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ORIGINAL CONTRIBUTION

Novel bioactive formulation derived from the conditioned medium of mesenchymal stromal cells reduces under-eye dark circles in human volunteers

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Abstract

Background: Under-eye dark circles are a common condition observed in dermatology practice. Mesenchymal stromal cell-derived conditioned medium (MSC-CM) contains an array of growth factors and cytokines reported to promote periorbital rejuvenation and may be useful in removing the dark circle around the eyes.

Aims: The aim of the present study was to evaluate the safety and efficacy of developed bioactive formulation containing mesenchymal stromal cell-derived conditioned medium in reducing the under-eye dark circles.

Patients/Methods: We tested the safety profile of MSC-CM along with antioxidants, in vitro using human melanocytes cultures. The bioactive formulation containing MSC-CM was developed and tested for physicochemical parameters. The dermatological safety was evaluated by primary irritant patch-test under complete occlusion on healthy human subjects. To elucidate its safety and efficacy, monocentric, open-label, single-arm study was carried out in 20 Indian female subjects for the duration of 12 weeks. Parameters such as eye puffiness, radiance, skin smoothness, even skin tone, periorbital fine lines and wrinkles, crow's feet, whitening, pigmentation, skin tightening, and refreshing/soothing effect were used to investigate the rejuvenating property of the bioactive formulation.

Results: Mesenchymal stromal cell-derived conditioned medium along with antioxidants decreased the melanin content compared to the CM alone in the melanocyte cultures. Besides, the bioactive formulation was safe and emerged as a non-irritant product. Improvement in the majority of the clinical parameters assessed through efficacy study was observed within 4 weeks of topical application of the formulation twice daily, and showed continued improvement for 12 weeks as evaluated by the dermatologists as well as self-assessment by the subjects.

Samatha Bhat and Muthukumar Amirthalingam contributed equally to this work.

Conclusion: The bioactive formulation containing MSC-CM was safe and effective in reducing the under-eye dark circles and was beneficial in improving the overall appearance of the eye area.

KEYWORDS
bioactive formulation, human volunteer study, in vitro study, under-eye dark circles

1 | INTRODUCTION

Dark circles under the eyes are one of the top beauty concerns worldwide. Dark circles are defined as bilateral, homogeneous hyperchromic macules, and patches primarily involving the lower eyelids but also sometimes extending toward the upper eyelids, eyebrows, malar regions, temporal regions, and the lateral nasal root.[1,2] It is also termed as 'periorbital melanosis', 'infraorbital skin discoloration', 'infraorbital darkening', 'idiopathic cutaneous hyperchromia of the orbital region', or 'infraorbital hyperchromia'.[3,4] The causative factors include genetic, dermal/epidermal melanin deposition, excessive pigmentation, excessive vascularity, periorbital edema, post-inflammatory hyperpigmentation secondary to atopic, and allergic contact dermatitis, tear trough associated with aging and shadowing due to skin laxity.[4,5] In humans, melanin is the primary factor contributing to skin color. Melanin synthesis occurs in the melanosome, an organelle containing pigment-producing enzymes, and is known to be regulated by several factors such as cytokines, growth factors, hormones, differentiation factors, and compounds from natural origin.[6,7] Mesenchymal stromal cell-derived conditioned medium (MSC-CM) contains several growth factors and cytokines (GFs/CKs), enzymes, chemokines, small molecules, and extracellular matrix proteins, which play a significant role in skin rejuvenation probably by reducing melanin synthesis.[8,9] These factors secreted by MSCs are used as ingredients in the preparation of many biopharmaceuticals and personal care products due to their wide range of biological functions.[10,11] MSC-CM containing secreted factors has great potential to be used as a cosmetic ingredient, and when formulated with suitable excipients, could greatly reduce the appearance of dark circles around the eye area. MSC-CM is known to promote skin regeneration, hair growth, and wound healing.[12] Formulation for skin rejuvenation is developed by mixing CM obtained from adult human bone marrow-derived mesenchymal stromal cells (BM-MSCs) along with suitable excipients developed into a cosmetic serum. Previously, we have identified 30 GFs/CKs secreted by BM-MSCs in our culture conditions, a few of which have been involved in repair and regeneration of damaged tissue.[13,14] Under Good Manufacturing Practice (GMP) conditions, using a novel production technology, MSC-CM is produced and used for developing the cosmetic product. Previously, we have developed and tested anti-aging cosmetic skin serum containing MSC-CM and reported its beneficial effect in skin rejuvenation.[12]

The available treatment options for under-eye dark circles include topical (e.g., chemical peels, demelanizing agents, sunscreens, moisturizers, gels, and anti-aging lotions) and surgical treatment (e.g., facelifts, laser surgery, dermal fillers, and dermabrasion). Most of these treatments are used for treating melasma.[5] However, very few studies have tested employing MSC-CM containing GF/CKs as a bioactive ingredient for the treatment of under-eye dark circles. In the present study, the safety of MSC-CM along with antioxidants such as kojic acid and ascorbic acid (excipients of the formulation developed) was studied in vitro using melanocyte cultures. The effect of MSC-CM with antioxidants in reducing the melanin content was investigated. In vitro skin permeation was carried out to determine the penetration of formulation into the skin. The under-eye dark circle formulation containing MSC-CM as the active ingredient was developed, and its safety and efficacy were evaluated in a clinical study.

