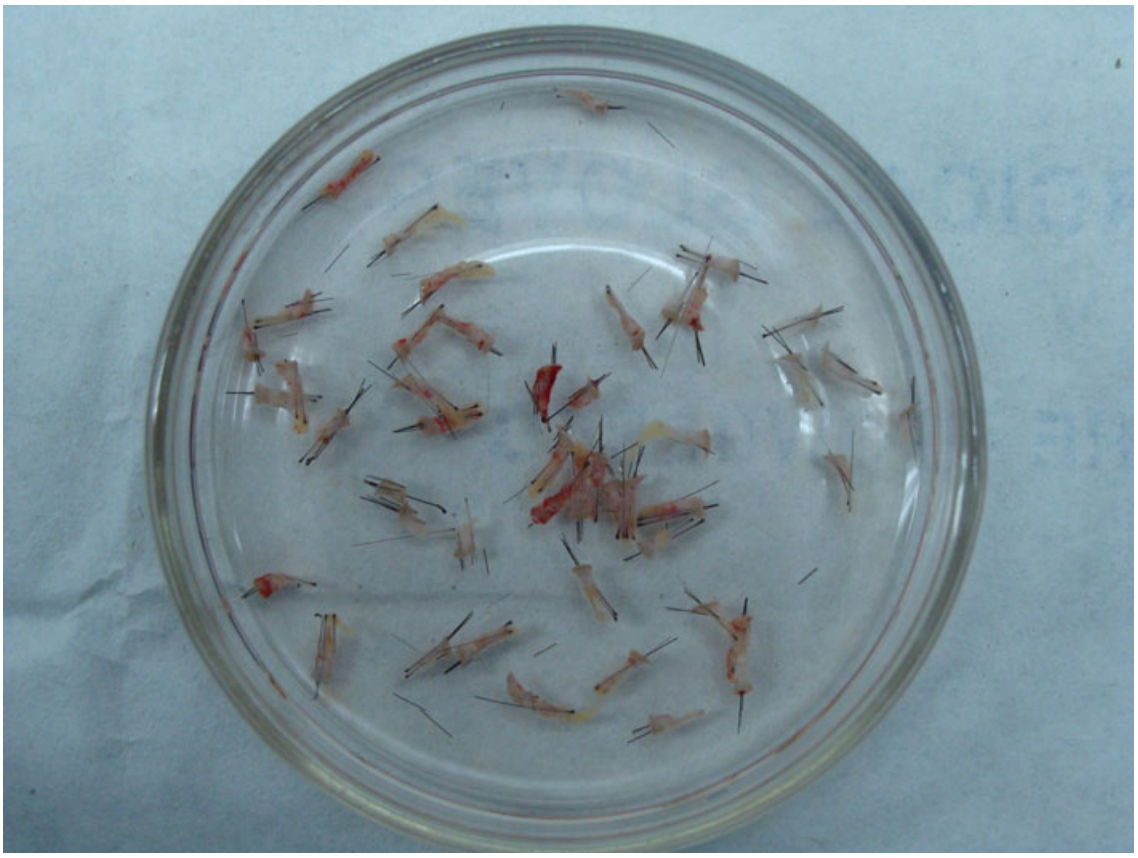
Fig. 4. (a)Histopathology of follicular units before trypsinization- outer root sheath cells and perifollicular dermis are attached (H&E, X400) with densely packed cells of the hair shaft in the centre. (b) After trypsinization, only few cells of the hair shaft are left (H&E, X400). ORS-Outer Root Sheath, HS- Hair Shaft

