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Therapeutic Approach for Hair Growth and Regeneration Using Bioactive Formulation Containing Mesenchymal Stromal Cell-Derived Conditioned Medium

Pawan Kumar Gupta[1*], Samatha Bhat[2], Suresh Kannan[1], Raviraja N. Seetharam[2], Udaykumar Kolkundkar[1]

[1]Stempeutics Research Pvt. Ltd., 3rd Floor, Manipal Hospitals Whitefield Pvt. Ltd., Bengaluru, India
[2]Stempeutics Research Pvt. Ltd., 4th Floor, Shirdi Sai Baba Cancer Hospital, Manipal, India
Email: *pawan.gupta@stempeutics.com

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Abstract

Background and Aims: Androgenetic alopecia (AGA) is a common form of hair loss in both men and women. Despite its high prevalence and associated patient morbidity, the approved therapeutic options are limited to finasteride and minoxidil. The present study is aimed at assessing the efficacy of hair serum formulation, Trichosera® containing Bone marrow-derived mesenchymal stromal cells conditioned media as an active ingredient, for hair fall control and hair regrowth in healthy Indian human volunteers. **Methods:** The product was made using a 20% concentration of 10X Conditioned Media along with excipients. The final product was tested for physicochemical parameters, biomarkers, total protein content and microbial limits as per our in-house specifications. **Results:** The primary irritation patch test showed that the product is non-irritant and dermatologically safe. A clinical study on 40 subjects was conducted to evaluate the effectiveness of the bioactive formulation in hair fall control and hair regrowth in healthy volunteers. Phototrichogram measurement showed hair density and hair growth rate increased significantly by 11.54% and 18.66% at week 24. Hair tensile strength also increased significantly by 41.10% at 12 weeks follow-up. Hair pull test, to see a reduction in pulled hair and comb's test to show a decrease in hair fall significantly improved from week 4 onwards. There were no significant adverse events in response to the product application. **Conclusion:** It is concluded that the hair serum product is completely safe on direct application to the scalp and showed significant improvement in the hair growth rate, hair density, scalp condition and reduction in hair fall.

Keywords

Trichosera®, Bone Marrow Derived Mesenchymal Stromal Cells, Conditioned Media, Hair Fall, Hair Regrowth, Human Volunteer Study

1. Introduction

Androgenetic alopecia (AGA) is a common form of hair loss in both men and women. In men, this condition is also known as male-pattern baldness. In women, the hair becomes thinner all over the head, and the hairline does not recede. AGA is androgen dependent, has a hereditary inheritance pattern and is associated with non-scarring, progressive miniaturization of hair follicles and shafts [1]. The result is alteration in hair cycle with decrease in duration of anagen phase and increase in telogen phase. This leads to shorter hair as anagen phase determines hair length, eventually leading to bald appearance in men [2] [3]. AGA in women rarely leads to total baldness. AGA can have psychosocial complications, including depression, low self-esteem altered self-image, and less frequent and enjoyable social engagements. In patients with AGA due to continuous disease progression, quality of life gets impaired. Therefore, treatment and counselling is very important in these cases to reduce the psychological impact of alopecia.

Despite its high prevalence and associated patient morbidity, the approved therapeutics options for AGA is limited to oral dihydrotesterone synthesis inhibitor finasteride [4] [5] and topical potassium channel opener minoxidil [6] [7]. These drugs have proven to be effective; however, discontinuation of them carries the risk of increased hair loss. Other treatment modalities include surgical hair transplant and laser therapy, but the limitation is that number of hair strands that could be transplanted during each surgical procedures is limited to a maximum of 2000 [8]. In addition transplants are associated with increased risk of bleeding and infection [9]. Thus to overcome these challenges an effective treatment strategy needs to be established.

Mesenchymal stromal cells (MSC) are progressively being used in regenerative medicine and offers hope to cure many unmet medical needs and cause repair/regenerate due to their paracrine factors, immunomodulatory and anti- inflammatory properties [10] [11] [12]. The various growth factors and cytokines (GFs/CKs) secreted by the MSC in the spent media of harvested cells, otherwise known as conditioned media (CM), are being used for making novel cosmetic products for hair care, skin care and under eye dark circles [13]. The paracrine factors secreted *in vitro* include vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin like growth factor-1 (IGF-1), transforming growth factor-beta (TGF-beta) and stromal cell derived growth factor (SDF-1) [14] [15]. These growth factors and cytokines possibly modulate the host environment and play a significant role in hair growth [16]. Published stu-

dies have shown that use of MSC derived CM significantly increased human hair regrowth and hair density [17] [18] [19] [20].

Formulation for hair growth serum, Trichosera®, is developed by mixing CM obtained from adult human bone marrow derived mesenchymal stromal cells (BM-MSCs) along with excipients. Previously we have identified 40 GFs/CKs secreted by BM-MSCs in our culture conditions [21] a few of growth factor families including the VEGF, Basic fibroblast growth factors (bFGF), Macrophage Stimulating Protein (MSP), IGF-I, HGF, and Platelet-derived growth factor (PDGF) have been shown to be crucial for the regulation of the hair cycle and hair growth [22]. Using a novel pooling technology of BM-MSCs [23], the CM is produced under Good Manufacturing Practice (GMP) conditions and used for developing the different cosmetic products including hair serum. Previously we have reported beneficial effects of anti-aging cosmetic skin serum and serum reducing under eye dark circle which has been developed using MSC-CM [24] [25].

Few studies have tested BM-MSC conditioned media containing GFs/CKs as a bioactive ingredient for the hair regrowth and increasing hair density [22]. The present study is aimed at assessing efficacy and safety of hair serum formulation, Trichosera® containing BM-MSCs conditioned media as active ingredient, for hair fall control and hair re-growth in healthy Indian human volunteers as determined by dermatological assessment using photonumeric scale and measurement of hair area using phototrichogram imaging.

2. Materials and Methods

Production of conditioned media derived from cultured bone marrow derived mesenchymal stromal cells

Stempeucel®, a pooled, allogeneic BMMSC product, was obtained from bone marrow aspirates from consenting donors who were not human leukocyte antigen matched to the recipients. Briefly, 60 ml of bone marrow aspirate was diluted (1:1) with knockout DMEM (Thermo Fisher Scientific Life Sciences, Waltham, MA, http://www.thermofisher.com), and was centrifuged at 1800 g for 10 minutes to remove the anticoagulant. Bone marrow mononuclear cells (BMMNCs) were isolated by the density gradient centrifugation (1.077 g/ml) method. BMMSCs were isolated by plastic adherence from the donor's BMMNCs and cultured until passage 1. A donor master cell bank constituted from MSCs from an individual BM sample was created and maintained under cryopreserved conditions. Subsequently, a working cell bank (WCB) was prepared by combining MSCs from three donors and cryopreserved for manufacturing Stempeucel® and further expanding the pooled WCB for additional passages for manufacturing Stempeucel® (U.S. patent number 8956862, dated February 17, 2015). For the work described in this article, pooled BMMSCs from three different individual donors were cultured, harvested, and expanded as described previously for production of conditioned media [26].

Briefly, the cells were grown up to passage 5 in complete medium containing 1 x Dulbecco's Modified Eagle's Medium-Knockout™ (Gibco) supplemented with 10% fetal bovine serum (FBS) (Hyclone), 1 x Glutamax™ (GIBCO), 2 ng/ml bFGF (Sigma Aldrich) and incubated in a 5% CO_2 incubator (Binder) at 37˚C. The total duration of the culture from WCB to the final product stempeucel® is around 3 to 4 weeks. The pooled BM-MSCs at passage 5 were analysed for the cell surface marker expression of CD markers by flow cytometry. The following markers were analysed using Guava Express Pro software (Guava Technologies) CD90-PE, CD105-PE, CD73-PE, CD34-PE, CD45-FITC (BD Pharmingen, San Diego, USA). The trilineage differentiation potential of the pooled BM-MSCs into adipocytes, osteocytes and chondrocytes were analysed by staining the corresponding differentiated cultures with Oil Red O, Alizarin Red S, and Alcian Blue staining respectively. For induction of differentiation, the cells were cultured in Stempro Adipogenesis Kit, Osteogenesis Kit and Chondrogenesis Kit for 21 days and were stained. CM was collected from cells at passage 5 upon attaining 80% - 90% confluency. The CM was concentrated 10 times using Millipore 1 KDa cut-off filters by Tangential Flow Filtration (TFF) technology (Merck-Millipore) as described elsewhere [24] and was stored at −80˚C till further use.

