PLAN OF THESIS

TRANSPLANTATION OF AUTOLOGOUS NONCULTURED EXTRACTED HAIR
FOLLICLE OUTER ROOT SHEATH CELL AND AUTOLOGUS NONCULTURED
EPIDERMAL CELL SUSPENSION IN COMBINATION AS A NOVEL METHOD IN
VITILIGO SURGERY

SUBMITTED IN PARTIAL FULFILLMENT OF THE DEGREE

OF

MD (DERMATOLOGY, VENEREOLOGY AND LEPROLOGY) OF THE
POST GRADUATE INSTITUTE OF MEDICAL EDUCATION AND RESEARCH
CHANDIGARH

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SUMMARY OF PROPOSED RESEARCH

Vitiligo is a complex disease that causes a selective, often progressive, loss of functioning melanocytes from basal layer of epidermis, leaving white patches on the skin and occasionally mucosae.\(^1\) Worldwide prevalence of vitiligo is around 1% whereas in India it is around 3-4%, ranging from 0.46% to 8.8%.\(^2\)

Neural crest derived melanocytes are located mainly in the basal layer of the epidermis and in the matrix of hair follicles.\(^3\) Melanocytes synthesize melanin pigment, transfer mature melanosomes to basal keratinocytes and are responsible for skin color.\(^4\) Etiopathogenesis of vitiligo is multifactorial and polygenic consisting of genetic, immunological and environmental factors. Environmental and genetic factors act in concert to destroy melanocytes.

The key clinical finding in vitiligo is the acquired onset of an increasing number of initially hypopigmented and then depigmented macules, patches and later even wide spread involvement of skin. It is often associated with leucotrichia. Vitiligo is divided into three types: localized, generalized, and universal. Localized vitiligo is further sub typed into focal, segmental and mucosal. Generalized vitiligo may be acrofacial, vulgaris or mixed. Universal vitiligo involves more than 80% of the skin. Generalized vitiligo is the most common type, and vulgaris is the most common subtype. The sites of predilection for vitiligo vulgaris are the fingers and wrists, axillae and groin, and body orifices, such as the mouth, eyes, and genitals.\(^5\) Though the condition is non-contagious and asymptomatic, it has significant psychosocial implications and can lead to an exaggerated sense of humiliation, loss of self-esteem and job discrimination among patients.\(^6\)

Various modalities of treatment (both medical and surgical) have been described for vitiligo. Although medical treatment is the mainstay of treatment, it is not effective in all and residual lesions require surgical treatment. Amongst the surgical options, replenishing melanocytes selectively within vitiliginous macules by autologous melanocyte transplantation, in the form of either tissue graft or cellular graft, is a promising treatment. Surgical method of vitiligo treatment doesn’t alter the natural course of the disease and treatment is mainly symptomatic. Transplantation of autologous noncultured extracted hair follicle outer root sheath cell and autologous noncultured epidermal cell suspension in combination, hereafter denoted as FCS + NCES (a mode of cellular grafting technique) is a novel surgical method for the treatment of vitiligo.
This pilot study is planned to introduce FCS + NCES as a novel method in vitiligo surgery and compare its outcome with NCES in the same stable vitiligo patient with regard to extent of repigmentation, color matching of repigmented area, patient satisfaction and any adverse events if any. There are studies comparing effectiveness of FCS or NCES separately in vitiligo surgery giving comparable results. But, this is the first study using FCS + NCES as a new modality in vitiligo surgery.
Vitiligo, the most common depigmenting disorder is an ‘idiopathic’, acquired pigmentary disorder caused by the loss of functional melanocytes from the basal layer of epidermis. The term is coined from the Latin word ‘vitelius’ that means calf, where the characteristic lesions of skin resemble the milky white macules of spotted calf. The disease runs an unpredictable course but is often progressive with phases of stabilized depigmentation. It usually begins during childhood or young adulthood. In approximately 50% of all cases, vitiligo appears before the age of 20 years, and 70–80% of patients develop the disease by the age of 30 years. A large series on childhood vitiligo conducted in India reported a prevalence of 2.4%. The presence of vitiligo on exposed areas of body leads to social embarrassment, psychological turmoil, and cosmetic disfigurement in those affected.

Vitiligo is known since times immemorial. The oldest information on vitiligo comes from the period of Aushooryan (2200 BC), in the classical Tarikh-e-Tib-e-Iran. The disease is mentioned as ‘Shweta kushtha’ in the ancient Indian sacred book ‘Atharva Veda’ (1400 BC). Its prevalence is 1%, ranging from 0.1 to > 8.8% in different countries of the globe. The highest incidence of the condition has been recorded in inhabitants of the Indian subcontinent. The reported difference in incidence between different communities may be due to a higher reporting of vitiligo in certain populations, where an apparent color contrast and stigma attached to the condition may force the patients to seek early medical advice. Both sexes are equally affected although the greater number of reports among females is probably due to the greater cosmetic concern and social consequences to women affected by this condition.

**CLASSIFICATION**

*Classification of vitiligo according to the distribution of lesions*

1. Localized
   a) Focal
   b) Segmental
   c) Mucosal
2. Generalized
   a) Vulgaris
   b) Acrofacial
   c) Mixed
3. Universal
Classification of vitiligo with emphasis on segmental vitiligo

1. Segmental (unilateral)
2. Non-segmental (bilateral)
   - Localized
      a) Focal
      b) Mucosal
   - Generalized
      a) Vulgaris
      b) Acrofacial
      c) Universal
3. Mixed: segmental and non-segmental

There are two main clinical presentations of vitiligo: unilateral (segmental, asymmetric) and bilateral (nonsegmental, symmetric).

Clinical features of segmental vitiligo

Segmental vitiligo, a subtype of vitiligo, is characterized by its early onset, rapid stabilization and unilateral distribution. The reported prevalence of segmental vitiligo ranges from 3.5% to 20.5% of all patients with vitiligo. Associated autoimmune diseases in patients with segmental vitiligo and their family members are reported less frequently than in generalized vitiligo. Face is the most common site of segmental vitiligo regardless of the gender of the patient. The trunk, neck, extremities, and scalp are involved in descending order. So far, several hypotheses for segmental vitiligo have been put forward, including (i) neuronal mechanisms, (ii) somatic mosaicism and (iii) microvascular skin homing, whether or not leading to an autoimmune destruction of melanocytes. Most of the patterns of segmental vitiligo did not follow a dermatomal distribution. High rates of repigmentation with surgical techniques are frequently achieved.

Clinical characteristics of non-segmental (bilateral) vitiligo

Bilateral vitiligo is a slowly developing condition, with a tendency to progress throughout life. Arrest of the condition may occur in a small percentage of these individuals. Focal vitiligo exhibits one or few macules in one area, most commonly in the distribution of trigeminal nerve, although neck and trunk are also commonly involved. Focal vitiligo is a starting point leading to other types of vitiligo. Mucosal vitiligo affects mucosae of the mouth.
and genitalia. Acrofacial vitiligo encompasses depigmentation of the distal parts of the extremities (hands rather than feet) and facial orifices, the latter in a circumferential pattern. Lip-tip vitiligo is a variety in which tips of fingers, toes, nipples, penis and lips become depigmented. Vitiligo vulgaris is composed of several scattered macules and is the most common form of the disease. Depigmented patches are widely and usually symmetrically distributed. Universal vitiligo implies loss of pigment over the entire body surface area and complete or nearly complete depigmentation can be noted.