2 | MATERIALS AND METHODS

2.1 | Production of conditioned medium derived from mesenchymal stromal cell cultures

Equal proportions of human BM-MSCs from three different healthy voluntary donors were pooled, cultured as described previously for production of conditioned medium.[15] Briefly, the cells were grown up to passage 5 in complete medium containing 1× Dulbecco's Modified Eagle's Medium-Knockout™ (Gibco) supplemented with 10% fetal bovine serum (FBS) (Hyclone), 1× Glutamax™ (GIBCO), 2 ng/ml bFGF (Sigma Aldrich) and incubated in a 5% CO_2 incubator (Binder) at 37°C. Conditioned medium was collected from cells (passage 5), upon attaining 80%–90% confluency. One portion of the CM was concentrated 10 times (10×) using Millipore 1 KDa cut-off filters by employing the Tangential Flow Filtration (TFF) technology (Merck-Millipore) as described previously.[12] The non-concentrated and 10× concentrated CM (10× CM) were stored at −80°C until further use. The control medium (complete medium not used for BM-MSC culturing) was also concentrated 10 times and stored at −80°C until use.

2.2 | Melanocyte culture and MTT assay

The safety profile of 10× CM was tested in vitro using human melanocyte cells. Cells (5×10^3 per well) were seeded in a 96-well microtiter plate with Ham's F-10 medium supplemented with 5% FBS,

0.1 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich), 10 ng/ml basic fibroblast growth factor (bFGF) (Sigma-Aldrich) and 85 nM 4-Phenylbutyric acid (PBA) (Sigma-Aldrich). Cells were incubated in a CO_2 incubator at 37°C. The next day, the cells were treated with different concentrations of 10× CM (5%, 10%, 25%, and 50% diluted with DMEM-KO) alone or in combination with 0.5% ascorbic acid and kojic acid, or kojic acid + ascorbic acid without CM. The treated cells were further incubated in a CO_2 incubator for 48 h. MTT assay was carried out to assess the viability of cells post-treatment. 5 mg/ml MTT (Thermo Fisher Scientific) was added to individual wells, incubated for 4 h at 37°C. Subsequently, supernatant was discarded, formazon crystals were solubilized in DMSO (Sigma-Aldrich) and absorbance was measured at 570 nm using a Varioskan™ microplate reader (Thermo Fisher Scientific). The experiment was performed in triplicates, and the results are represented as mean ± standard deviation.

2.3 | Determination of melanin content

The potential of 10× CM in reducing melanin content in vitro was tested in human melanocytes as described previously.[8] Briefly, cells (50 × 10^3 per well) were seeded in a 6-well microtiter plate and cultured as described above. The next day, cells were treated with different concentrations of 10× CM (10% and 25% diluted with DMEM-KO) alone or in combination with 0.5% ascorbic acid and kojic acid or 0.5% kojic acid + ascorbic acid without CM. The treated cells were incubated in a CO_2 incubator for 48 h. Following this, the cells were washed with 1×PBS, lysed using 1 N NaOH containing 10% DMSO, and centrifuged for 15 min at 3500 rpm. The supernatant was collected, and the absorbance was measured at 490 nm using Varioskan™ microplate reader. The experiment was performed in duplicates, repeated twice and the results are represented as mean ± standard deviation.

2.4 | In vitro skin permeation study

The skin of Wistar rats weighing approximately 200–250 g was used for the study. The experimental protocol was approved by the institutional animal ethics committee. The back portion of the Wistar rat was shaved on the previous day of the experiment without causing any damage to the skin. On the day of the experiment, the skin was dissected out and the fat was removed carefully. The skin was mounted in a vertical type diffusion cell with the stratum corneum facing the donor compartment (where the formulation was applied), whereas the dermis facing the receptor compartment. The latter compartment was filled with 0.2 M phosphate buffer of pH 7.4, which served as receptor fluid. The receptor phase was stirred constantly at 300 rpm and 37°C with the help of a stirrer bar on magnetic stirrer.[16] A portion of 0.5 g of the formulation was spread evenly over the entire membrane area. The volume of the medium receptor was 20 ml, and the area available for diffusion was 1.96 cm^2. The stirring system was activated, and samples of 2.0 ml of the receptor phase were collected at different intervals of time. Fresh buffer solution was added to receptor compartment to maintain the initial volume. The amount of kojic acid (KA) and ferulic acid (FA) (two of the excipients present in the formulation) permeating into receptor compartment across the skin was quantified using HPLC. At the end of the skin permeation study, the amount of KA and FA retained in the skin was assessed. Following withdrawal of samples of in vitro permeation study, the skin was cleaned and completely homogenized using a high-speed homogenizer (IKA T 25 digital) in phosphate buffer of pH 7.4. The solution was passed through 0.45 μm membrane and subjected to HPLC analysis to estimate the content of KA and FA.[16,17]

2.5 | Preparation of bioactive formulation

We have reported the enhanced anti-aging effects of 10× BMMSC CM at a concentration of 5% and 10% in our previous publication.[13] Based on the preformulation and in vitro cell line safety results, the 10× CM at 10% concentration was selected in the present study for formulation development. The in vitro cell line efficacy studies showed that the 10× CM, when combined with the antioxidants was effective in inhibiting the melanin content than CM alone. The formulation was developed using 10% of 10× CM as an active ingredient along with antioxidants and a unique combination of the cosmetic excipients. First, the polymer xanthan gum was dissolved in purified water along with allantoin and DL-panthenol. To this mix, solan E dissolved in sterile water was added. Following this, other excipients such as glycerin, methyl gluceth-20, Optasense-RMA IS, vitamin-E and Euxyl PE 9010, kojic acid, ferulic acid, ascorbic acid, and eyedeline was added and mixed well to form a cosmetic formulation. Finally, the 10× conditioned medium was added and mixed for 20 min to get a homogenous bioactive formulation (Table 1).