Secretome analysis of the conditioned medium for identifying hair growth promoting factors:

The conditioned medium was analyzed for growth factors and cytokines by antibody-based growth factor array. Various growth factors in CM were analyzed either using human specific ELISA kits (R&D systems, Minneapolis, USA) or multiplexing using Millipore's proprietary Luminex® Technologies. The reading was captured on Luminex 200™ (Austin, TX, USA) and based on their relative position and intensities, values were generated. The values in picograms/milliliter (pg/ml) so obtained were interpolated from the standard graph generated by the inbuilt system software.

Gene expression profiling of BM MSC to screen the expression of hair growth promoting factors

Gene expression analysis was performed on BM-MSC to analyse the expression of different genes (BMP4, BMP6, BMP7, C-Met, EGFR, FGF-7, FGF-10, FGFR2, NOG, PDGFa, TIMP1, TIMP2, TGFbRII, TGFbRI, WIF1, WNT5a) involved in the various stages of hair growth cycles and compared it to the dermal papilla cells. The expression level of these genes was analysed using real time PCR (StepOnePlus-Thermo Fischer) and the quantification was done using the ΔΔCt method after normalizing to the β-actin housekeeping gene. Briefly, the total RNA from BM-MSCs and dermal papilla cells were isolated using RNA Qiagen RNeasy kit by following the kit instructions. The concentration of RNA was measured using spectrophotometer and 1 µg of RNA was used for cDNA synthesis. The reverse transcription reaction was carried out using Superscript II kit (Invitrogen) following the manufacturer's protocol. For real time PCR, 1 µl of

cDNA was used and the expression was measured using SYBR green chemistry.

Proliferation of human dermal papilla cells with various concentration of concentrated condition medium

For analyzing the proliferation of human DP cells, the cells were plated at density of 2000 cells/well in 96 multiwell plate in complete media. The next day, the media was replaced with serum free media for 24 hrs, followed by treatment with various concentration of CM. After 48 hrs of addition of CM or control medium, the cells were pulsed with BrdU and left for 24 hrs. The BrdU assay was carried out following the manufacturer's instruction (Calbiochem, USA) and the percentage of proliferation was calculated from OD values over control. The CM concentration which showed the maximum proliferation of DP cells over the corresponding control media was chosen and the excipient Taurine was tested on the proliferation of DP cells with BrdU assay.

Preparation of bioactive formulation

Based on the *in vitro* studies (DP proliferation), the 20% concentration of 10X CM was selected to be used as an active ingredient for the hair formulation. The *in vitro* studies showed that 20% concentration of 10X CM with taurine as one of the excipient induced proliferation of DP cells. The formulation was developed using unique combination of excipients which contains stabilizing agents, hydrating agents and viscosity increasing agents in order to ensure that the product is appropriately formulated for hair application. First Carbopol 974 P NF is dissolved in de-mineralized water (DM) and added to propylene glycol. To this mix, taurine dissolved in DM followed by hydrolyzed silk protein was added. To this mix, Cyclopentasiloxane (and) Dimethiconol (DC1501), PEG-12 Dimethicone (DC193C) and fragrance was added. The pH of the cosmetic formulation was adjusted between 7.5 and 8.0 using triethanolamine. Finally, 20% of the 10X conditioned medium was added and mixed well to formulate the final product—Trichosera®.

Testing the final developed formulation for physicochemical parameters, biomarker, total protein content and Microbial limits for all pathogens

The pH of the final formulation was measured using a pH meter (ThermoFisher Scientific). The viscosity was measured using a viscometer (Brookfield). The total protein content in the formulation was estimated spectrophotometrically by the Bradford method at 595 nm. The microbial assays such as total aerobic microbial count (TAMC), total yeast and mould count (TYMC) and the test for the pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Candida albicans* in 1 g/mL product and Salmonella sps. in 10 g/mL was carried out using microbial limit test method as per Indian pharmacopeia guidelines (https://www.pharmaguideline.com/2010/01/sop-for-microbial-limit-test-of-raw.html). Briefly, the formulation was mixed with sterile soybean casein digested agar for TAMC and sterile sabouraud dextrose agar for TYMC in petri plates. The plates were incubated at 30˚C - 35˚C and 20˚C - 25˚C for 5 days. The microbiological quality was assessed in accordance with the acceptance criteria

specified in Indian Pharmacopoeia. All the tests were carried out in duplicates and repeated twice.

Clinical studies

Primary Irritation Patch Test (PIPT)

The single application occlusive patch test is the most common technique to evaluate a product tolerance on human skin. The dermatological safety of Trichosera® at different concentrations of CM (5%, 10% & 20%) was evaluated by 24 hrs patch test under complete occlusion on healthy human subjects. The method was based on the Bureau of Indian Standards method 4011:1997 Amendment-2 (14th Jan. 2005) modified in terms of site of application. 40 µl of the test product 1 (Placebo) and test products 2, 3 & 4 (Trichosera®—5%, 10% & 20% respectively) were placed in the allotted IQ ultra-chamber prefixed to a micropore tape. Positive control (3% w/w Sodium Lauryl Sulphate) and negative control (Vaseline) were also placed inside the allotted IQ ultra-chamber prefixed to a micropore tape. A total of 24 healthy male and female subjects in the age group of 18 - 55 years were enrolled in the study as per the inclusion-exclusion criteria after obtaining written informed consent. The total duration of the study was 9 days. The study was approved by an independent ethics committee and was conducted in accordance of good clinical practice guidelines. Subjects with Fitzpatrick skin type III to V, willing to maintain the patch test in position for 24 hours, with healthy skin on the studied anatomic unit (free of eczema, wounds and inflammatory scars) and willing to come for regular follow-up visits were enrolled in the study. The test site was between the scapulae and waist of the subjects and was free of pigmentation, pimple, coarse hair, mole or any dermatological conditions that could interfere with the reading. The patch system was applied at the test site of the study subjects starting with the lower edge of the patch system and slowly pressing upwards till the top edge in order to squeeze out the air. The patch at the test site was retained till 24 h post-application and thereafter removed. The test sites were assessed for erythema/dryness/wrinkles and oedema as per the Draize scale for scoring at the treatment site at 0 h (for irritation reactions), 24 h (for immediate reactions) and 7 days (for delayed reactions) after patch removal.

Human volunteer study design

An open label, monocentric, single arm study for efficacy evaluation of hair serum for hair fall control, and hair re-growth in healthy Indian human volunteers was carried out. The study population consisted of 40 subjects in the ratio of 1:3 (10 male: 30 female) aged between 18 to 65 years. The primary end point of the study was to see improvement in hair re-growth and reduction of hair fall in comparison to baseline using photo numeric scale and measurement of hair area using Phototrichogram imaging. The secondary end points were to determine safety of the product by assessment of site application reaction and improvement in overall condition of hair. The main inclusion criteria for selection of the subjects were males and females falling under Grade 3 to Grade 8 of hair

loss severity grade as per photo numeric 10 point scale, subjects complaining of hair fall and damage and subjects willing to refrain from any type of hair treatment like perming, straightening etc. during the study duration. The main exclusion criteria were subjects who were undergoing hair growth treatment within 3 months before screening into the study, having any active scalp disease, who were undergoing any form of cancer treatment, who have had hair transplant, taking pharmaceutical product which cause hirsutism (eg. phenytoin) and finasteride for androgenic alopecia and any history of underlying uncontrolled medical illness. The study was conducted in accordance with GCP guidelines and approved by Independent Ethics committee. Informed consent was obtained from all the subjects participated in the study. The subjects were asked to apply the product once daily at night at a volume of 1 ml on each half of scalp. The subjects were also asked to use Neutral shampoo thrice weekly on alternate days. The subjects were allowed to miss maximum of 10% of total dose and were considered dropped out of the study if they exceed the missed dose range.

Subject assessment visits

The study was conducted for a period of 24 weeks approximately for each subject and included total of 13 visits. Visit 1 was the screening visit during which subjects were evaluated based on the inclusion/exclusion criteria and Visit 2 (Day 0) was the baseline visit, followed by Visit 3 (Day 2), Visit 4 (Day 28), Visit 5 (Day 30), Visit 6 (Day 56) ,Visit 7 (Day 84), Visit 8 (Day 86),Visit 9 (Day 126),Visit 10 (Day 128),Visit 11 (Day 147), Visit 12 (Day 168) & Visit 13 (Day 170). Various parameters were assessed which included hair pull test (assessment of hair fall reduction and strengthening of hair) which was done on visit 2, 4, 7, 9 and visit 12, subject self-assessment of hair by comb test and subject self-assessment questionnaire (assessment of improvement in hair fall reduction) done on visit 2, 4, 7, 9 and visit 12, hair tensile strength done on visit 4, 7, 9 and visit 12. Phototrichogram Imaging of hair (assessment for improvement in follicular density and hair length assessment on specified trimmed site for growth rate) was done on all visits. The assessments for phototrichogram included the following parameters—hair density, hair growth rate, hair thickness & Anagen to Telogen ratio.