More important is to differentiate between active and stable vitiligo for the initiation of appropriate therapy. Active vitiligo usually requires medical therapy. Surgical therapy is indicated when medical therapy fails and could actually be considered as the first therapeutic option for the treatment of stable vitiligo.\textsuperscript{15} Active vitiligo is characterized by increase in size of old lesions, development of newer lesions, and appearance of white macules after trauma (Koebnerisation).

In patients affected by segmental vitiligo, the causative factor(s) usually disappears, leaving well-defined depigmented lesions. Even generalized vitiligo can enter long phases of clinical remission in which the size and number of lesions are stationary for several years and the Koebner phenomenon is absent. This stage of the disease is referred to as stable vitiligo.\textsuperscript{16}

Sometimes vitiligo is a slowly spreading disease or is limited to a specific anatomic region, and on other occasions it becomes aggressive dermatosis developing in a relatively short period of time. Fortunately, most patients have a slow and prolonged course over several years, but progression is the rule, especially with vitiligo vulgaris.\textsuperscript{17}

Various hypotheses, not mutually exclusive, have been proposed for pathogenesis of vitiligo. Of these, the most accepted theories include genetic, autoimmune, neurogenic, and the melanocyte self-destruction hypothesis.\textsuperscript{18} So far, no convincing model describing the interplay of these contributing factors has been formulated. A multi-factorial etiology has been proposed based on existing research.

ETIOLOGY

Though vitiligo is an ancient disease, the exact etiology still eludes us. There appears to be a combination of genetic predisposition and a number of potentially precipitating factors.

Heritability

Vitiligo is a heritable condition, upto 30\% of the patients have a positive family history and 20\% have an affected first degree relative. The pattern of inheritance points to a polygenic
trait with the involvement of 3 or more diallelic alleles.\textsuperscript{19} HLA studies have shown an aggregation of HLA-DR4 in blacks and HLA-B13 in Moroccan Jews.\textsuperscript{9} HLA-B12 has also been shown to be associated with vitiligo.\textsuperscript{18} Recently HLA-A2 has been correlated with vitiligo.\textsuperscript{20} It has been suggested that the genetic background of these patients may render them more susceptible to melanocyte damage and hence to vitiligo.

Precipitating factors

Patients frequently attribute the onset of vitiligo to a specific life event such as an accident, crisis, physical or emotional stress. It may follow a cut or abrasion due to Koebner phenomenon.

PATHOGENESIS

The importance of the disease makes the understanding of the pathophysiological, biological and molecular events leading to melanocyte death or dysfunction, crucial to the outcome. Multiple theories have been postulated to explain the appearance of vitiligo patches. The proposed causative factors are not mutually exclusive.

Autoimmune hypothesis

It is the most popular hypothesis. Vitiligo is considered as an autoimmune disease due to the following features: presence of auto-antibodies directed against melanocytes and related structures in patient’s sera,\textsuperscript{21} the associations of vitiligo with other autoimmune conditions, the detection of organ specific antibodies in patient’s sera, the detection of auto-antibodies in first degree relatives of subjects with vitiligo and the association of the disease with HLA – DR4, HLA-DR1.

In vitiligo, there is production of auto antibodies against melanocyte antigens. These anti melanocyte antibodies have different target antigens on the surface of melanocytes. The titer of these antibodies correlates with the activity and extent of the disease.\textsuperscript{22} Recently anti-tyrosinase antibodies were also detected. Baharav et al\textsuperscript{22} demonstrated these antibodies to be more in extensive widespread vitiligo than in localized vitiligo. Whether these antibodies represent a primary event or are secondary to the release of antigens from previously damaged melanocytes is not yet known. In a recent study conducted in Mumbai, antibodies were detected against tyrosinase, tyrosine hydroxylase, thyroid peroxidase, thyroglobulin and keratinocytes at frequencies of 11\%, 22\%, 18\%, 24\% and 27\% respectively. Overall, antibodies were more common in patients with nonsegmental vitiligo (50–67\%) than in those with segmental disease (0–17\%), and were detected more frequently in patients with shorter
disease durations (<10 years).\textsuperscript{23} Patients with vitiligo have been reported to have a reduced number of lymphocytes and helper T-cells and an increased number of natural killer cells in the serum whereas in inflammatory vitiligo there is an increase in T – cell infiltration, predominantly CD-8 + T-cells at the periphery of the lesions. A statistically significant decrease in Helper T – cells/Suppressor T-cells ratio was obtained in the study as compared to controls.

**Neural hypothesis**

Proposed by Lerner\textsuperscript{24} about 40 years ago, this theory states that there is liberation of a neurochemical mediator that is toxic to the melanocytes from nearby nerve endings. Support for this hypothesis comes from a number of observations namely: vitiligo in neurologically compromised skin, vitiligo sparing paralyzed limbs, onset of vitiligo following peripheral nerve injury and vitiligo limited to a single dermatome, though strictly not following a particular nerve course.\textsuperscript{25} The neural hypothesis is based on the presence of segmental vitiligo. An ultrastructural study of normal dermal nerves was performed recently. Subtle ultrastructural differences were observed between biopsies taken from marginal and central parts of vitiliginous skin and non vitiliginous skin. The most consistent feature seen in all the biopsies from vitiliginous skin was an increase in thickness of basement membrane of Schwann cells. This change was seen in approximately ¾ of dermal nerves in vitiligo biopsies and ¼ of dermal nerves of normal control biopsies. About half of the dermal nerves showed minor axonal damage, whereas indicators of regeneration predominated in others. In addition, relation between the nervous stem cell and epidermal melanocyte has recently been provided.\textsuperscript{26} Abnormalities reflecting possible nerve mediated aberrations in beta-endorphins and met-encephalin secretion in vitiligo patients and increased immunoreactivity to neuropeptide Y and vasoactive intestinal polypeptide in vitiligo skin have been reported. Although little is known about the effects of neuropeptides on human melanocytes, the nervous system may exert a tonic effect on melanocytes in normal or diseased human skin, especially through calcitonin related peptide secretion\textsuperscript{26}, yet the role of the nervous system in the pathogenesis is yet to be elucidated.

Biochemical support for this hypothesis arises from the observation that acetylcholine has been shown to have an inhibitory effect on DOPA oxidase activity in marginal melanocytes in vitiligo and acetylcholinesterase activity has been shown to be absent in depigmenting skin and present in repigmenting skin.\textsuperscript{14}
Self destruct hypothesis\textsuperscript{27} or autocytotoxic hypothesis
This postulates that an intermediate metabolite of melanin synthesis, particularly quinine is toxic to melanocytes. Melanin repigmentation is produced in the melanocytes through the tyrosinase activity. Tyrosinase gene family consists of tyrosinase enzyme, TRP-1&2, Calnexin and LAMP-1. It has been postulated that mutation in TRP-1 protein is involved in cell degeneration or death, associated with faulty scavenging of intermediates of melanin pigmentation leading to apoptosis of melanocytes.

Compartmentalization of melanosomes normally protects melanocytes from destruction by such substances. However, it is thought that leaky melanosomes or high quinine : melanin ratio could damage the pigment cell. The free radical scavenging function of melanin may be insufficient to prevent damage by highly toxic quinine.\textsuperscript{19}

Biochemical theory
It has also been shown that both lesional and non-lesional epidermis has decreased catalase activity, that leads to an increase in peroxidase concentration in it. Hydrogen peroxide functions as a reversible inhibitor of human tyrosinase.