2.6 | Testing the developed formulation for pH, total protein content (TPC), total aerobic microbial count (TAMC), total combined yeasts, and molds count (TYMC), pathogens

The pH value of the developed formulation was measured using a pH meter (ThermoFisher Scientific). The total protein content in the formulation was estimated by the Bradford method at 595 nm spectrophotometrically. The microbial assays such as TAMC, TYMC, and the test for the pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *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 30–35°C and

TABLE 1 Composition of the bioactive formulation and its functions

S. No.	Ingredients	Function	Percentage (%)
1.	Concentrated conditioned Medium (10× CM)	Cosmetic active ingredient	10
2.	Xanthan gum	Bulking agent	1
3.	Allantoin	Soothing agent/skin conditioner	0.2
4.	Solan E	Skin conditioner	0.2
5.	Glycerin	Humectant	2
6.	Methyl gluceth-20	Humectant	2
7.	Optasense RMA IS	Skin conditioner	0.2
8.	Euxyl PE 9010	Preservative	1
9.	DL-Panthenol	Skin conditioner	0.2
10.	Ascorbic acid	Antioxidant/skin conditioner	0.5
11.	Ferulic acid	Antioxidant/skin conditioner	0.3
12.	Kojic acid	Antioxidant/skin conditioner	0.5
13.	Propylene glycol	Solvent	5
14.	Vitamin E	Skin conditioner	0.6
15.	Eyedeline	Skin conditioner/Eye puffiness reducer	2.5
16.	Purified water	Solvent	73.8

TABLE 2 Draize scale for scoring at the treatment site for patch test

Score for erythema	Reaction	Score for edema	Reaction
0	No reaction	0	No reaction
1	Very slight erythema/dryness with shiny appearance	1	Very slight edema
2	Slight erythema/dryness/wrinkles	2	Slight edema
3	Moderate erythema/dryness/wrinkles	3	Moderate edema
4	Severe erythema/wrinkles/scales	4	Severe edema

20–25°C for 5 days for TAMC and TYMC, respectively. 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.

2.7 | Primary irritant patch test (PIPT)

The dermatological safety of the bioactive formulation was evaluated using a closed patch test under complete occlusion for a period of 9 days on healthy human subjects. The study method was based on the Bureau of Indian Standards method 4011:2018 (third revision). Sodium lauryl sulfate (SLS) (1% w/w solution) was used as a positive control, and normal saline (0.9% w/w solution) was used as a negative control. A total of 24 healthy subjects in the age group of 18–65 years, satisfying the inclusion-exclusion criteria were enrolled in the study after obtaining the written informed consent. The study was approved by an independent ethics committee and conducted in accordance with good clinical practice guidelines. Subjects with normal, dry, oily skin type, or combination of these were included in the study after satisfying the inclusion/exclusion criteria. The test site was between the scapulae and waist of the subjects, free of pigmentation, pimple, coarse hair, mole, or any dermatological conditions that can interfere with the reading and was examined for baseline skin condition before patch application by a dermatologist. The patch at the test site was applied using the Dermaproof® aluminium finn chamber and was retained till 24 h post-application. Positive and negative control was also applied to the designated sites. The patch was removed after 24 h, and the test site was assessed for erythema/dryness/wrinkles and edema, post-patch removal by dermatologists at 0 h patch removal for irritation reactions, at 24 h post-patch removal for immediate reactions, and 7 days post-patch removal for delayed reactions as per the Draize scale for scoring (Table 2).

2.8 | Clinical study design

A monocentric, open-label, single-arm, and efficacy study was carried out in Indian female (n = 20) subjects in the age group of 18–65 years. The inclusion criteria were as follows: subjects having one or more of the periorbital skin conditions, which include moderate to severe form of under-eye dark circle, wrinkles, eye puffiness, pigmentation, periorbital fine lines, and wrinkles, and crow's feet. The

exclusion criteria were as follows: (1) Subjects who are undergoing treatment for the under-eye dark circles (other than topical application) within 3 months before screening into the study, (2) Pregnant and lactating women, (3) History of hypersensitivity to any ingredients of the test product, (4) Subjects on any concomitant therapy, dermatological disorder or surgical treatment on the face that may interfere with the study results. The primary objective was to evaluate the efficacy of (0.1 g per dose) application of bioactive formulation twice daily over 12 weeks period and assessing the reduction in clinical parameters related to periorbital skin conditions (such as under-eye dark circles and eye puffiness). The secondary objective was to assess the product safety, tolerance, and acceptability. The study was carried out in accordance with International Conference on Harmonization-Good Clinical Practice (ICH-GCP) guidelines and approved by the institutional ethics committee. Informed consent was obtained from all the subjects involved in the study. Subjects were asked to stop the usage of any other facial product and were instructed to apply the test product twice daily (morning and evening) as per the directions for use. The first application of the product was conducted under the supervision of the study personnel at the site.

2.9 | Subject assessment and visits

The study was conducted for a period of approximately 12 weeks for each subject which included a total of 4 visits - visit 1 (screening visit), visit 2 (1st follow-up visit at 4 weeks post-treatment), visit 3 (2nd follow-up visit at 8 weeks post-treatment), and visit 4 (3rd follow-up visit at 12 weeks post-treatment). Various clinical parameters such as eye puffiness, even skin tone, radiance, periorbital fine lines and wrinkles, crow's feet, skin brightening/whitening, pigmentation, skin tightening, skin smoothness and texture, skin refreshing/soothing, overall appearance of the eye area were assessed by the dermatologists. Subjects were asked to rate the condition of their periorbital skin using a self-assessment questionnaire. The grading scale for the evaluation of clinical parameters by dermatologists is given in Table 3. Grading by dermatologists was noted, and a change from baseline was calculated as improvement according to the grading scale. A shift in subject's self-assessment scores from no change to improvement (slight, moderate, or large) was considered as positive, and the percentage of subjects showing improvement was calculated.

2.10 | Statistical analysis

The data analysis was performed using Graph Pad Instat (Trial Version). The statistical analysis for the in vitro studies was performed using paired and unpaired *t*-test. The clinical study data analysis was carried out using Microsoft Excel and Graph Pad Instat and summarized with summary statistics, including mean and standard deviation. The *p*-value less than 0.05 ($p < 0.05$) was considered as statistically significant.