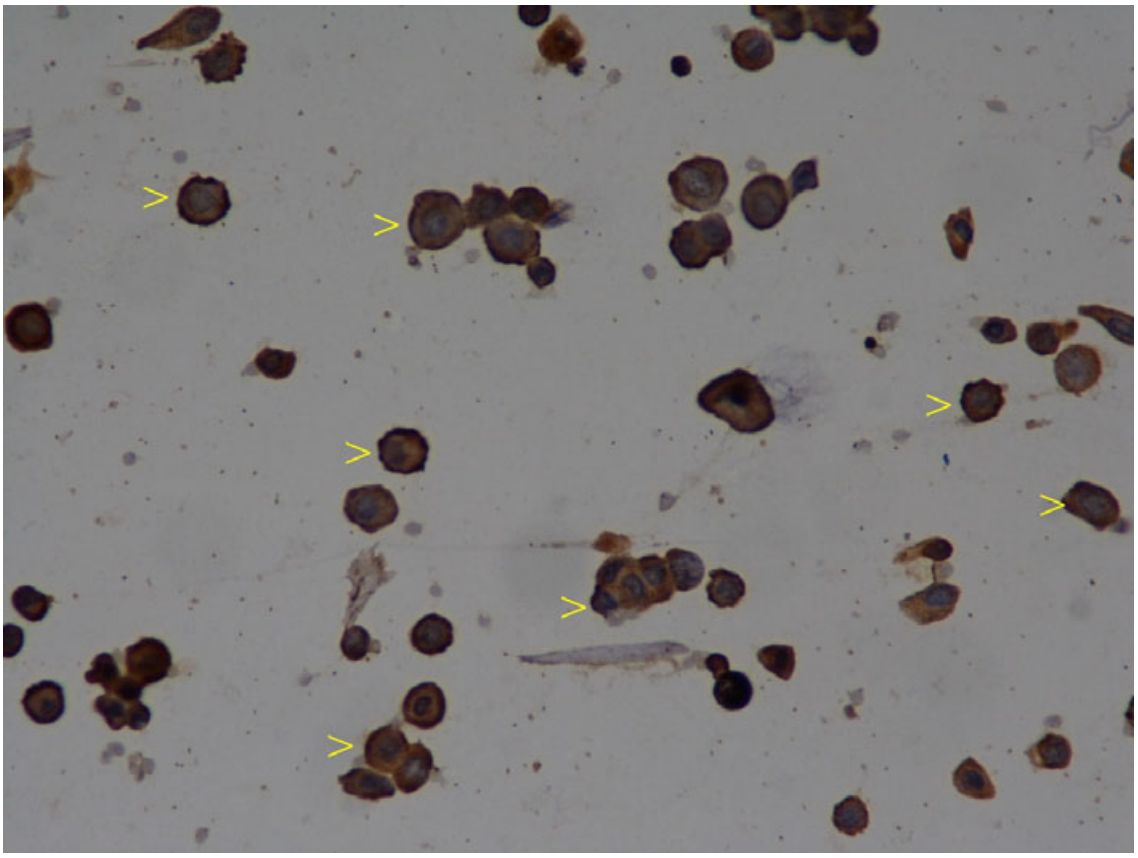
Fig. 5 (a). Vitiligo patch on left foot chosen for transplantation; vitiligo patch on right foot was kept as control (b) Excellent repigmentation on transplanted side.

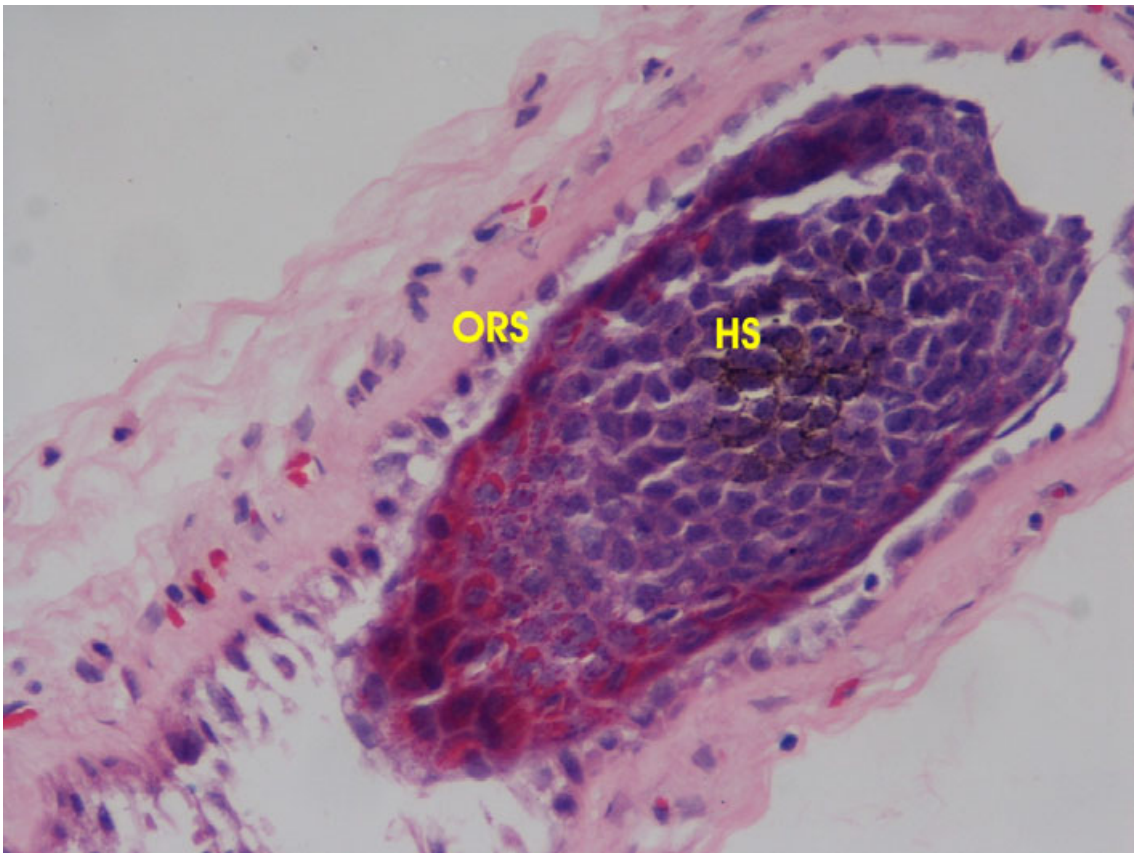
S No	Gender	Age (in years)	Area transplanted	Site	Cell Number Transplanted	Type	Follow-up period(in months)	S n
1.	Male	20	7X4 cm	Lt leg	28000	Segmental	8	2
2.	Female	18	5X3 cm	Lt knee	25000	Acrofacial	8	3
3.	Female	22	3X2 cm, 2X2 cm	Lt hand	37000	Vulgaris	3	3
4.	Female	21	14X6 cm	Lt foot	50000	Acrofacial	7	1
5.	Male	25	4X3 cm	Neck & Chin	30000	Segmental	14	2
6.	Female	23	12X8 cm	Lt foot	39000	Valgaris	6	8
7.	Female	20	8X4 cm	Trunk	38000	Valgaris	15	1
8.	Female	20	2X2cm, 3X4cm	Arm pit & breast	27000	Vulgaris	6	1
9.	Female	28	4X6 cm	Trunk	17000	Vulgaris	7	1
10.	Female	17	10X4 cm	Lt leg	40000	Vulgaris	5	1
11.	Female	26	2X7 cm	Breast	43000	Vulgaris	3	1
12.	Female	17	4X4 cm	Rt breast & Rt shoulder	40000	Segmental	10	8
13.	Female	32	5X4 cm	Lt foot	50000	Vulgaris	4	5
14.	Male	30	6X4 cm	Neck	44000	Acrofacial	1	4