Role of Liver X receptor (LXR) expression in vitiligo
LXR regulate a variety of cellular functions, they have robust anti-inflammatory activity in skin, but they also modulate epidermal proliferation, carcinogenesis, differentiation and permeability barrier function. Kumar et al\textsuperscript{28} demonstrated in their study that expression of LXR-\(\alpha/\beta\) at both mRNA and protein level was significantly higher in perilesional skin as compared to the normal skin of vitiligo patient.

The new hypotheses:
The melanocyte growth factor deficient theory – Defective growth and passage capacity of melanocytes derived from uninvolved and perilesional skin could be due to decreased concentration of melanocyte growth factors in vitro.\textsuperscript{29}

Decreased melanocyte lifespan hypothesis – Several cytokines, such as interleukin-1 and interferon-gamma, mainly produced and released by keratinocytes, may induce apoptosis of melanocytes due to deficiency in survival signals by interfering with the melanocyte membrane tyrosine kinase receptor, C-KIT.\textsuperscript{30} Reduced levels of C-KIT receptors in vitiligo melanocytes or of growth factors could induce premature apoptosis and decreased melanocyte survival.
New Integrated Theory of Non Segmental Vitiligo (NSV): - A Melanocytorrhagic disorder

The new integrated theory takes into account melanocyte detachment and transepidermal elimination, neural-biochemical and autoimmune hypotheses. This new theory proposes that NSV is a primary melanocytorrhagic disorder with altered melanocyte responses to friction and possibly other types of stress, inducing their indolent detachment and subsequent transepidermal loss. Further it was shown that melanocytorrhagy and apoptosis is seen only in patches of unstable vitiligo.

1. Melanocyte defective adhesion: - The melanocyte adhesion system is less well organized and far weaker than the system which firmly holds epidermal keratinocytes bound to each other and to the basement membrane. No melanocyte – keratinocyte adhesion structures can be detected by electron microscopy.

2. Loss of dendricity: - Dendrites are critically important for melanosome transfer, because one melanocyte contacts several keratinocytes in the epidermis through dendritic cell processes. Moreover, ultrastructural observations suggest that dendrites, independently of structural junctions, may dramatically increase the adhesion and anchoring of melanocytes within the basal layer of the epidermis. It has been suggested that the loss of dendricity induced either by oxyradicals (impaired redox status hypothesis) or by increased release of catecholamines (neural biochemical hypothesis) exaggerates transepidermal loss induced by minor mechanical trauma. This loss of dendricity could also affect melanosome transfer and contribute to depigmentation.

3. Weakening of melanocyte attachment and melanocyte detachment after friction: - Ultrastructural abnormalities of the basement membrane have been observed frequently in vitiligo, namely multiple replication or layering of the basement membrane directly beneath melanocytes and focal gaps in the basement membrane. As a result of the weakening of their basal anchoring, melanocytes could be detached by mechanical or chemical injury. Human skin is repeatedly exposed to mechanical stimuli usually grouped under the term friction. During friction, an alternation of stretching and relaxation sometimes results in epidermal disruption, degeneration of keratinocytes and widening of intercellular spaces. Extracellular granular material deposits are found ultrastructurally in NSV skin after severe frictional injury of normal skin. Altered synthesis of extracellular matrix components (such as tenascin) may be produced by damaged keratinocytes.
4. **Transepidermal elimination of melanocytes**: After their detachment, melanocytes are seen in a mid-spinous location as early as 8 hours after friction. Twenty-four hours later, some melanocytes reach the stratum corneum.

The ultimate consequence of all the pathogenic mechanisms described above is melanocyte destruction, and therefore the final outcome is absence of pigmentation. Initially only epidermal melanocytes are affected, but as the condition progresses, the most important pigment cell reservoir, the hair follicle, may also become involved and leucotrichia develops, thus making repigmentation difficult. How and to what extent this phenomenon occurs is dependent on the individual response of the affected patient and the aggressiveness of the pathogenic process.

**HISTOPATHOLOGY**

There is a marked absence of melanocytes and melanin in the epidermis. Histochemical studies show a lack of dopapositive melanocytes in the basal layer of the epidermis. Recent immunohistochemical studies with a large panel of antibodies show only an occasional melanocyte in lesional skin.\(^{33}\) Electron microscopy studies confirm the loss of melanocytes, which appear to be replaced by Langerhans’ cells. Areas around the margins of vitiligo show abnormalities of keratinocytes as well as degenerating melanocytes. In inflammatory vitiligo, where there is a raised erythematous border, there is an infiltrate of lymphocytes and histiocytes. This infiltrate is also found in the marginal areas of some biopsies.\(^{34}\)

**Mechanisms of repigmentation in vitiligo**

Some vitiligo patients show spontaneous repigmentation even though all have a permanent melanocyte loss. Spontaneous repigmentation of the vitiligo patches is a regular feature when exposed to sun. After therapy, repigmentation can occur in four ways: follicular, marginal, diffuse and combined. Repigmentation usually occurs in the follicular pattern, suggesting that follicular melanocytes colonize vitiliginous skin. In most patients of repigmenting vitiligo, studies also argue for a proliferation of melanocytes, followed by their migration; however less commonly, repigmentation might occur from residual intraepidermal melanocytes. Based on follicular repigmentation, the existence of a melanocyte reservoir has been postulated. The existence of a population of intraepithelial cells that have immunopathological characteristics of mature melanocytes within the upper epidermal region has been shown.\(^{35}\) These KIT (+), BCL-2(+), TRP-1(-) cells may contribute to the precursor melanocyte reservoir of human skin.
During repigmentation, melanocytes migrate from the outer root sheath of the hair follicle to the basal layer of the epidermis just above the basement membrane. Because keratinocytes are attached to each other by desmosomes and to the basement membrane by hemidesmosomes, migration of melanocytes involves several complex processes that are not yet understood.

**Treatment options**

A number of therapeutic options for vitiligo are available but there is still a need for a treatment that is promptly effective. There is no curative treatment for this condition. Management of vitiligo is a real challenge for a dermatologist.

**Medical therapies:**

Corticosteroids (Topical, intralesional and systemic)\(^{36}\), Oral mini pulse\(^{37}\), PUVA (topical and systemic)\(^{38}\), NBUVB\(^{39}\), calcipotriol\(^{40}\) and tacrolimus\(^{41}\) are used most widely. Some of the less commonly used medical modalities include phenylalanine\(^{41}\), khellin\(^{41}\), topical minoxidil\(^{42}\), levamisole\(^{43}\) and melagenina.\(^{44}\) Recently oral minocycline was shown to be effective in treating vitiligo.\(^{45}\)

Most of these therapies aim to restore melanocyte function by their anti-inflammatory or immunomodulatory action and by preventing melanocyte auto destruction so that normal skin appearance and function is restored.

**Surgical therapies:**

All patients with vitiligo should be initially treated with medical methods. Surgical methods are important solutions for stable vitiligo refractory to medical treatment. High repigmentation rates are obtained with all procedures so far described in most anatomic locations, but they are of little help for acral areas and bony prominences. Unilateral vitiligo is the clinical form with the best response to grafting and transplant methods, and a good proportion of patients with stable bilateral disease also respond adequately. Nevertheless, appropriate patient selection is important to achieve maximal results.\(^{14}\) However none of the surgical modalities developed so far is uniformly effective in all patients and body sites and there is need for constant research and innovations for better surgical therapeutic options for vitiligo.

**Aims of various surgical procedures:**\(^{46}\)
A) Camouflage Tattooing: Introduction of artificial pigments into the lesions for permanent camouflage.