TABLE 3 Dermatological assessment of various clinical parameters to determine the efficacy of the test product

Grade	Reaction
0	No improvement
1	1%–24% improvement
2	25%–49% improvement
3	50%–74% improvement
4	75%–100% improvement

3 | RESULTS

3.1 | hBM-MSC-CM along with antioxidants was non-toxic to melanocyte cultures and reduced the melanin content in vitro

The in vitro safety study of BM-MSC-CM on melanocyte culture showed that the viability of melanocytes was not affected by treatment with CM. MSC-CM (10×) alone or in combination with antioxidants did not reduce the cell viability after 48 h of treatment when compared to control confirming that the MSC-CM was non-toxic to melanocytes (Figure 1A). However, treatment with antioxidants reduced the cell viability when compared to control. The role of BM-MSC-CM in skin pigmentation has not been well explicated. Melanin inhibition assay was conducted to investigate the (1) effect of BM-MSC-CM on melanogenesis and (2) to understand the extent of inhibition of the melanin content when treated along with antioxidants. Primary melanocytes were treated with 10× CM derived from BM-MSCs alone, along with ascorbic acid, and kojic acid (antioxidants used in the formulation) or ascorbic acid + kojic acid without MSC-CM. Cells incubated with MSC-CM along with antioxidants or antioxidants without CM showed reduction in the melanin content than that of MSC-CM alone although not significant. However, cells incubated with MSC-CM along with antioxidants or antioxidants without CM showed significant reduction in the melanin content when compared to control medium (Figure 1B). Collectively, these results suggested that BM-MSC-CM, along with antioxidants reduced the melanin content in vitro without inducing any cytotoxicity.

3.2 | In vitro skin permeation study results

The amount of KA and FA permeating across the rat skin and the amount of these actives deposited within the skin was determined by HPLC and the results are shown in Figure 1C. The amount of KA and FA permeated at the end of 12 h ($Q_{12\ h}$) was observed to be 49.10 ± 2.83 and 7.46 ± 0.25 μg/cm^2, respectively. Between the two actives tested, highest skin permeation was observed with KA compared with FA. After 12 h, skin content of KA was observed to be 65.18 ± 0.25 μg/cm^2, while that of FA was 10.88 ± 0.48 μg/cm^2. It is