Hair pull test, assessment by dermatologist and self-assessment of hair by comb test was done to evaluate control of hair fall reduction with strengthening. Briefly, in hair pull test, bundle of hairs (around 60 strands) were grasped between the thumb, index finger, and middle finger from the base near the scalp. The hairs were firmly, but not forcibly, tugged away from the scalp as fingers slide along the hair shaft. Afterwards the number of extracted hair was counted. The same procedure was repeated in four scalp sections. If more than 10% of grasped hairs, or six hairs, are pulled away from the scalp, this constitutes a positive pull test and implies active hair shedding. If fewer than six hairs were pulled out, this is considered normal physiologic shedding. In Comb test subjects were instructed to do a middle partition of the hairs and were asked to comb their hair slowly from the top of their head throughout the hair length five times each

on left and right side. Number of fallen hairs were counted and evaluated in different visits. Hair tensile strength was evaluated using Chatillon Motorized Force hair tensile tester (TCM 100). The instrument displayed the peak tensile force for the test and the break force for the test based on the break criterion. The amount of force applied until the breakage point of hair was recorded. Self-assessment of hairs was evaluated using a questionnaire in which subjects were asked to assess their hair and scalp conditions and were told to score on certain parameters like hair fall rate, current hair texture, hair volume and application site reactions. The visit specific schedule for conduct of specific assessments is given in Table 1.

Statistical analysis

Demographic characteristics and results of the study were summarized with summary statistics, including average and standard deviation (SD) for continuous variables and frequency and percentages for categorical variables. Any AEs were summarized with the number and percentage. Paired t-test was performed to find out efficacy in comparison to baseline on continuous scales such as dermatological assessment and instrumental assessments. Z proportion test was performed to find the significance of change in proportion of outcome from baseline to final visit on categorical type such as subject's assessment. The p value less than 0.05 ($p < 0.05$) was considered as statistically significant.

Table 1. Visit specific schedule for conduct of specific assessments.

Parameter	Treatment											
	Week 0		Week 4		Week 8	Week 12		Week 18		Week21	Week 24	
	Visit 2 (Day 0)	Visit 3 (day 2)	Visit 4 (day 28)	Visit 5 (day 30)	Visit 6 (day 56)	Visit 7 (day 84)	Visit 8 (day 86)	Visit 9 (day126)	Visit 10 (day 128)	Visit 11 (day 147)	Visit12 (day 168)	Visit 13 (Day 170)
Hair trimming	√	-	√	-	-	√	-	√	-	-	√	-
Dermatological Assessment for efficacy	√	-	√	-	-	√	-	√	-	-	√	-
Scalp Imaging (Canon D70)	√	-	√	-	-	√	-	√	-	-	- √	-
Dermatological assessment for site application reaction. (Local intolerance)		√*	√	√	√	√	√	√	√	√	√	√
Comb test	√		√			√		√			√	
Hair tensile tester	√		√			√		√			√	
Subject Self-assessment questionnaire of hair &scalp	√		√			√		√			√	
Subject assessment for site application reaction	-	√*	√	√	√	√	√	√	√	√	√	√
Phototrichogram Imaging	√	√	√	√	√	√	√	√	√	√	√	√
Densitometer for hair thinning	√	-	√	-		√	-	√			√	-

*This evaluation is prior to first application to record the baseline scalp condition. **Window period**: Between screening (Visit1) and enrolment visit (Visit 2) +4 days, and ±3 days for Visit 4, 6, 7, 9, 11 and 12. No window period for Visit 3, 5, 8, 10 and 13.

3. Results

BM-MSCs characteristics

The BM-MSCs showed distinctive MSCs morphology of being spindle shaped and express CD 73, CD 90 and CD 105 (>90% for all markers) but do not express CD 34 and CD45 (shown in Figure 1(a)). The trilineage differentiation culture conditions reveal their ability to differentiate into adipocytes, osteocytes and chondrocytes as evident by their staining to Oil Red O, Alizarin Red S, and Alcian Blue respectively (shown in Figure 1(b)).

Secretome analysis of the conditioned medium:

Secretome analysis revealed the presence of hair growth promoting factors in the conditioned medium (shown in Figure 2(a)). The growth factors and cytokines that have been shown to play a role for hair growth promotion includes PDGF, VEGF, prostaglandin E2 (PGE2), FGF7, HGF, keratinocyte growth factor (KGF) and IGF-1. Few of the selected cytokines/growth factors in CM responsible for hair growth were quantified and shown in Figure 2(b). These factors are produced by the stem cells and are enriched in the conditioned medium. So, the conditioned medium is a suitable active agent for hair growth promotion and to maintain the healthy scalp.

Gene expression profile of BM-MSCs

Analysis of Gene expression profile of BM-MSCs revealed that most of the genes involved in the hair growth phase like Bone morphogenetic protein 6 (BMP6), tyrosine-protein kinase Met (C-met), epidermal growth factor receptor (EGFR), fibroblast growth factor 7 (FGF-7), FGF-10 and platelet derived growth factor alpha (PDGFα) were highly expressed in BM-MSCs compared to dermal papilla cells (shown in Figure 2(c)). Most of these secreted growth factors by the stem cells are present in the conditioned medium as well. Hence, the conditioned medium possesses the potential to prevent hair loss, induce hair growth and thickness.

Proliferation of dermal papilla cells using 10X CM

10X conditioned medium significantly induced the proliferation of dermal papilla cells at both 50% and 25% concentration (shown in Figure 2(d)). Hence, the conditioned media have the potential to enrich the hair follicle and induce hair regrowth.

Bioactive formulation test results

The prepared bioactive formulation was subjected to various tests such as pH, viscosity, VEGF, total protein count, total aerobic microbial count (TAMC), total combined yeasts and molds count (TYMC) and tests for pathogens. The pH of the formulation was between 5 and 8.5, viscosity ≤ 15,000 cPS and total protein content was between 2 and 16 ng/ml. The VEGF level as measured by ELISA was between 0.5 to 12 ng/ml. The TAMC and TYMC were $\leq 10^3$ CFU/g and ≤ 100 CFU/g respectively. The pathogens such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* were absent in 1 g/ml and Salmonella sps. in 10 g/ml product suggesting that the microbial count was

within the acceptable limit as per specific monograph defined in Indian pharmacopeia. Altogether, these results indicated that the total protein content and VEGF levels were stable at acidic to mild alkaline pH, and the formulation was free from the microbial contaminants.