B) Excision: Removal of the depigmented areas, e.g. excision with primary closure and covering with thin Thiersch's graft.

C) Melanocyte transplantation: Commonly used methods of autologous transplant of melanocytes are

- Tissue grafts:
  1. Thin and ultra-thin split thickness grafts (STSG)
  2. Suction blister epidermal grafts (SBEG)
  3. Mini punch grafts (MPG)
  4. Hair follicular grafts (HFG)

- Cellular grafts:
  5. Noncultured epidermal cell suspension (NCES)
  6. Cultured “pure” melanocytes (CM)
  7. Cultured epithelial grafts (CE)
  8. Autologous noncultured extracted hair follicle outer root sheath cell suspension also called follicular cell suspension (FCS)

D) Therapeutically wounding the lesion to stimulate the melanocytes from the periphery and the black hair follicles to proliferate, migrate and re-pigment the lesion, e.g. therapeutic dermabrasion, laser ablation, cryosurgery (liquid nitrogen spraying), needling and local application of phenol or trichloroacetic acid.

Every method has its own advantages and disadvantages. As there are no specific data available from the prospective studies in this field, it is not easy to recommend which surgical approach to vitiligo offers the best result.

Several points need to be assessed in patients when surgical treatment is planned.

Stable Disease:

The most important factors indicating stability are

1. No progression of lesions for at least 1 year.
2. Spontaneous repigmentation.
3. A positive minigrafting test disclosing repigmentation around four to five minigrafts (1.0 or 1.2 mm), implanted 3 to 4 mm apart within an achromic lesion, is an indication
of future recovery by surgery. So far, this test is the most accurate evidence of vitiligo stability.

(4) Absence of new koebner phenomenon (KP), including the donor site for the minigrafting test.\textsuperscript{14}

(5) Unilateral vitiligo is almost a synonym of stable disease with an excellent repigmentation response.

However, these criteria may be challenged by clinical observations in which KP and minigraft testing are discordant. Data obtained from minigraft testing in case series suggest that the minigraft test provides a reflection of the stability of defined individual lesions, which does not necessarily reflect global stability of the disease.\textsuperscript{49}

‘Vitiligo global issues consensus conference, 2011’ convened by Vitiligo European Task Force (VETF), concluded that assessment of ‘overall’ stability is inaccurate and unreliable, whereas individual lesion stability is more reliable, especially when used in the context of surgical intervention.\textsuperscript{50}

**Methods and Size of Lesions:**

Depending on the size of the treated area, the method may vary. Simple methods such as minigrafting and suction epidermal grafting are useful for small or medium sized lesions. On the contrary, for extensive depigmented defects, cellular transplants may be required.

**Age:**

Because of the invasive nature of surgical procedures, they are not recommended in children; nevertheless, highly motivated preadolescents can be treated under sedation or general anesthesia. Also, it is not surprising to see patients beyond the age of 50 years who may be interested in surgical repigmentation.

**Psychological Aspects:**

Some patients with high emotional trauma because of depigmentation may seek advice for invasive procedures. However, a psychological evaluation may be needed to ascertain the real need for surgical treatment.

**Photographic Record:**

*Illustrations are recommended to help in determining the percentage of improvement, quality of repigmentation and possible side effects.*
**Patient's Expectations:**

Repigmentation is not always comparable with normally pigmented skin and the final results vary considerably from patient to patient. However, most individuals are pleased with the achieved results; minor imperfections are far less important than the noticeable repigmentation of vitiliginous skin, mainly in ethnic skin patients with a dark complexion; sometimes surgical repigmentation may look even better than what is observed in many patients after medical therapy.

**Method and Donor Site:**

Appropriate training with a specific method is an important prerequisite for surgical therapy. Donor site should be as hidden as possible and the gluteal region may be suitable for this purpose in most patients.

**Serial Procedures:**

Most procedures require more than one intervention and several sessions may be needed to accomplish full recovery or to complete repigmentation of minor depigmented defects. Combination methods may be of value for this purpose.

**Difficult Areas for Surgical Treatment:**

With surgical procedures much improvement is achieved, particularly in unilateral vitiligo, but certain areas are difficult to repigment, such as joints, lips, eyelids, genitalia, cutaneous folds, the dorsum of hands and feet, especially fingers and toes. In some of these areas, inadequate immobilization prevents a good take of grafts and repigmentation is difficult to achieve; some of these areas may need regrafting, and recovery is possible in some patients. Nevertheless, other factors not known at present may prevent a good repigmentation response.

**Success rates of different surgical options:**

Among all procedures, suction blister epidermal grafts and thin and ultra-thin split-thickness grafts seem to be the most effective procedures, with overall success rates of 80.3% (CI 76.4–84.2%) and 77.9% (CI 72.2–83.6%) respectively. But, a recent randomized study directly comparing NCES and SBEG showed NCES is significantly better than SBEG. Among cellular grafts, all techniques seem to be equally effective with success rates of 61.1% (CI 56.1–66.1%), 63.6% (CI 57.2–70%), and 63.6% (CI 55.8–70.6%) for noncultured epidermal cell suspension, cultured melanocytes and cultured epidermis respectively. The mean repigmentation with noncultured extracted outer root sheath cell suspension is about 65.7%. 
Cases with more extensive vitiligo vulgaris, involving greater than 30% body surface area, are generally considered unsuitable for transplantation procedures as chances of retention of the pigment are less. Extensive areas may be best treated with cellular grafts. Theoretically, culture methods would provide an unlimited number of cells/tissue for transplantation, while NCES would provide up to 8–10 times donor-to-recipient expansion. Therefore, it seems that larger areas may be treated with cellular grafts and thin and ultra-thin split-thickness grafts and moderate areas may be treated with cellular grafts and minigrafts. Smaller areas may be easily treated with suction blister epidermal grafts which gives good aesthetic results and is technically less challenging. Overall, better results are reported in focal and segmental vitiligo (75%–95%) than in generalized vitiligo. Young, dark complexioned patients have better results. Comparatively, acral areas, malleoli, knees, and elbows are less responsive to surgery. Smaller patches respond better. Addition of PUVA/PUVASOL therapy enhances repigmentation and increases the repigmentation rate (90-95%).

**Adverse events:**

No serious adverse events have been reported with any of the transplantation methods. Cellular grafts appear to have the least frequency of adverse events. Adverse events reported at recipient sites are infection, milia, scarring, rejection of the graft. Complication at donor sites reported are infection, milia, scarring and pigmenitary changes. Commonly recipient site is prone to secondary bacterial infections if asepsis is not followed. This can be minimized by following strict asepsis in procedure. If dermabrasion or skin harvesting is too deep it will lead to milia (keratin filled cysts), scarring and pigmenitary changes. Rejection of graft (failure to repigment after surgery) is seen if surgery is done on unstable vitiligo patches or resistant sites. Cultured melanocytes and NCES have a mean of 0.0 and 0.08 adverse events at recipient site respectively, and 0.01 and 0.009 at the donor site respectively. Tissue grafts are reported to be associated with more adverse effects and the maximum number of adverse events on the recipient site is seen with MPG (0.7) and STSG (0.5).