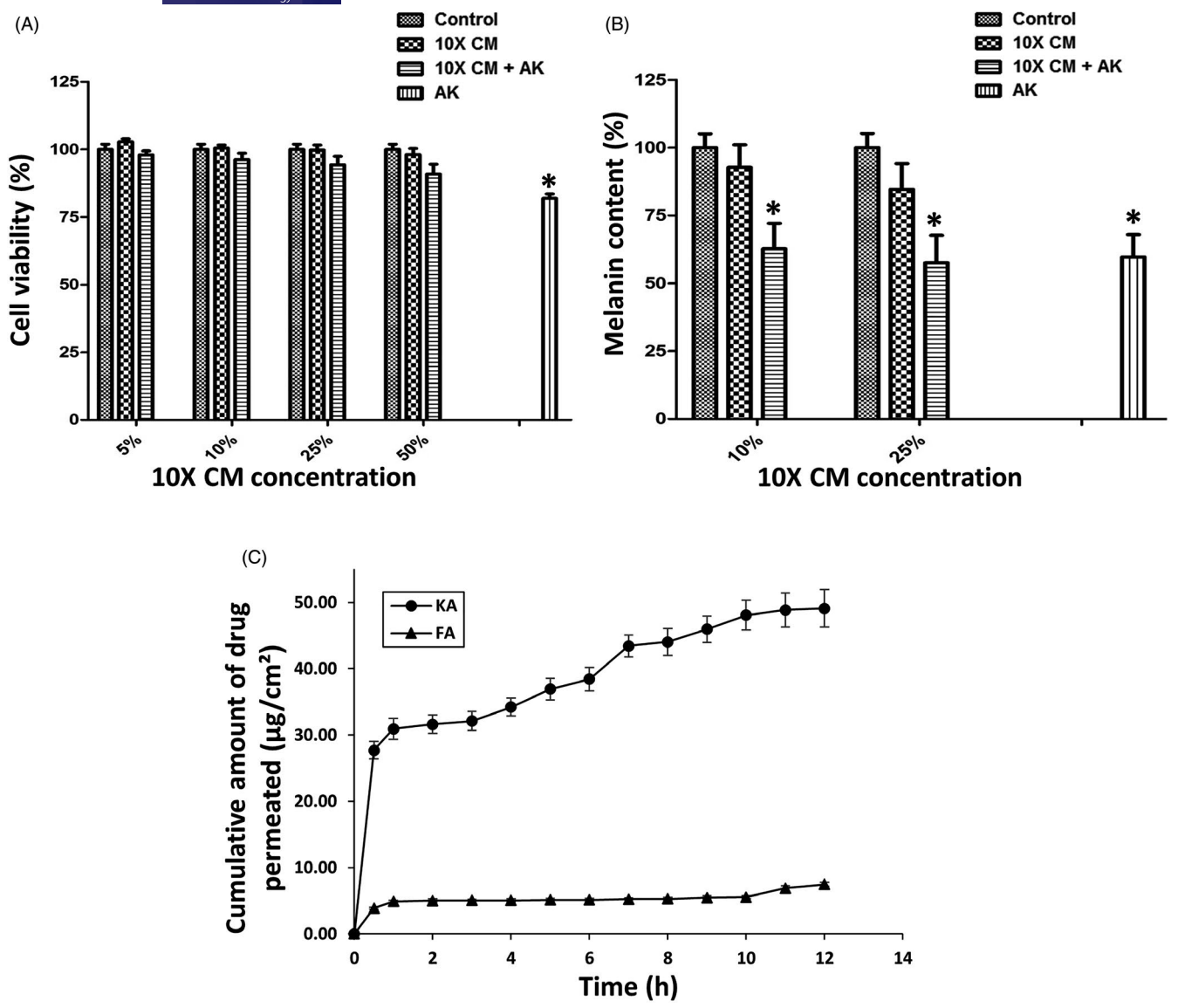

FIGURE 1 In vitro Cell viability assay and melanin inhibition assay of 10× conditioned medium (10× CM) along with antioxidants in melanocyte culture. (A) Bar graph showing the viability of melanocytes after treatment with 10× CM alone or in combination with antioxidants at the concentrations of 5%, 10%, 25%, and 50%. The viability of cells was not affected at the concentration tested, indicating that CM was non-toxic to the cells. (B) Bar graph showing the percentage of melanin content after treatment with 10× CM alone or in combination with antioxidants (ascorbic acid + kojic acid) at 10% and 25% concentration. A significant decrease in the melanin content was observed when treated with 10× CM along with antioxidants compared with the CM alone in the melanocyte cultures. The data are represented as mean ± SD ($n = 2$; $^*p < 0.05$). 10× CM, 10× conditioned medium; 10× CM+AK, 10× conditioned medium with ascorbic acid and kojic acid; AK, Ascorbic acid + Kojic acid. (C) In vitro skin permeation study of kojic acid (KA) and ferulic acid (FA) present as excipients in formulation across Wistar rat skin determined by HPLC

quite evident that both KA and FA were preferentially deposited in the skin rather than undergo permeation which is beneficial in mediating the required local therapeutic activity.

3.3 | Bioactive formulation results

The prepared bioactive formulation was subjected to various tests such as pH, TPC, TAMC, TYMC, and test for pathogens. The pH of the formulation was 4.1 ± 0.02. The total protein content was 2.14 ± 0.16. The TAMC and TYMC were <10 CFU/g, and the pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans* were absent in 1 g/ml product and *Salmonella* sps. in 10 g/ml 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 was stable at acidic pH, and the formulation was free from the microbial contaminants.

3.4 | Bioactive formulation was dermatologically safe and qualifies into non-irritant category as assessed by patch application test

All 24 subjects enrolled in the study completed the study. The average mean irritation score for each tested formulation is mentioned in Table 4. The mean irritation score for the bioactive formulation was 0.08, 0, and 0 at 0 h, 24 h, and 7 days post-patch removal, respectively, indicating that the formulation was non-irritant for all skin types (Table 4). The mean irritation score for negative control was 0 and for positive control was 2.33, 2.33, and 0.17 at 0 h, 24 h, and 7 days post-patch removal confirming as an irritant at 0 and 24 h post-patch removal (Table 4). Thus, the investigational bioactive formulation emerged as non-irritant product for human application and deemed to be dermatologically safe.

3.5 | Bioactive formulation was effective in reducing the under-eye dark circles as evaluated by clinical study

A total of 20 female subjects were enrolled in the study, out of which 16 subjects completed all three follow-up visits, three of the subjects completed only first two follow-up visits, and one subject completed only the first follow-up visit. The subjects were assessed for 10 clinical parameters associated with periorbital skin conditions during each follow-up visit post-application of the formulation. The subjects were observed for the clinical significance and toxicity symptoms in comparison with the baseline and graded by the dermatologists as per Table 3. The parameters evaluated for all the subjects, and its grading by dermatologists is given in Table 5. The subjects who could not complete the 2nd and/or 3rd follow-up visit were also included in calculating the improvement from baseline visit. The majority of the subjects showed improvement within 4 weeks of usage with significant continuous improvement during subsequent weeks compared with the baseline. There was a significant reduction in under-eye dark circles (Figure 2A–D), eye puffiness (Figure 2D), periorbital fine lines, wrinkles, and crow's feet (Figure 2C,D) (dermatological assessment). The percentage of subjects who showed good improvement (grade 1 and above) from baseline for each of the parameters as assessed by dermatologists is shown in Figure 3A–I. The percentage of subjects with improvement in the overall appearance of the eye area assessed by dermatologists and the percentage of subjects who felt overall improvement as determined by self-assessment questionnaire is shown in Figure 4A,B, respectively. The percentage of subjects showing improvement of 50%–100% (Grade 3 and above) is shown in Figure 4C. The adverse events such as erythema, scaling, and pruritus were not observed in any of the subjects during the study period, and the product was considered overall safe. Hence, based on the 12 weeks safety and efficacy data, it can be concluded that the test product is safe and effective in reducing the under-eye dark circles, eye puffiness, and other associated skin conditions.

4 | DISCUSSION

Under-eye dark circle is a common condition affecting both men and women of all ages globally. Being an aesthetic concern, the under-eye dark circle is reported to have a negative emotional impact on the well-being of individuals. Under-eye skin is one of the first areas to show the symptoms of aging. Various factors linked with induction of the under-eye dark circle include hyperpigmentation, poor sleeping habits, allergies, reduction in the fatty tissue in and around the skin of the eyes, and skin thinning.[2] A variety of treatment modalities is currently being used to treat the under-eye dark circle condition such as chemical peeling, autologous fat transplantation, and laser treatment. However, these methods have several disadvantages, which include cost, needs multiple visits, less effective in patients having thin and translucent skin.[2,5] This suggests the need for an alternative and cost-effective method to reduce dark circles.