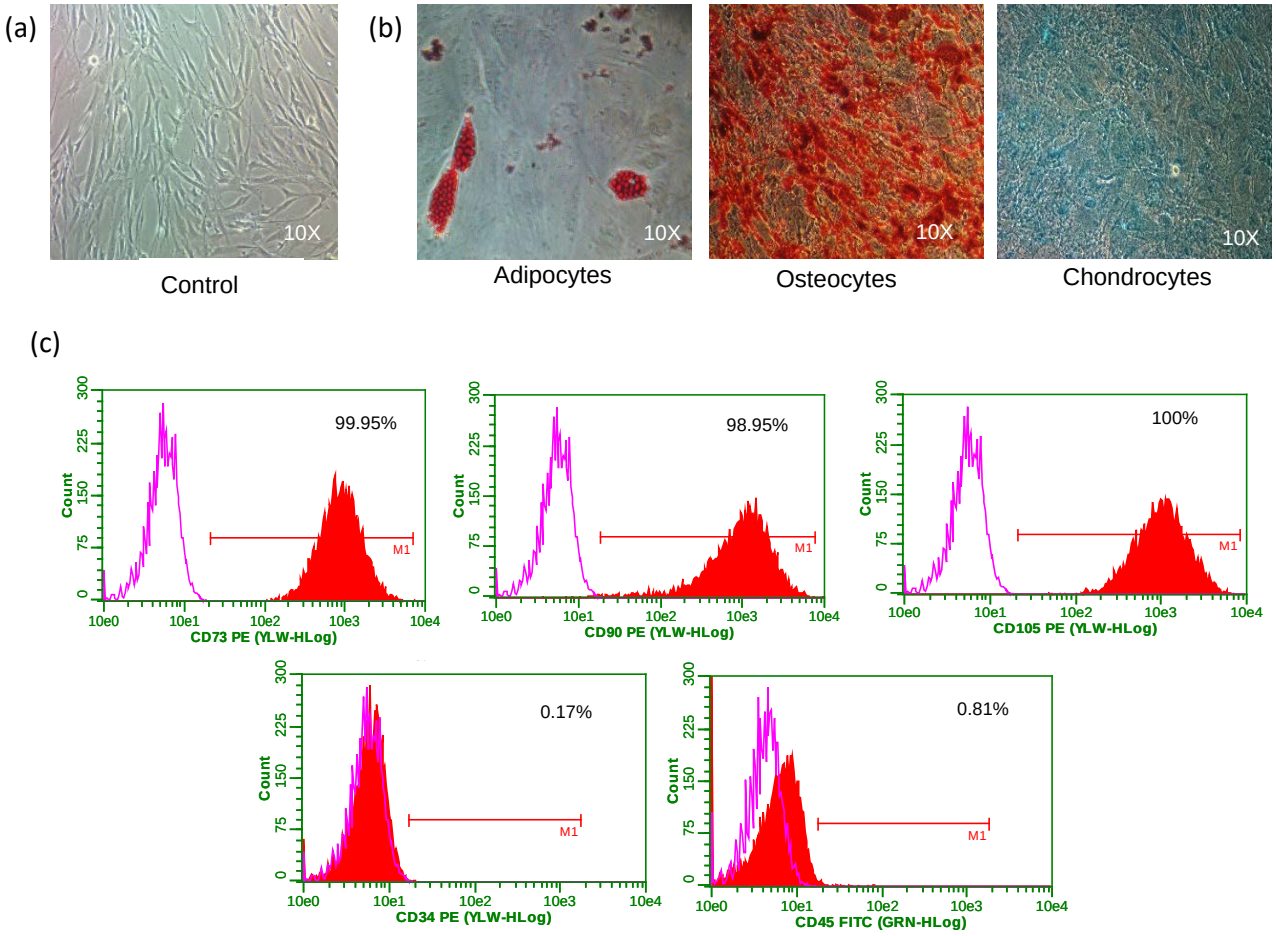
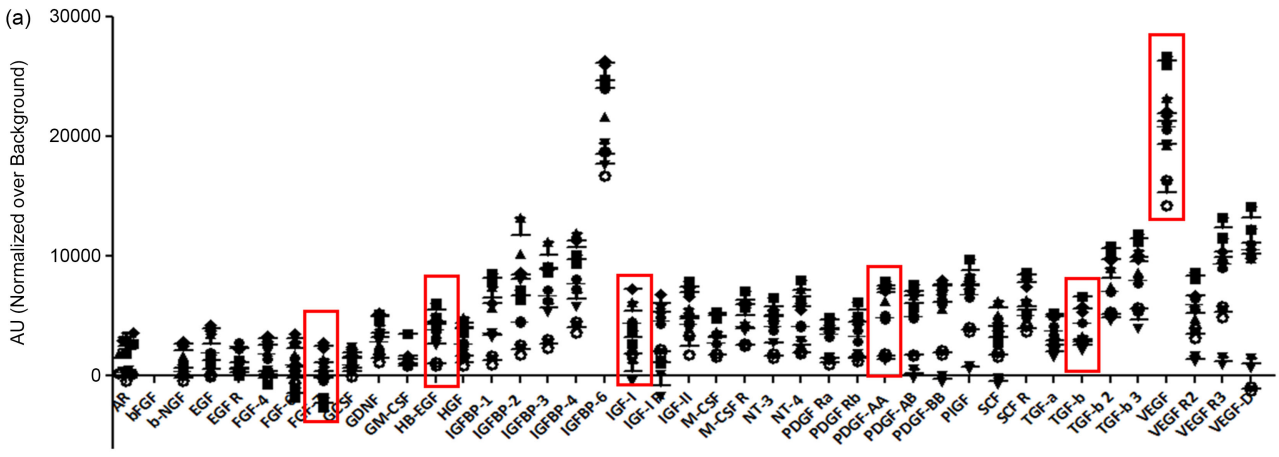


Figure 1. Characteristics of pooled BM-MSCs. (a) Cells appeared spindle shaped in culture (b) differentiation into adipo, osteo and chondro lineages as evident by Oil Red O, Alizarin Red S, and Alcian Blue stain (c) cells are positive for CD 73, 90 105 and negative for CD 34 and 45.



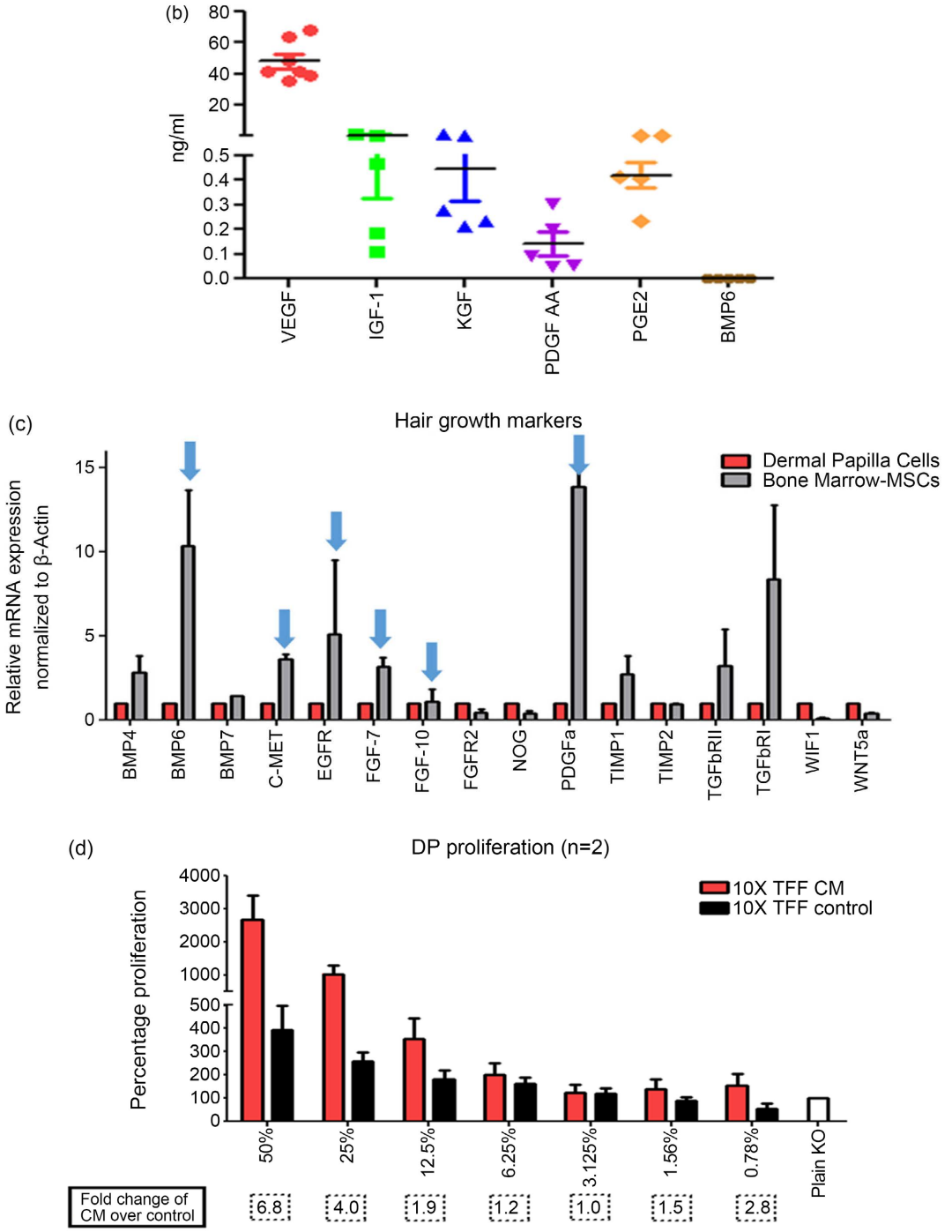


Figure 2. *In vitro* assays of BM-MSCs derived conditioned medium (CM). (a) Secretome analysis of CM for identifying hair growth promoting factors. Various growth factors such as Platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor-7 (FGF7), hepatocyte growth factor (HGF) and insulin like growth factor-1 (IGF-1) that are related to hair growth promotion were identified in the CM. (b) Dot plot showing the quantification of few of the selected growth factors/cytokines involved in hair growth promotion. (c) Gene expression profiling of BM-MSCs and dermal papilla revealed that the genes such as Bone morphogenetic protein 6 (BMP6), tyrosine-protein kinase Met (C-met), epidermal growth factor receptor (EGFR), fibroblast growth factor 7 (FGF-7), fibroblast growth factor 7 (FGF-10) and platelet derived growth factor alpha (PDGF*α*) involved in hair growth promotion were highly expressed in BM-MSCs. (d) Proliferation of dermal papilla cells was analysed using various concentration of 10X CM and it was observed that 10X CM at concentration of 25% and 50% significantly induced proliferation of dermal papilla cells.