**Response:**

The treated area appears bright pink immediately after removal of the dressing. The earliest pigmentation was noticed 3 weeks post surgery. Many patients showed hyperpigmentation which gradually blended with the surrounding skin over 6–8 months. The donor area healed rapidly and soon became indistinguishable from the surrounding skin. Occasionally, the donor area healed with hyperpigmentation.
NONCULTURED EPIDERMAL CELL SUSPENSION

The technique of noncultured epidermal suspension was pioneered by Gauthier et al.\textsuperscript{52} The suspension was prepared by incubating the donor skin obtained from the scalp in trypsin 0.25\% for 18 hrs. The suspension was injected into blisters raised by cryo-therapy. Eight out of the 12 patients treated had > 70\% repigmentation at the vitiligo site. It was proposed that the presence of keratinocytes in the suspension supplies essential growth factors for melanocyte growth. They stated that this technique could emerge as simple and effective alternative to the costly cultured melanocyte transplantation technique.\textsuperscript{52}

Olsson and Juhlin\textsuperscript{53} first used the M2 medium for suspension of the noncultured epidermal cells. A total of 20 vitiligo patients were included and results showed 100\% repigmentation in all 3 patients with segmental vitiligo and 80 \% repigmentation in 12 patients of stable generalized vitiligo. The research group found the transplantation as effective as transplantation of cultured melanocytes. However, several practical problems surfaced with the procedure. Usage of cryo-therapy to raise blisters damaged the melanocytes resulting in hypopigmentation. Significant run-off of suspension from recipient site was associated with the high fluidity of suspension. Blisters were difficult to raise at the bony prominences by the use of cryo-therapy.

Van Geel et al\textsuperscript{54} added hyaluronic acid to the cellular suspension to improve the viscosity and fixation, CO\textsubscript{2} laser was used to obtain a depth-controlled and precise dermabrasion at the recipient site and adjuvant PUVA or UVB therapy was added 3 weeks after grafting to stimulate and homogenize the repigmentation. First, a pilot study was conducted in 4 patients all of whom achieved 80 \% pigmentation. The therapeutic value of this procedure was increased with the further evaluation in 28 patients in a double blind placebo controlled study. 70\% or more repigmentation was observed in 55\% of the patients.

It is concluded that the transplantation of noncultured epidermal cell suspension is an efficacious and safe procedure. The technique requires special laboratory equipment. However, in comparison to cultured melanocytes, this is an inexpensive and simple OPD procedure requiring 4-6 hrs. Large areas, 8-10 fold the size of donor skin, can be treated with this procedure. A temporary color mismatch is observed in all patients, which improved over 5-6 months. No scarring is observed at the donor or recipient site. They stressed on proper aseptic precautions and the use of prophylactic antibiotics to prevent postoperative infections. The use of post-operative UVB therapy helped to achieve uniform pigmentation. In one
patient it was observed that leucotrichia adjacent to depigmented eyebrow was also repigmented supporting retrograde migration of pigment cells.\textsuperscript{54, 55}

Transplantation of noncultured melanocytes is the method that results in least hyperpigmentation. This is because the cells are diluted and are transplanted in smaller numbers than in other methods. Halo phenomenon that noted in transplantation of cultured melanocytes is noted even in grafting of noncultured epidermal cell suspension. The technique shows excellent results in segmental vitiligo, focal stable vitiligo and piebaldism. Age and gender seem to have no significant effect on repigmentation. Acral and periorificial vitiligo has the poorest response.\textsuperscript{56}

Mulekar et al\textsuperscript{57} conducted an extensive study recruiting 49 patients with segmental vitiligo and 15 with focal vitiligo. They used Hams F-12 medium for suspension. 95-100\% repigmentation was observed in 41 patients of segmental vitiligo and 11 patients of focal vitiligo. The percentage of patients with segmental vitiligo showing an excellent response was 84\%, while 6\% of the patients had a good response to treatment. 10\% of patients failed to produce any pigmentation. In focal vitiligo 73\% showed an excellent response, while 20\% had poor repigmentation at the end of the respective follow-up period. Response to the treatment on the lips was not encouraging. Patients with both focal and segmental vitiligo had retained the pigment at the end of respective follow-up periods (5 yrs). Repigmentation failed to be produced in 10\% of patients in the segmental vitiligo group and 20\% in the focal vitiligo group.

Pandya et al\textsuperscript{58} abraded the recipient area with a high speed motor dermabrader and the denuded area was covered with saline moistened gauze piece. The suspension was poured evenly from the pipette and covered with a collagen dressing. This is covered with a small gauze piece moistened with MK medium. The dressing was kept in place by a Tegaderm dressing.

In Swedish procedure of melanocyte transplantation pioneered by Olsson and Juhlin,\textsuperscript{59} they used phosphate buffered saline (PBS) to wash the denuded area and they put PBS moistened gauze over denuded area.

**NONCULTURED EXTRACTED HAIR FOLLICLE OUTER ROOT SHEATH CELL SUSPENSION (FCS)**

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. This reservoir of melanocytes and melanocyte stem cells are important in the treatment of vitiligo as the initial repigmentation in vitiligo patches often occurs around the hair follicles 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. These include basal cells high in α6-integrin/keratin 14 (K14) expression, suprabasal cells low in α6-integrin/K14 expression, hair germ cells expressing Lgr5, P-cadherin and S100A4, bulge cells expressing CD34 and CD200 and a more distal population expressing MTS24. 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.

Vanscheidt et al, in a small case series 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 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. 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. 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.

The dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes such as trypan blue, eosin or propidium whereas dead cells do not. Mohanty et al successfully used trypan blue dye exclusion method to show viability of melanocytes in their study.

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. 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. It can be used to cover large depigmented areas.
In an open labeled pilot study Mohanty et al performed FCS in fourteen patients of vitiligo and achieved >75% pigmentation in 9 patients. The procedure involved removal of only 15-25 follicular units, which provide 25000 to 50000 cells sufficient to treat up to 25 cm². From the experience gained from NCES, the authors recommended the desired number of cells for repigmentation as 2000 cells/cm². There are no control studies that have determined the precise number of melanocyte concentration required for achieving pigmentation. In follicular melanin unit, there is one melanocyte for every five keratinocytes in the hair bulb, 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 melanocytes are larger, more dendritic, produce larger melanosomes and with more extensive golgi and rough endoplasmic reticulum. Hair melanocytes have remarkable synthetic capacity so that a relatively small number of melanocytes can potentially produce sufficient melanin to pigment up to 1.5 m of hair shaft. 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. Because of the above mentioned factors FCS might require lesser melanocyte concentration compared to epidermal cell suspension.
Justification for the proposed study

FCS + NCES – added benefit of two successful methods

Same patient – no confounding factor

Similar anatomical area – no question of koebnerisation or other local factors

Both FCS and NCES are proved to be efficacious for vitiligo surgery in terms of extent of pigmentation and colour matching. Recent randomized study comparing effects of FCS and NCES in two different groups of patients showed result more favouring to NCES. Here, we are doing NCES in one group of vitilgo patches and FCS + NCES in other group of patches in the same patient. So we are treating the vitiligo patches with the modality with proven efficacy in one area, and in other area we are combining two modalities which proved their efficacy while used exclusively.

Theoretically one will expect more repigmentation with FCS, due to the presence of various melanocyte stem cells, better melanocyte – keratinocyte ratio and morphological properties of melanocytes in hair follicle, compared to NCES. But, in various studies there is no statistically significant superior result with FCS, and there are even studies showing inferior results with FCS than NCES. It was proposed that the presence of keratinocytes in the suspension supplies essential growth factors for melanocyte growth. Melanocytic homeostasis is modulated via a complex network of autocrine and paracrine factors. Melanocyte proliferation, melanogenesis, migration, dendricity, and differentiation are influenced by keratinocytes and fibroblasts, as well as the melanocyte-derived growth factors and cytokines. Keratinocyte derived factors that help in melanogenesis include Endothelin-1 (ET-1), Stem cell factor (SCF), also known as steel factor, Basic fibroblast growth factor (bFGF), Nerve growth factor (NGF) etc. So there may be keratinocyte growth factors lacking if we use FCS alone, that is contributing to inferior outcome, which can be overcome by combining NCES to FCS.