One of the most common causes of an under-eye dark circle is hyperpigmentation due to excessive synthesis of melanin. The basic, preclinical, and clinical studies have shown that the mesenchymal stromal cell secretome has an intrinsic property to promote tissue repair and regeneration.[18,19] Currently, systemic and topical application of stem cell-derived products are extensively being used for cosmetic applications.[20] The secretome of the conditioned medium derived from the MSCs contains growth factors, chemokines, cytokines, extracellular matrix proteins, enzymes, miscellaneous proteins, besides other unknown factors, which plays major role in tissue regeneration and repair.[8,9] Hence in the present study, we evaluated the safety and efficacy of novel bioactive formulation containing mesenchymal stromal cell-derived conditioned medium

TABLE 4 Average mean irritation score as per Draize scale for scoring irritation in patch test

S. No	Investigational product	Mean irritation score—0 h	Irritancy assessment	Mean irritation score—24 h	Irritancy assessment	Mean irritation score—7 days	Irritancy assessment
1	Perioptisera	0.08	Non-Irritant	0.00	Non-Irritant	0.00	Non-Irritant
2	Positive control (1.0% w/w dilution)	2.33	Irritant	2.33	Irritant	0.17	Non-Irritant
3	Negative control (0.9% normal saline)	0.00	Non-Irritant	0.00	Non-Irritant	0.00	Non-Irritant

TABLE 5 Dermatological assessment grading of subjects for the clinical parameters evaluated to determine the efficacy of the bioactive formulation

Clinical parameters	Eye puffiness			Even skin tone			Radiance			Skin smoothness and texture			Periorbital fine line and wrinkles and crow's feet			Brightness/ whitening effect			Pigmentation			Skin tightening effect			Refreshing Effect			Overall appearance of the eye area		
Follow-up visits (weeks)	4	8	12	4	8	12	4	8	12	4	8	12	4	8	12	4	8	12	4	8	12	4	8	12	4	8	12	4	8	12
Sub 1	-	-	-	2	2	3	2	2	3	1	2	3	1	1	2	2	2	3	2	2	3	1	1	1	2	2	3	2	2	3
Sub 2	1	2	3	2	2	3	2	2	3	2	2	3	2	2	3	2	2	3	2	2	3	-	-	-	2	2	2	2	2	3
Sub 3	-	-	-	2	2	2	-	-	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Sub 4	1	3	3	1	3	3	1	2	2	1	2	2	1	2	2	1	3	3	1	3	3	1	2	2	1	2	2	1	2	3
Sub 5	1	2	2	1	2	2	1	2	3	1	2	2	1	1	2	1	2	3	1	2	2	1	2	2	1	2	2	1	2	2
Sub 6	-	-	-	1	2	3	2	2	3	2	2	2	1	1	2	2	2	2	2	2	2	0	1	1	0	2	2	2	2	2
Sub 7	1	2	3	1	2	3	1	2	3	1	1	2	1	1	1	1	1	2	1	2	3	1	2	3	1	2	3	1	2	3
Sub 8	-	-	-	1	2	-	1	2	-	1	2	-	1	2	-	2	2	-	1	1	-	1	1	-	1	1	-	1	2	-
Sub 9	2	2	3	2	2	3	2	2	3	2	2	2	2	2	2	2	2	3	2	2	3	2	2	2	2	2	2	2	2	3
Sub 10	1	1	-	1	1	-	1	1	-	1	1	-	1	1	-	1	1	-	1	1	-	-	-	-	1	2	3	1	1	-
Sub 11	-	-	-	1	2	4	1	1	4	1	2	3	1	1	1	1	2	4	1	2	4	-	-	-	1	2	2	1	2	4
Sub 12	1	2	3	1	2	3	2	2	3	1	2	2	2	3	3	2	3	4	2	3	4	1	2	2	1	2	2	2	3	3
Sub 13	1	1	1	1	2	3	1	2	3	1	1	2	1	2	3	1	2	3	1	2	3	1	1	2	1	1	2	1	2	3
Sub 14	-	-	-	0	1	2	0	1	2	0	1	1	0	1	2	1	1	2	1	1	2	-	-	-	0	1	1	0	1	2
Sub 15	-	-	-	1	2	2	1	2	2	1	2	2	1	2	2	1	2	2	1	2	2	1	2	2	1	2	2	1	2	2
Sub 16	1	2	-	1	2	-	1	1	-	1	1	-	1	2	-	2	2	-	2	2	-	1	1	-	1	2	-	1	2	-
Sub 17	1	-	-	1	-	-	1	-	-	1	-	-	1	-	-	1	-	-	1	-	-	1	-	-	1	-	-	1	-	-
Sub 18	1	1	3	1	1	3	1	2	3	1	1	1	1	1	1	2	2	3	2	2	3	1	1	3	1	1	3	1	2	3
Sub 19	1	1	2	1	1	2	1	1	2	1	1	2	-	-	-	1	2	2	1	2	2	-	-	-	1	2	2	1	2	2
Sub 20	2	4	4	2	4	4	2	4	4	2	4	4	1	2	3	2	4	4	2	4	4	-	-	-	2	4	4	2	4	4

[a]4, 8, 12 corresponds to follow-up visits at 4 weeks (visit 2), 8 weeks (visit 3), 12 weeks (visit 4), respectively.

[b]Out of 20 female subjects, 16 subjects completed all three follow-up visits, Three subjects completed only first two follow-up visits (Sub 8, Sub 10, and Sub 16) and 1 subject completed only the first follow-up visit (Sub 17).

[c]Eye puffiness, even skin tone, radiance, skin smoothness and texture, periorbital fine lines and wrinkles, brightening/whitening effect, pigmentation, skin tightening effect, refreshing/soothing effect and overall appearance of the eye was evaluated in 13, 20, 19, 20, 19, 20, 20, 14, 20, and 20 subjects, respectively, during the visit.