PATCH test classifies bioactive formulation as dermatologically safe and qualifies into non-irritant category

Twenty nine subjects were enrolled in the study, out of which 24 subjects completed the study. The average mean irritation score for each tested formulation is mentioned in Table 1. The mean irritation score for 5%, 10% and 20% bioactive formulation at 0h were 0.21, 0.08, and 0.04; at 24 h were 033, 0.17 and 0.08 and at 7 days were 0.08, 0.04 and 0 post patch removal, respectively indicating that the formulation was non-irritant for all skin types (Table 2). The mean irritation score for placebo was 0.21, 0.33 and 0.04 at 0 h, 24 h and 7 days post patch removal. The mean irritation score for negative control was 0 and for positive control was 3.83, 4.17 and 1.58 at 0 h, 24 h, and 7 days post patch removal confirming as mild irritant at 0 h, irritant at 24 h and nonirritant at 7 days post patch removal (Table 1). Thus, the investigational bioactive formulation at all concentrations emerged as non-irritant product for human application and deemed to be dermatologically safe.

Clinical study evaluation showed that bioactive formulation was effective in hair fall control and hair re-growth in healthy volunteers

The study included a total of 40 subjects (10 male and 30 female) aged between 18 - 65 years, out of which 9 male and 27 female subjects completed the study. During all the 13 visits, assessments were carried out by dermatologists in addition to instrumental evaluation (Phototrichogram), subject self-assessment and image assessment. The subjects involved in the study were photographed at each visit and representative images displaying enhanced hair coverage were shown in Figures 3(a)-(c).

Table 2. Average mean irritation score as per Draize scale for scoring irritation in patch test.

Investigational Product	Mean Irritation Score—0 Hrs	Irritancy assessment	Mean Irritation Score—24 Hrs	Irritancy assessment	Mean Irritation Score—7 days	Irritancy assessment
Product 1 - Placebo	0.21	Non irritant	0.33	Non irritant	0.04	Non irritant
Product 2 - Hair growth Product with 5% CM	0.21	Non irritant	0.33	Non irritant	0.08	Non irritant
Product 3 - Hair growth Product with 10% CCM	0.08	Non irritant	0.17	Non irritant	0.04	Non irritant
Product 4 - Hair growth Product with 20% CCM	0.04	Non irritant	0.08	Non irritant	0.00	Non irritant
Product 5 - Positive control (3% Sodium Lauryl Sulphate solution)	3.83	Mild Irritant	4.17	Irritant	1.58	Non irritant
Product 6- Negative control (Vaseline)	0.00	Non irritant	0.00	Non irritant	0.00	Non irritant

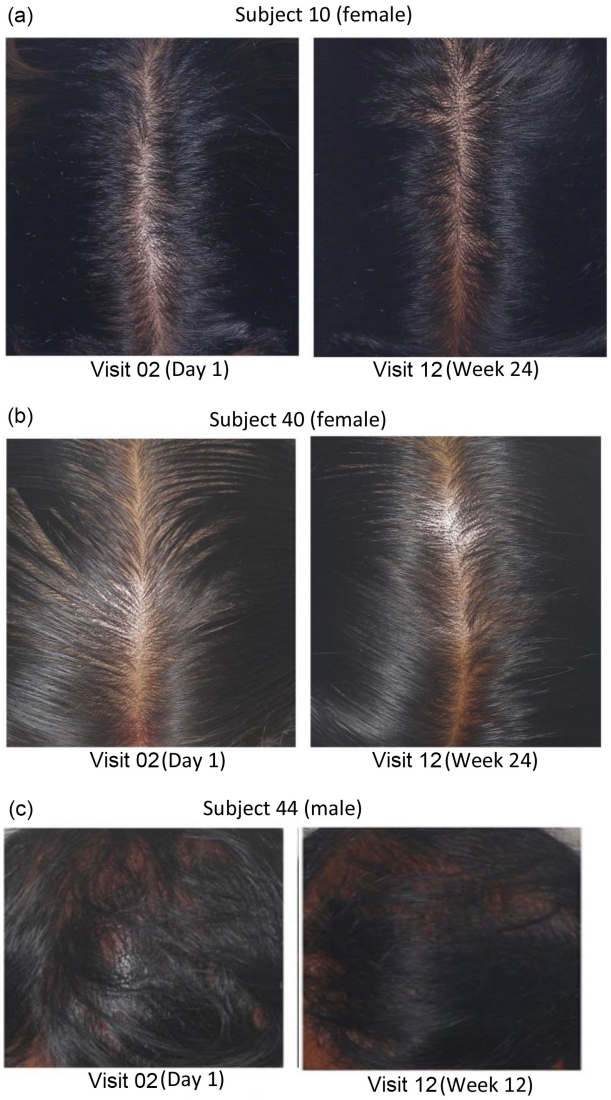


Figure 3. (a, b, c) Representative pictures of female and male subjects showing enhanced hair coverage at start and end of study.

Instrument measurement using Phototrichogram involves taking a close-up photograph of a well-defined scalp area and then analyzing it using special digital analyzing software Trichosan®. The assessment included parameters like-hair density, hair growth rate and hair tensile strength. Hair density analysis showed statistically significant improvement in hair density from week 12 which progressed till the end of the study in comparison to the baseline (shown in **Figure 4(a)** and **Figure 4(b)**). The percentage of hair density increased with test product application at 4, 12, 18 & 24 weeks with value of 5.15%, 10.93%, 11.07% and 11.54% respectively (**Table 3(a)**). There was a suggestively significant improvement in hair density noted at 4 week which progressed to show statistically significant improvement at week 12, week 18 and week 24 in comparison to the baseline. Significant improvement in hair growth rate at week 4 and week 12 was observed which progressed to show statistically significant improvement at week

18 and week 24 in comparison to the baseline (shown in Figure 4(c) and Figure 4(d)). The percentage of hair growth rate increased with Trichosera® product application at 4, 12, 18 & 24 week was 10.31%, 10.86%, 17.47% & 18.66% respectively (Table 3(b)). Significant improvement in hair tensile strength from week 18 was observed which progressed till the end of the study in comparison to the baseline. The percentage hair strength increased with test product application at 4, 12, 18 & 24 weeks and it was 4.11%, 17.81%, 19.185% & 41.10% respectively (Table 3(c)). There was a progressive improvement in hair tensile strength with the product application and the improvement was suggestively significant at week 12 and statistically significant at week 24 in comparison to baseline.

Table 3. (a): Hair density (hair/cm^2)—Mean and percentage change over baseline; (b): Hair growth rate (μm/day)—Mean and percentage change from baseline; (c): Hair tensile strength—Mean and percentage change from baseline; (d): Presence of scales—Mean and percentage change from baseline; (e): Number of hairs pulled—Mean and percentage change from baseline; (f): Hair density measured by densitometer—Mean and percentage change from baseline; (g): Combs test with bulb—Mean and percentage change from baseline.