Also, there are studies showing keratinocyte damage in vitiligo. Bhawan J et al performed light and electron-microscopic studies on the amelanotic and adjacent normal-appearing skin in patients with vitiligo. The amelanotic skin revealed complete loss of pigment and absence of melanocytes. In addition to severe degenerative changes of melanocytes, varying degree of damage was also seen in the keratinocytes of normal-appearing skin adjacent to amelanotic skin. Kumar R et al proposed that oxidative stress may also manifest as the ultrastructural and functional changes observed in keratinocytes.
extracted from the perilesional and normal skin of NSV patients. There are notable morphological alterations like swollen mitochondria with disrupted cristae, a pathognomonic feature of apoptosis in keratinocytes. So, by combining NCES to FCS, we are replenishing healthy keratinocytes to the vitiligo patches.

The drawback of many efficacy studies comparing various modalities in vitiligo surgery is, they used different patient population for comparing. Every individual is different. Especially in the case of vitiligo, there may be alteration in outcome based on patient characteristics such as type of vitiligo, immunological profile, disease activity, family history, adherence to the medical advice etc. Moreover, there may be difference in results due to the quality and concentration of suspension used, care taken during surgery; if different modalities of vitiligo surgery are done on different time. Here, we are performing both methods of surgery on the same patient and at the same sitting, so that avoiding above mentioned confounding factors.

The success of vitiligo surgery also depends on the anatomical area of vitiligo patch. Acral areas, joints, lips show poor response to any therapeutic method. In the literature, various anatomical areas are selected randomly for comparing different surgical methods. We selected vitiligo patches which are bilaterally symmetrical or different patches on the similar anatomical sites for the comparison of outcome. This overcomes the issue of koebnerisation, lack of quiescence after surgery at areas like joints and the innate resistance of acral areas to vitiligo surgery.
AIMS AND OBJECTIVES

AIM: To compare the effect of transplantation of autologous noncultured extracted hair follicle outer root sheath cell suspension and epidermal cell suspension in combination v/s transplantation of autologous noncultured epidermal cell suspension alone in stable vitiligo in the same patient using primary and secondary outcome parameters.
MATERIALS AND METHODS

Subjects will be recruited from the patients attending Pigmentary and Dermatosurgery Clinic of Department of Dermatology, Venereology and Leprology; Postgraduate Institute of Medical Education and Research, Chandigarh, India. A total of 30 subjects of stable vitiligo (lesional stability defined as individual lesions not increasing in size for the last 1 year), who are satisfying inclusion/exclusion criteria would be recruited. In one group of vitiligo patches, (say, right side lesions if bilaterally symmetrical vitiligo or proximal/medial lesions if more than one vitiligo patch in same anatomical region), FCS + NCES will be done and in other group of vitiligo patches (say, left side lesions if bilaterally symmetrical vitiligo or distal/lateral lesions if more than one vitiligo patch in same anatomical region) NCES alone will be done. This will be exercised using random number table. Follow ups will be done at day 8, week 4, week 8 and week 16. Patient assessment will be done by digital photographs in the same settings with respect to patient positioning, background, lighting and camera settings; and questionnaire to know extent of repigmentation and compare the efficacy of both methods using primary and secondary outcome parameters.

**Primary outcome:**
- Extent of repigmentation.

**Secondary outcomes:**
- Pattern of repigmentation.
- Extent of repigmentation and type of vitiligo
- Extent of repigmentation and site of lesions
- Color matching of repigmented area.
- Patient satisfaction (patient global assessment).
- Adverse events if any.

**INCLUSION CRITERIA:**

1. Subjects with clinical diagnosis of focal, segmental or generalized vitiligo which has been stable for more than 1 year.
2. Vitiligo patches should be in same anatomical region bilaterally or two or more patches in the same anatomical region separated by a stretch of normal skin (at least 1cm)
EXCLUSION CRITERIA:

1) Age less than 10 years
2) Pregnancy
3) Patient with actively spreading vitiligo
4) Appearance of new lesions
5) History of Koebnerization
6) History of hypertrophic scars or keloidal tendency
7) Bleeding disorders
8) Patients with unrealistic expectation

At the first visit, a pro forma is filled noting the baseline characteristics, history and examination findings. Informed consent is taken for the procedure.

**Difficult and Simple types of vitiligo**

Repigmentation in generalised vitiligo (VV) and acrofacial vitiligo (AFV) is variable and often more disappointing while results in segmental (SV) and focal vitiligo (FV) is in general consistently high. So we planned to assess the extent of repigmentation separately for difficult type of vitiligo defined as VV and AFV and simple type of vitiligo defined as SV and FV.

**Subcategorisation based on sites of surgery**

Acral lesions as well as bony areas, joints and eyelids are inherently resistant to any method of surgery while face, trunk and proximal limbs show good response. A separate analysis of extent of repigmentation will be carried out for the lesions on resistant sites defined as fingers, hands, feet, joints, bony areas and eye lids; also for those on easy sites defined as face, trunk, arms and legs.

**TECHNIQUE OF TRANSPLANTATION:**

**Noncultured Epidermal Cell Suspension Method**

Harvesting the graft:
1. About one-tenth the size of recipient area will be selected as the donor site, usually on non-cosmetically important site like the thighs.

2. Donor area will be shaved, cleaned with betadine and surgical spirit and anaesthetized with mixture of 2% lignocaine and normal saline, NS (1:1).

3. Split thickness skin graft will then be taken with the help of a shaving blade held firmly by a straight artery forceps.

4. Haemostasis is established and the area will be dressed with Bactigras gauze.

5. Suitable antibiotic and analgesic will be prescribed.

**Preparing noncultured epidermal cell suspension:**

1. Split thickness skin specimen will be transferred under aseptic conditions to a container with NS and transferred to laboratory. There, the skin graft will be transferred to Trypsin-EDTA solution (0.25% trypsin and 0.02% EDTA) in a Petri dish and incubated at 37°C in 5% CO₂ for one hour to separate the epidermis from the dermis.

2. Afterwards, the Trypsin-EDTA solution will be removed and PBS will be added and pipetted well so as to separate the cells from the tissue.

3. The solid waste of tissue will be removed and the suspension will be centrifuged at 1000 rpm for 5 minutes.

4. The supernatant will then be discarded and the pellet, containing cells from the stratum basale and lower half of the stratum spinosum that are rich in melanocytes will be taken.

5. The melanocytes will be stained with trypan blue and counted simultaneously with Neubauer's chamber under the light microscope. This will help to identify whether the melanocytes are viable as the dead cells would pick up the blue stain.

6. Phosphate buffer saline is added to make suspension of noncultured epidermal cells.

**Transplantation procedure:**

1. The recipient site will be shaved, cleaned with betadine and surgical spirit and anaesthetized with mixture of 2% lignocaine and NS (1:1).

2. Dermabrasion will be done until tiny pinpoint bleeding spots are seen which imply that the dermo-epidermal junction has been reached. Dermabrasion will be extended 5mm beyond margins to prevent halo phenomenon.

3. The denuded area will be washed with PBS and covered with a PBS moistened gauze piece.
4. The noncultured epidermal cell suspension will be carefully transferred to a tuberculin syringe.