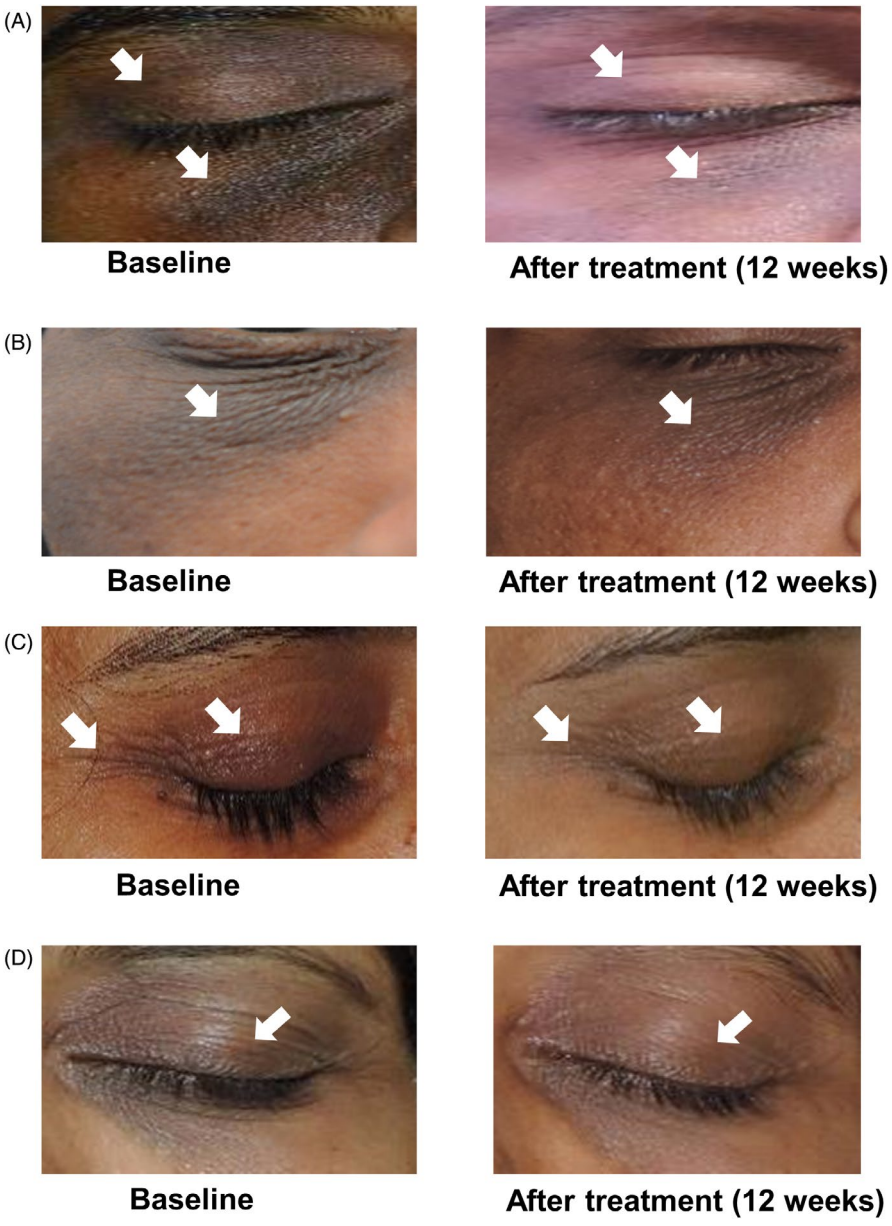

FIGURE 2 (A, B) Reduction in under-eye dark circles with Grade 4 improvement 12 weeks post-application of the formulation. (C) Reduction in under-eye dark circles, wrinkles, and crow's feet (Grade- 3 improvement) (D) Reduction in under-eye dark circles, eye puffiness, and wrinkles (Grade- 4 improvement). Grade 0: Baseline, Grade 1: 1%–24% improvement, Grade 2: 25%–49% improvement, Grade 3: 50%–75% improvement, Grade 4: 75%–100% improvement

in reducing the under-eye dark circles. The benefit of the present formulation over the previous ones is the use of MSC-CM along with antioxidants such as kojic acid and ascorbic acid in the formulation. Our study demonstrated that the novel formulation decreased the melanin content, was safe and emerged as a non-irritant product for use against under-eye dark circles in humans. The topical application of the formulation twice daily has shown significant improvement over 12 weeks. Several previous studies have demonstrated that the excessive synthesis and deposition of melanin in the skin is one of the vital causes of the under-eye skin darkening and hyperpigmentation.[1,2] Thus, targeting the excessive melanin production/deposition or enhancing the degradation of the deposited melanin can be used as a treatment strategy to overcome the under eye skin darkening problem. Previous studies have reported the in vitro skin permeation and longer retention of formulations containing kojic acid within porcine ear skin.[16] Also, slower skin permeation of ferulic acid when formulated within a carbopol gel system is reported, emphasizing the importance of the formulation additives in modulating the drug permeation and retention.[21] The decreased permeation and increased retention of ferulic acid within skin are attributed to the effect of pH of the formulation, with the unionized form preferentially being deposited within the skin.[22] Hence, the greater skin retention of ferulic acid and kojic acid in the present study can be attributed to the lipophilic constituents within the formulation that improve the localization of the bioactives and hence achieve therapeutic concentrations within the skin.

Previous studies have shown that the treatment of melanoma cells with MSC-CM reduced the expression of microphthalmia-associated transcription factor (MITF), which regulates melanin biosynthetic pathways. The expression of melanogenic enzymes involved in the development, proliferation, and survival of melanocytes is regulated by MITF.[8,9] Another study reported that the CM secreted by adipose