(a)

Hair Density (hair/cm^2)					
	Baseline	Week 4 (Day 30)	Week 12 (Day 86)	Week 18 (Day 128)	Week 24 (Day 168)
Mean ± SD	129.23 ± 37.22	135.89 ± 30.38	143.35 ± 31.66	143.53 ± 29.43	144.14 ± 32.54
P value over Baseline	-	0.0779	0.0050	0.0036	0.0020
% Changed over Baseline	-	5.15	10.93	11.07	11.54

(b)

Hair Growth rate (μm/day)					
	Baseline	Week 4 (Day 30)	Week 12 (Day 86)	Week 18 (Day 128)	Week 24 (Day 168)
Mean ± SD	227.55 ± 70.46	251.02 ± 67.09	252.27 ± 67.18	267.31 ± 62.29	270.00 ± 68.86
P value over Baseline	-	0.0878	0.068	0.003	0.003
% Changed over Baseline	-	10.31	10.86	17.47	18.66

(c)

Hair tensile strength					
	Baseline	Week 4 (Day 30)	Week 12 (Day 86)	Week 18 (Day 126)	Week 24 (Day 168)
Mean ± SD	0.73 ± 0.29	0.76 ± 0.21	0.86 ± 0.32	0.87 ± 0.18	1.03 ± 0.21
P Value over baseline	-	0.584	0.0743	0.0181	<0.0001
% Change over baseline	-	4.11	17.81	19.18	41.10

(d)

Evaluation of presence of scales (Scale: 1 = None, 2 = Mild-few loose scales/flakes, 3 = Moderate-loose and/or adherent scales, 4 = Severe-loose and/or adherent scales)					
	Baseline	Week 4 (Day 30)	Week 12 (Day 86)	Week 18 (Day 126)	Week 24 (Day 168)
Mean ± SD	1.72 ± 0.57	1.44 ± 0.50	1.19 ± 0.40	1.19 ± 0.40	1.22 ± 0.42
% Change over baseline	-	16.28	30.81	30.81	29.07
P value Over baseline	-	0.0057	<0.0001	<0.0001	<0.0001

(e)

No. of hair pulled (number of hair strands) 1 = none (0), 2 negligible (1 - 2), 3 = mild (3 - 4), 4 = Moderate (5 - 6), 5 = severe (>6)					
	Baseline	Week 4 (Day 30)	Week 12 (Day 86)	Week 18 (Day 126)	Week 24 (Day 168)
Mean ± SD	0.74 ± 0.43	0.53 ± 0.33	0.47 ± 0.23	0.44 ± 0.20	0.38 ± 0.17
P value Over baseline	-	<0.0001	0.0002	0.0002	<0.0001
% Change from baseline		28.38	36.49	40.54	48.65

(f)

Densitometer (evaluated on a scale of 1 to 10 with score of 1 in normal hair density & 10 with severe hair thinning)					
	Baseline	Week 4 (Day 30)	Week 12 (Day 86)	Week 18 Day 126)	Week 24 Day 168)
Mean ± SD	7.14 ± 0.54	7.13 ± 0.53	7.00 ± 0.52	6.91 ± 0.59	6.86 ± 0.61
% Change over baseline	-	0.10	1.95	3.21	3.89
P Value over baseline	-	0.3239	0.0001	<0.0001	<0.0001

(g)

With Bulb (hair fall count)					
	Baseline	Week 4 (Day 30)	Week 12 (Day 86)	Week 18 (Day 126)	Week 24 (Day 168)
Mean ± SD	16.75 ± 10.12	13.72 ± 9.48	13.89 ± 7.86	11.69 ± 11.1	10.14 ± 5.82
P value Over baseline	-	0.0042	0.0267	0.0069	<0.0001
% Change over baseline	-	18.09	17.07	30.21	39.46

Assessment by dermatologist included visual evaluation of scalp condition, presence of scales, hair pull test and densitometer evaluation for hair density. At baseline, 91.67% of the population had normal scalp, 5.56% had oily scalp and 2.78% had scaly scalp. 100% of the subjects had normal scalp and none of the subjects had dry, oily and scaly scalp from week 12 to week 24. There was a statistically significant improvement in the evaluation of scales at all evaluated time points over baseline. At the end of the study, with 24 weeks of product application, scales/flakes reduced by 29.07% over baseline (**Table 3(d)**). To carry out

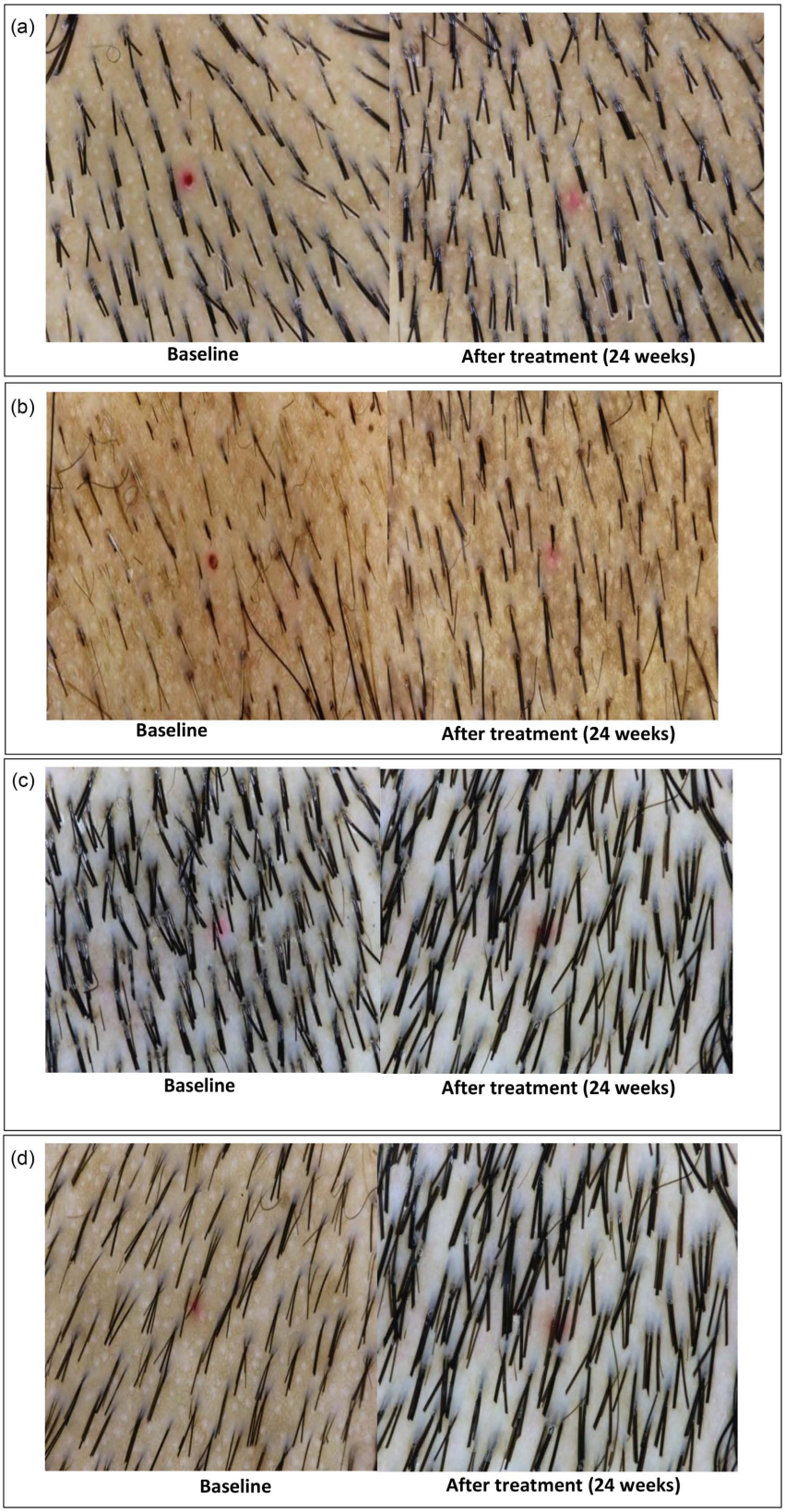


Figure 4. (a & b) Representative pictures showing increase in hair density 24 weeks post-application of the formulation. (c & d) Representative pictures showing increase in hair growth rate 24 weeks post-application of the formulation.

hair pull test—a bundle of hair (around 60 strands) was grasped between the thumb, index finger, and middle finger from the base near the scalp. The hair was firmly, but not forcibly, tugged away from the scalp as fingers slide along the hair shaft. The number of extracted hair was counted and scored with score of 0 with no hair strand extracted and maximum score of 5 with more than 6 hair strands extracted. Trichosera® application showed statistically significant reduction in number of pulled hairs from week 2 to week 24 in comparison to baseline. The percentage reduction of pulled hairs with test product application at 4, 12, 18 & 24 week was 28.38%, 36.49%, 40.54% & 48.65% respectively (**Table 3(e)**). There was a progressive improvement observed with less hair count by hair pull test in comparison to the baseline. The noted improvement was statistically significant at all evaluated time points in comparison to baseline. Hair density—it was evaluated using the microscopic images on a linear scale of 1 (best) to 10 (worst) by visual examination. Decrease in the mean score is a sign of improvement. There was a progressive improvement in hair density with the product application and the improvement was statistically significant at week 12, week 18 and week 24 in comparison to baseline (**Table 3(f)**).