5. With 18g needle attached to this syringe, few small drops of suspension will be placed over the denuded surface which will be then spread evenly with the help of needle.

6. This will be covered with sterile Vaseline gauze or Bactigras after washing with NS. Once again small drops of suspension will be placed over this gauze and spread evenly.

7. After washing with NS a meshed collagen sheet (Kollagen M) will be put over the gauze with suspension.

8. This will be then covered by a small gauze piece moistened with PBS.

9. Tegaderm will be placed over this so that an artificial blister will be formed which holds melanocytes with PBS over the recipient site. At difficult areas like lips surgical glue will be used to put Tegaderm in place.

10. Over this, surgical pad is put and the dressing will be stabilized by placing the elastic plaster (Dynaplast).

   The patient will be observed for 1 hour after procedure and then allowed to go home. The dressing will be removed at the first follow-up visit after 5-7 days in the hospital.

**Noncultured Extracted Hair Follicle Outer Root Sheath Cell Suspension and Noncultured Epidermal Cell Suspension Combination**

Anagen hairs are extracted from occipital area of scalp. 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. Follicular unit extraction method (FUE) is used for hair follicle tissue harvest. Samples are sent to the laboratory for processing within 15-20 min of harvesting of the tissue.

**Follicular unit extraction (FUE):**

1. Hairs are trimmed to a length of approximately 2 mm.

2. Field block anaesthesia is given with 2% lignocaine, which is infiltrated in the skin encircling the area chosen for FUE.

3. To obtain follicular units, 1-mm punch is rotated till mid-dermis in the direction of hair follicle. Care is taken not to go up to subcutaneous space to avoid transaction of the hair follicle.
4. Then follicular unit is pulled out gently using hair follicle holding ring forceps by holding the skin surrounding the hair shaft(s).

5. Transacted hair follicles are discarded. Depending on the area to be transplanted, approximately 15-25 pigmented follicles are extracted per subject and collected in NS.

6. The procedure of FUE takes approximately 25-30 minutes.

**Preparation of single cell suspension:**

1. The extracted hair follicles are transported to the laboratory under sterile conditions and washed three times with phosphate buffered saline containing the antibiotics and antimycotics.

2. The follicles are then incubated with 0.25% trypsin-0.05% EDTA at 37 ºC for 90 minutes to prepare the single cell suspension.

3. Cells started loosening up within 15-20 minutes. After every 30 minutes the hair follicles are placed in a new tube of trypsin EDTA and the reaction in the previous tube is terminated by adding the trypsin inhibitor (Sigma-Aldrich).

4. This is done to prevent digestion of separated cells by trypsin. After cell separation only thin keratinous shafts of the hairs are left, which are discarded.

5. The cell suspensions of all the three tubes are added in a single tube and then filtered through a 70μm cell strainer to prepare a single cell suspension. Finally, the cell suspension is centrifuged for 5 minutes at 1000rpm to obtain a cell pellet, which is re-suspended in a small amount of PBS and transported to the operation theatre for transplantation, where NCES is added to FCS and gently mixed to prepare FCS + NCES combination.

6. The whole procedure of preparation of cell suspension took approximately 2-3 hours.

**Transplantation procedure:**

1. The recipient site will be shaved, cleaned with betadine and surgical spirit and anaesthetized with mixture of 2% lignocaine and NS (1:1).

2. Dermabrasion will be done until tiny pinpoint bleeding spots are seen, which imply that the dermo-epidermal junction has been reached. Dermabrasion will be extended 5mm beyond margins to prevent halo phenomenon.

3. The denuded area will be washed with PBS & covered with a PBS moistened gauze piece.

4. The FCS + NCES combination will be carefully transferred to a tuberculin syringe.
5. With 18g needle attached to this syringe, few small drops of suspension will be placed over the denuded surface which will be then spread evenly with the help of needle.
6. This will be covered with sterile Vaseline gauze/Bactigras after washing with NS. Once again small drops of suspension will be placed over this gauze and spread evenly.
7. After washing with NS a meshed collagen sheet (Kollagen M) will be put over the gauze with suspension.
8. This will be then covered with a small gauze piece moistened with PBS.
9. Tegaderm will be placed over this so that an artificial blister will be formed which holds melanocytes with PBS over the recipient site. At difficult areas like lips surgical glue will be used to put Tegaderm in place.
10. Over this, surgical pad is put and the dressing will be stabilized by placing the elastic plaster (Dynaplast).

The patient will be observed for 1 hour after procedure and then allowed to go home. The dressing will be removed at the first follow-up visit after 5-7 days in the hospital.

**FOLLOW UP:**

The patients will be asked to follow up at the clinic on day 8, week 4, week 8 and week 16 after the transplantation procedure and percentage of repigmentation will be assessed by blinded investigator (Dr. Davinder Parsad) subjectively by serial digital photographs in the same settings with respect to patient positioning, background, lighting and camera settings and objectively by serial paper markings. No intermittent treatment will be given during this post-surgery period.

Repigmentation will be assessed as follows:

- **≤25%** Minimal repigmentation
- **26-50%** Mild repigmentation
- **51-75%** Moderate repigmentation
- **76-90%** Marked repigmentation
- **>90%** Excellent repigmentation

Also, the repigmentation pattern will be noted as ‘diffuse’, ‘perifollicular’ or ‘migrating from the borders’. A note will also be made of the colour matching of repigmented skin as ‘somewhat lighter than’, ‘same as’ or ‘somewhat darker than’ normal skin.
At each visit, patient will be assessed for any complications at the donor and recipient sites. Patient will be asked to fill a patient satisfaction questionnaire at week 16. Hence, both objective and subjective evaluation of the results shall be done.
Sample size was estimated based on the previous study, assuming excellent (> 90%) repigmentation in one group as 71% and in the other group as 27%. Our sample size came out to be 31 lesions per group at a power of 95% and confidence interval of 95%. It was decided to include extra subjects for the possible lost to follow up cases. Finally, we included 42 lesions per group for the proposed study.

The statistical analysis will be carried out using Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, version 16.0 for Windows). All quantitative variables will be expressed using measures of central tendency (mean, median) and measures of dispersion (standard deviation). Normality of data will be checked by Kolmogorov Smirnov test. For normally distributed data, means will be compared using student's t-test for outcome. For skewed data or scores Mann–Whitney test will be used. Qualitative or categorical variables will be described as frequencies and proportions. For significance of changes within the group over a period Wilcoxon signed-rank test will be used. Proportions will be compared between groups using Chi square test or Fisher's exact test whichever will be applicable. All statistical tests will be two-sided and performed at a significance level of p < 0.05.
ETHICAL JUSTIFICATION

This planned study is to be undertaken in stable vitiligo patients not responding to medical treatment. Informed consent will be obtained from all patients and they will be explained that surgical treatment is for the existing lesions of vitiligo and new lesions of vitiligo may still appear in future. Patients will not be denied of medical treatment and only those who failed medical treatment will be chosen for surgery. To detect any adverse effect at the earliest, periodic visits of the patient along with active intervention are planned at regular intervals. All necessary steps would be undertaken to ensure safety and convenience to the patients during entire study period. Moreover, the patients who may deny participating would be excluded from study without asking any reason thereof.