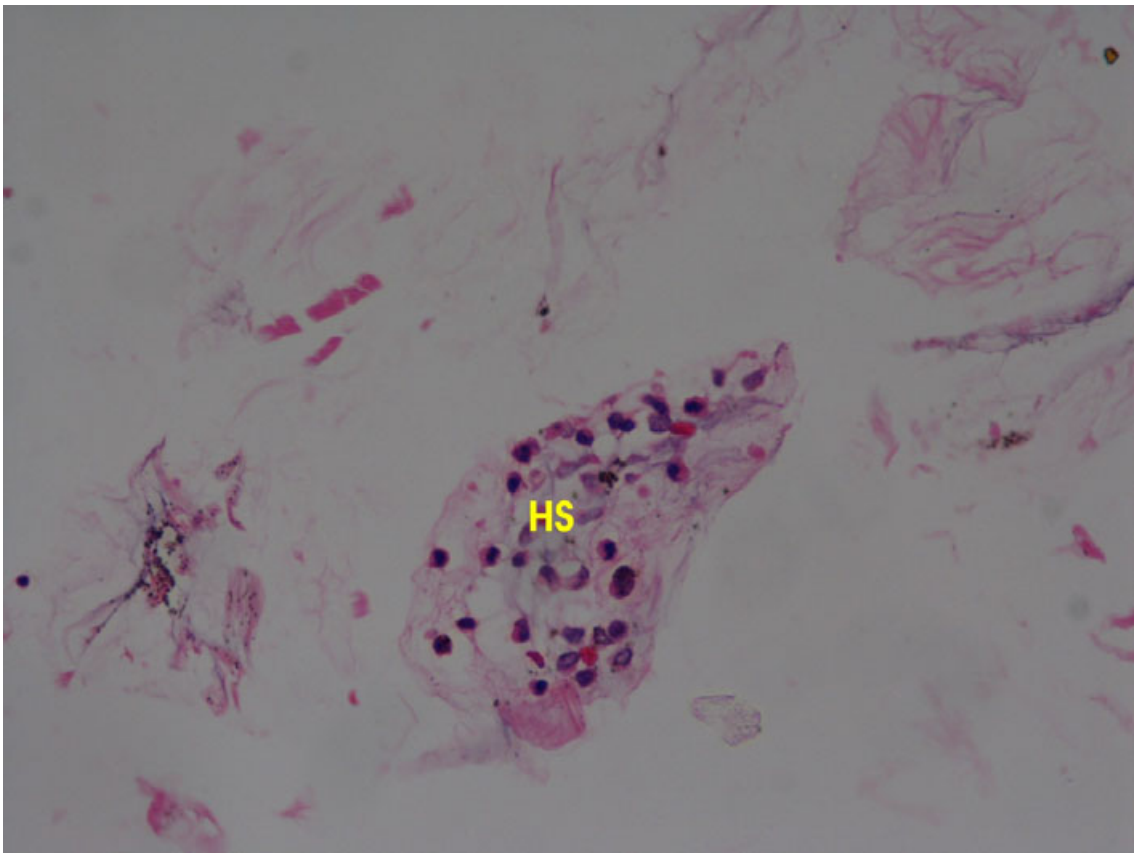
1: Details of vitiligo Patients Transplanted with Single Cell Suspension of Hair Follicle harvested by FUE Method and transplantation outcome:


HS
ORS






ORS
HS


HS