Subject self-assessment includes comb test and questionnaire assessment. Comb test with bulb-decrease in the mean value is a sign of improvement. The test product showed statistically significant improvement, as there was a decrease in hair fall due to combing at 4, 12, 18 and 24 weeks in comparison to baseline. At the end of the study, with 24 weeks of product usage, hair fall (with bulb) reduced by 39.46% over baseline (**Table 3(g)**). Subject self-assessment for product performance included hair fall rate which was considered on a scale of 1 to 5 with value of 5 is a sign of improvement. After 24 weeks of product application, 86.11% of the population showed less than 10 hair fall per day (shown in **Figure 5(a)**). Significant proportion of the population showed improvement in hair texture. The improvement was seen as decrease in roughness of hair and increase in normal and soft hair texture. After 24 weeks of product usage, 44.44% of the population perceived to have soft hair texture and 50% perceived to have normal hair texture (shown in **Figure 5(b)**). Significant proportion of the population showed improvement in hair volume at week 12, week 18 and week 24. After 24 weeks of product usage, 72.22% of the population perceived to have average hair volume and 13.89% of the population perceived to have good hair volume (shown in **Figure 5(c)**). Moreover, the most likable parameter of the test product was colour, texture and fragrance. About 86.11% of the population graded that the product was good in reducing the hair fall.

As per the safety evaluation with dermatological application site reaction, one subject perceived dryness (at visit 3) and one subject perceived oiliness (at visit 6) during the study period and the noted reactions were resolved subsequently without any intervention. None of the subjects had erythema, allergic reactions, folliculitis or any other reactions. Few subjects experienced dryness, itching, hair fall, oiliness, dandruff during the study. The noted reactions were not significant in response to the product application. None of the subjects experienced boils on

scalp, allergic reactions or any other reactions. Adverse events were noted in 4 subjects during the study. Two subjects had headache, one subject had fever while the other subject had cold /cough. The adverse events were not related to product application. There were no product related local intolerance or adverse events. The noted application site reaction did not require any clinical intervention and the product was overall safe.

Hence, based on the 24 weeks data it can be concluded that regular usage of test product [Hair Serum (Trichosera®), showed significant improvement in the hair growth rate, hair density, scalp condition and reduction in hair fall. The Trichosera® showed improvement in hair texture and hair volume and reduction in hair fall as assessed by the subject self-assessment. Figure 6 represents the outcome of the study.

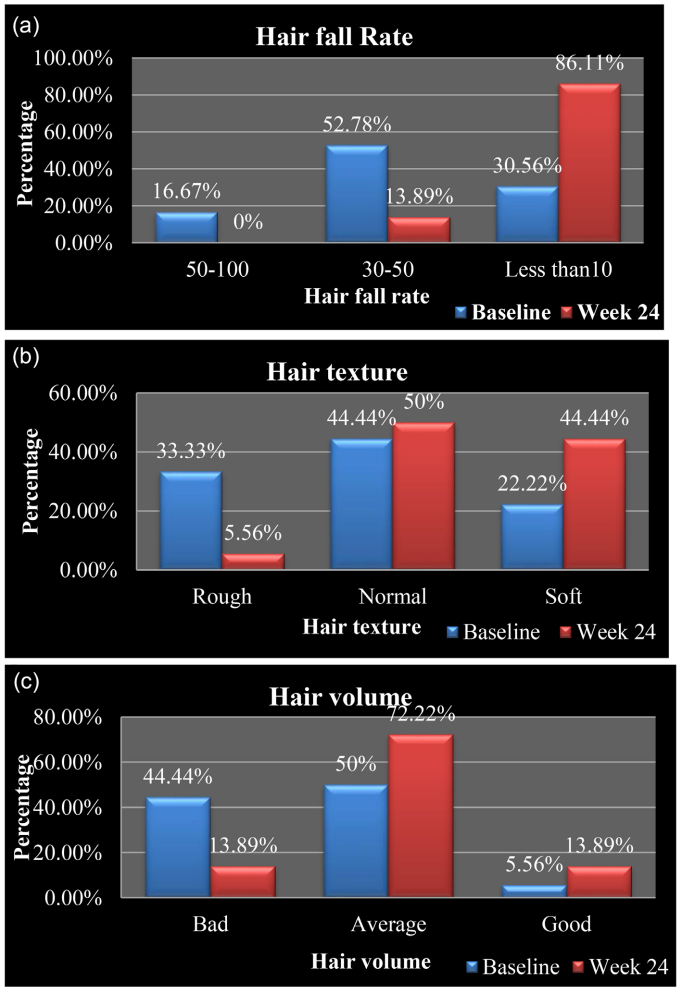


Figure 5. Dermatological assessment of parameters associated with hair condition. (a) Bar graph showing the percentage of subjects with decrease in the hair fall rate after 24 weeks of product application. 86.11% of the subjects showed less than 10 hair fall per day 24 weeks post application of formulation. (b) Bar graph showing the percentage of subjects with significant improvement in the hair texture from the baseline at the end of 24 weeks of product application. (c) Bar graph showing the percentage of subjects with improvement in the hair volume at the end of 24 weeks of product application.

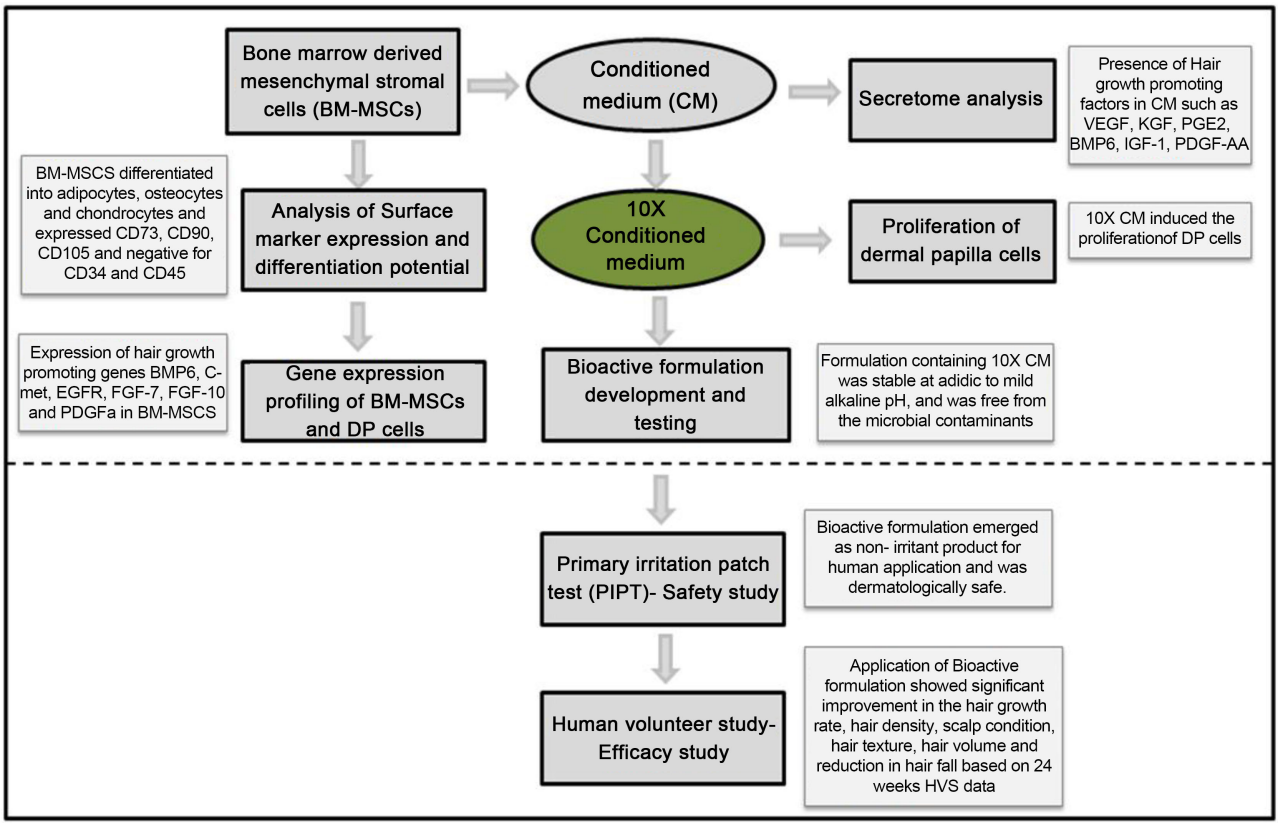


Figure 6. Pictorial representation explaining the findings of the study.