Nowadays surgical modalities have become treatment of choice for stable vitiligo not responding to medical treatment. Both autologous noncultured epidermal cell suspension (NCES) and autologous noncultured extracted hair follicle outer root sheath cell suspension (FCS) have been shown to be safe and efficacious in the repigmentation of stable vitiligo patches. So we hypothesise that FCS + NCES will result in better repigmentation in stable patches of vitiligo and can be used in areas that show inferior result to above two methods like acral areas. These surgical modalities are affordable to most of the patients and impart not much financial burden.

By this study our aim is to establish FCS + NCES as a novel method in the treatment of stable vitiligo using NCES as a control. This in the long run will be helpful to the patients in terms of cost-effectiveness and ultimately the outcome. Thus, the cost and risk of undergoing an invasive procedure by the patient can be ethically justified.
BIBLIOGRAPHY


**APPENDIX I**

**CONSENT PROFORMA**

**TRANSPLANTATION OF AUTOLOGOUS NONCULTURED EXTRACTED HAIR FOLLICLE OUTER ROOT SHEATH CELL AND AUTOLOGOUS NONCULTURED EPIDERMAL CELL SUSPENSION IN COMBINATION AS A NOVEL METHOD IN VITILIGO SURGERY**

| Name of the participant: ____________________________________________ |
| Name of the Principal (Co-) Investigator: ______________________________ |
| Name of the Institution: ____________________________________________ |
| Name and address of the sponsoring (funding) agency(ies): ________________ |

I, ________________, age ______ CR. No. ______ exercising my free power of choice, hereby give my consent to be included as a subject in “Transplantation of autologous noncultured extracted hair follicle outer root sheath cell and autologous noncultured epidermal cell suspension in combination as a novel method in vitiligo surgery.”

- I have been explained in a language understandable to me, the nature of the treatment, its expected benefits and possible side effects and I am willing to undergo any necessary investigations.
- I have been informed that for academic and scientific purposes, the white patches will be photographed before and after the study.
- I will allow the use of my photographs for presentation and publication purposes with the understanding that I will never be identified by name.
- I hereby give permission to the investigators to release the information obtained from me, as a result of participation in this study, to the sponsors, regulatory authorities, government agencies, and ethics committee. I understand that they may inspect my original records.
- I am aware that I will have to come to PGIMER, Chandigarh for follow up at least 4 times over a period of 16 weeks (weeks 1, 4, 8 and 16) for the proper conduct of study.
- I am also aware of my right to opt out of the study any time during the course trial without having to give the reason for doing so.
- My signature on this form indicates that I:
  - o Have carefully read and understood the information provided in this form
  - o Have been explained the nature of this study and give my consent for inclusion in the study.

| Name and signature of patient | Name and signature of physician |
| Date | Name and signature of witness |

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Follicular cell suspension and epidermal cell suspension are recently described surgical techniques with promising results for the management of stable vitiligo patches. By combining these two methods, we expect better repigmentation. This new method involves extraction of 15-25 hair follicles from the back of the head and also harvesting superficial skin from thigh under local anaesthesia, then treating them with various reagents to form a combined cell suspension. This cell suspension is rich in pigment forming cells called melanocytes. This cell suspension is transplanted into vitiligo area after abrading superficial areas of the skin under local anaesthesia. Melanocytes in the cell suspension home into the dermabraded area and causes pigmentation in 2-6 months.

If patient is not a case of stable vitiligo, there is chance to get new white patch at skin harvested site and also failure of repigmentation at treated vitiligo site. Hair will regrow from surroundings of extracted site, the back of the head, covering hairless patch within a month. Surgical treatment is for the existing lesions of vitiligo and new lesions of vitiligo may still appear in future.

The surgery and preparation of cell suspension is undertaken in strict aseptic precautions to minimize the chances of infection. Patient need to come to minor operation theatre in OPD on 2 consecutive days for surgery. First day, skin harvesting will be done and it will take only half an hour, then patient can go home. On the second day, hair follicle extraction will be carried out in the morning, then sample will be sent to laboratory; after getting cell suspension after 2 hours, dermabrasion at vitiligo site will be carried out followed by application of cell suspension over it. The surgery site will be dressed neatly, and patient can go home on the same day after surgery. Patient has to come after 5 days to remove dressings and to rule out any surgical site infection.

The result obtained from this study may establish a novel method in vitiligo surgery helping patients suffering from vitiligo.
APPENDIX II
DATA RECORD SHEET

Name: Age/ Sex: CR. No.: 
Occupation: Pt. No
Address:

Chief complaints:

Total duration:

Site of onset:

Progression of disease:

Precipitating factors:

Present status: stable/ unstable

Koebnerisation: present/ absent

History of past illness: H/o similar disease/ autoimmune disorder (pernicious anaemia, hyperthyroidism, hypothyroidism, alopecia areata, autoimmune hemolytic anemia and myasthenia gravis)/ atopy / diabetes/ hypertension/ tuberculosis / photosensitivity / any other disease.

Treatment history:

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Personal history:

Smoking
Alcohol
Addictions

Family history

GENERAL PHYSICAL EXAMINATION:

Pulse: BP: Weight:
Pallor: Edema: Clubbing:
Cyanosis: Icterus: Lymphadenopathy:

SYSTEMIC EXAMINATION:

CVS
RS
P/A

CUTANEOUS EXAMINATION:

% BSA involved:
Areas affected:
Head and neck/ trunk/ upper limb/ lower limb/ hands/ feet/ mucosae
Leukotrichia: Present/Absent
Mucosal involvement: Present/Absent
**INVESTIGATIONS:**

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<td>RBS</td>
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<tr>
<td>Bleeding time</td>
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<tr>
<td>Clotting time</td>
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<tr>
<td>HBsAg</td>
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<tr>
<td>HIV</td>
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</tbody>
</table>

**Donor site:**

**Size of the split thickness graft:**

**Number of hair follicles extracted:**

**Site of treated area:**

a) NCES  

b) FCS + NCES

**Size of treated area:**

a) NCES  

b) FCS + NCES
**CLINICAL EVALUATION:**

1 = NCES  
2 = FCS + NCES

<table>
<thead>
<tr>
<th>Evaluation</th>
<th>Day 8</th>
<th>4 weeks</th>
<th>8 weeks</th>
<th>16 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
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</tbody>
</table>

**Extent of pigmentation**
- <25%
- 26-50%
- 51-75%
- 75 -90%
- >90%

**Color match of grafted area with the normal skin**
- somewhat darker
- somewhat lighter
- same

**Pattern of repigmentation**
- Diffuse
- Perifollicular
- Migrating from the borders

**Complications / side effects**

a. **Recipient site**
- infection
- milia
- scarring
- rejection

b. **Donor site**
- Infection
- Milia
- Scarring
- Hypopigmentation
- Hyperpigmentation

**Evaluation**

<table>
<thead>
<tr>
<th></th>
<th>NCES</th>
<th>FCS + NCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Repigmentation at 16 weeks</td>
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</tbody>
</table>
APPENDIX III
PATIENT SATISFACTION QUESTIONNAIRE

1. Patients’ Global Assessment:

1) Grade the change in pigmentation in the transplanted area. (0 to 10)
   a) NCES                      b) FCS + NCES

2) Are you satisfied with the obtained result? (0 to 10)
   a) NCES                      b) FCS + NCES

3) Do you find the treatment worthwhile? (0 to 10)
   a) NCES                      b) FCS + NCES

4) Would you choose this treatment again? (yes / no)
   a) NCES                      b) FCS + NCES

For question 1, ‘0’ means ‘much worse’ and ‘10’ means ‘much improved’.
For question 2 and 3, ‘0’ means ‘not at all’ and ‘10’ means ‘very much’.