4. Discussion

Over the past several years about 300,000 products have claimed to improve hair regrowth. With the exception of minoxidil and finasteride, none of them was found to be effective in hair growth promotion [5] [7]. Currently, minoxidil (useful in both male and female pattern baldness) and finasteride (useful in male pattern baldness) are two US-FDA approved synthetic drugs finding concomitant use for treatment of androgenic alopecia, but their side effects have reduced their usage. The adverse events that commonly occur with minoxidil include contact dermatitis, dryness, pruiritis and facial hypertrichosis [27]. The most concerning side effects with use of finasteride includes sexual dysfunction, mood disorders and increased risk of prostate cancer in men and other long term use may lead to development of insulin resistance, type 2 diabetes, dry eye disease, non-alcoholic fatty liver disease and potential kidney dysfunction [28] [29]. Follicular unit transplantation is invasive and posing risk of infection, is limited by shortage of donor hair, reduced viability of cells and time consuming procedure. Another emerging treatment in alopecia is injection of autologous platelet rich plasma (PRP). PRP is a concentrate of human platelets containing number of growth factors like PDGF, transforming growth factor β (TGF-β), VEGF, epidermal growth factor (EGF), IGF and FGF [30] [31]. It is widely used for the treatment of alopecia but the outcomes are conflicting and difficult to interpret the efficacy of PRP [32] [33] [34] [35] [36]. Therefore novel therapeutics ap-

proach need for hair regeneration.

Conditioned media derived from MSCs may be potential, safe, and novel therapy for hair regeneration and this has fueled the field of hair research. Previous published reports suggests that CM is tested for hair restoration [18] [19] [37] [38] with promising results. CM mixed with appropriate excipients has its beneficial effects through its paracrine action via various cytokines and growth factors. Secretory factors derived from CM include PDGF, TGF-beta, VEGR, PGE2, FGF-7, HGF, KGF and IGF-1 as revealed by Secretome analysis of CM [21]. Several studies reported that function of various growth factors derived from CM. PDGF has been shown to promote proliferation of dermal papillae cells and maintain the anagen phase in the hair cycle [39], HGF facilitates hair elongation [40], VEGF increases hair growth and size by follicle vascularization [41] and IGF-1 improves the migration, survival, and proliferation of hair follicle cells [32] and KGF promotes hair growth by inducing the anagen phase in resting hair follicles and might be a potential hair growth-promoting agent [42]. Hair regeneration is a complex process involving the activation of the hair follicular stem cells, suggesting that group of growth factors rather than one single growth factor in the CM cocktail may be responsible for triggering the positive response [43].

Many animal studies have investigated the effects of conditioned media on hair growth and have shown its positive effects in promoting hair regeneration [40] [44] [45] but only a few clinical studies have investigated the effects of CM based therapies on the hair growth in humans. In one randomized, double blind, vehicle controlled study, 38 patients with androgenetic alopecia were assigned to the active group with twice self-application of adipose tissue derived stem cell constituent extract topically over the scalp. The phototrichogram at week 8 showed increase in hair count in the active group as compared to the control and remained significant till week 16 (28.1% vs. 7.1%) and significant increase in hair diameter was observed after 16 weeks (14.2% vs. 6.3%) [46]. In another retrospective, observational study in 27 patients with female pattern hair loss who were treated with adipose tissue derived stem cells derived conditioned media (ADSC-CM) showed efficacy in increasing hair thickness and hair density after 12 weeks of therapy [18]. The therapeutic potential of ADSC-CM was established both in male and female pattern hair loss through a pilot study in 52 subjects who received CM monotherapy using microneedles for 12 weeks [17]. Similar findings were reported in this study with daily application of the product for 24 weeks. Hair product Trichosera® showed significant improvement in hair growth rate (18.66%), hair density (11.54%), hair fall rate (48.65%), hair tensile strength (41%) and improvement in flaking from the scalp (29.07%) at week 24.

The use of CM as a medium for hair regeneration has numerous advantages. This includes its immuno-compatibility, being a cell-free state, it does not require donor-recipient match which may be pre-requisite in other cell therapies [43]. Other advantages include lower time and cost of production of CM with

use as off the shelf product with long shelf life, can achieve mass production, freeze drying, easy packaging, transportation and storage which makes it more economical and practical for clinical use [43] [47]. However, the use of CM for hair regeneration has several challenges which may need to overcome. The main challenge is the type and level of growth factors which may be variable, depending on the source of the cells, passage number and the culture conditions of the stem cells [48] [49]. So selecting the correct source of stem cells, age of donors and quantifying growth factors/cytokines at different passages may be important to obtain the correct set of factors at the correct stage for use of CM [43]. This may have to be standardized to obtain the CM with a consistent composition. Additionally, to raise the content of bioactive factors, culture conditions have to be optimized, such as use of normoxia/hypoxia conditions, use of monolayer/3D cultures/bioreactors. The main safety concerns of tumorigenicty potential which may be associated with stem cell transplantation will not be present on use of CM [50] [51]. To avoid transmission of pathogens it is recommended to use CM from cells cultured in xeno free media which will further ensure the safety of CM in clinical use [52]. The CM used for the manufacturing of the final product Trichosera® is produced from culturing of pooled bone marrow derived MSCs from different donors under GMP conditions. The levels of the important growth factors have been quantified which includes VEGF (2000 - 2500 pg/ml), IGF (50 - 55 pg/ml), PDGF (350 - 400 pg/ml), KGF (350 - 400 pg/ml) and level of these factors were consistent batch to batch. The CM used has also been tested for all possible infectious diseases like HIV, HCV, HBV, HTLV, CMV, EBV and parvovirus B19, hence the final product used is completely safe.

In this study, the hair density and hair growth rate increased significantly by 11.54% and 18.66% at week 24 when compared with baseline values. This improvement is comparable with the results of studies on the efficacy of conventional treatment in AGA. In the previous study of 157 patients with male pattern of hair loss, 5% minoxidil application for 48 weeks increased the hair density by 12.3% which is comparable with this study [53]. In another study on the use of finasteride in 779 patients with male pattern hair loss, it was found to increase the hair density by 11.0% after 52 months of treatment [54]. As the duration of treatment using Trichosera® was relatively short (24 weeks), it can be cautiously concluded that the efficacy of the product is not inferior to those of conventional available therapies for alopecia. Further it has been shown that minoxidil can be used as an adipose tissue stem cells (ASC) preconditioning agent for hair regeneration as minoxidil stimulates the secretion of growth factors by ASCs which may enhance hair growth by promoting dermal papillae proliferation [55]. So, CM derived hair regenerative products can be considered in alopecia treatment or as add-on-therapy with existing conventional treatment in patients with alopecia.

The safety of the product has also been proven in this study with no adverse events or no local intolerance reported. As per dermatologist assessment, each

subject showed dryness and oiliness during the study period. The noted reactions were resolved subsequently without any intervention. Further as per subjects evaluation few of them experienced dryness, itching hair fall, oiliness and dandruff during the study. But the noted reactions were not significant in response to the product application. So, no product related reactions were noted and the product was dermatologically safe to be used in the subjects.

This study is valuable as it has several advantages. First, this is one among the published studies which has shown positive results in the efficacy and tolerability of BM-MSCs derived CM in hair growth. Second, the study has used local non-invasive application of the product without any delivery tools like micro needles. Thirdly, the subjects were followed-up with daily application for 24 weeks which is sufficient to show the efficacy of the hair regenerative product. Lastly, the measurements of hair regeneration were done objectively using phototrichogram images that allowed more quantitative assessments than those by using photographs based analysis by the dermatologists or self-assessments by the subjects which are being used by other published studies [49] [56]. There are few limitations of the study. Firstly, the total number of subjects in the study was adequate to show statistical significance but the population of total male subjects enrolled may be considered small for verifying efficacy in male pattern hair loss. Second, our data may not be generalizable as the data is from a single centre study. Lastly, the study is an open label, single arm, and non-comparative study design lacking the placebo group. Further, no histological examination was done to demonstrate efficacy of the product.

Based on the existing data, it can be concluded that application of CM based Trichosera® solution directly to the scalp is safe and efficacious for treatment of alopecia and it also may be used as an add on therapy with the existing conventional treatment using either finasteride or minoxidil for alopecia. However, similar studies with larger sample size need to be done to demonstrate continued efficacy of use of CM based regenerative products for hair growth.

Statements

Statement of Ethics

The study was conducted in accordance with GCP guidelines and was reviewed and approved by Clinicom Ethics committee, approval number MSCR/SRHS/2016-01. Informed consent was obtained from all the subjects participated in the study.

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Author Contributions

Conceptualization: PKG, UK
Methodology: SB, RS

Investigation: SB
Formal Analysis: PKG, SK, RS, UK
Writing, Review & Editing: PKG, SB, SK
Approval of the final manuscript: PKG, SB, RS, SK, UK

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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