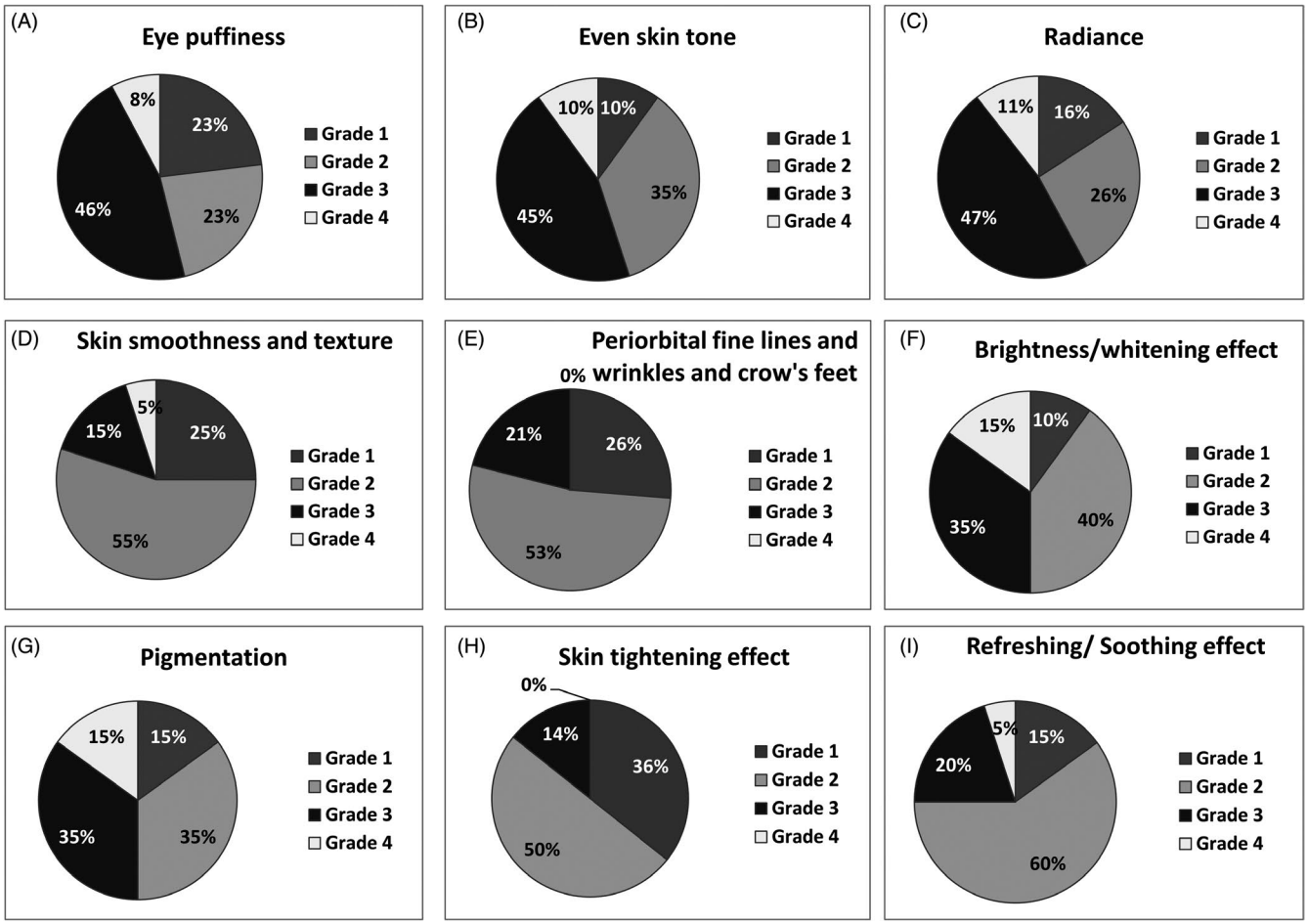

FIGURE 3 Dermatological assessment of clinical parameters associated with a periorbital skin condition. (A–I) Pie chart showing the percentage of subjects with significant improvement from the baseline at the end of 12 weeks for the nine clinical parameters evaluated. Grading is given by the dermatologists based on the percentage of improvement from the baseline for each subject. Reduction in the eye puffiness, periorbital fine lines, wrinkles, and crow's feet, pigmentation was felt by 70%, 26.7%, and 62.5% of the subjects, respectively. Improvement in the even skin tone, radiance, skin smoothness, and texture, skin brightness/whitening effect, skin tightening effect, refreshing/soothing effect was felt by 68.8%, 73.3%, 25%, 62.5%, 18.2%, and 29.5% of the subjects, respectively. Grade 0: Baseline, Grade 1: 1%–24% improvement, Grade 2: 25%–49% improvement, Grade 3: 50%–75% improvement, and Grade 4: 75%–100% improvement. The subjects who could not complete the 2nd and/or 3rd follow-up were also included in calculating the percentage showing improvement from baseline

stem cell (ASC) contained TGF-β1, IL-6, and TNFα. These cytokines were shown to downregulate the expression of melanin biosynthetic pathways genes, thereby inhibiting the pigment formation.[9] Our study showed that the treatment of melanocytes with MSC-CM along with antioxidants decreased melanin content, and application in human subjects reduced the under-eye dark circles. Besides, cell and molecular studies on melanocytes before and after treatment with MSC-CM are required to understand the precise molecular mechanisms and cell signaling pathways behind the inhibition of melanin synthesis. A study by Hwang and co-workers demonstrated that MSC-CM treatment enhanced depigmentation by downregulating the Wnt/β-catenin pathway and upregulation of Dickkopf-related protein 1 (DKK1). Thus, it is proposed that the inhibitor of Wnt/β-catenin signaling may be useful for decreasing melanin synthesis. Also, the inhibitory role of TGF-β1 on pigmentation independent of MITF is reported. Kim et al.[9] demonstrated that skin whitening upon treatment with ASC-CM is a TGF-β1 dependent event independent of MITF. This evidence suggests that the secreted factors present in the stem cell-derived CM contain active molecules that target multiple members of the melanin biosynthetic pathways and regulate the growth, proliferation, and survival of melanocytes, thereby promoting the depigmentation of the skin. In this direction, future studies are required to profile the secretome derived from MSC-CM, identify the key secreted molecules and their cellular targets involved in the inhibition of melanin production.

In this study, we showed that the application of the biocosmetic formulation showed early improvement in the periorbital skin conditions by reducing the under-eye dark circles. Rejuvenation of the skin in general and periorbital in particular is a complex process, which requires reduction in hyperpigmentation, fine lines, and wrinkles. Both environmental and genetic factors play critical role in the onset of hyperpigmentation and periorbital darkening.[5,23,24] This is one of

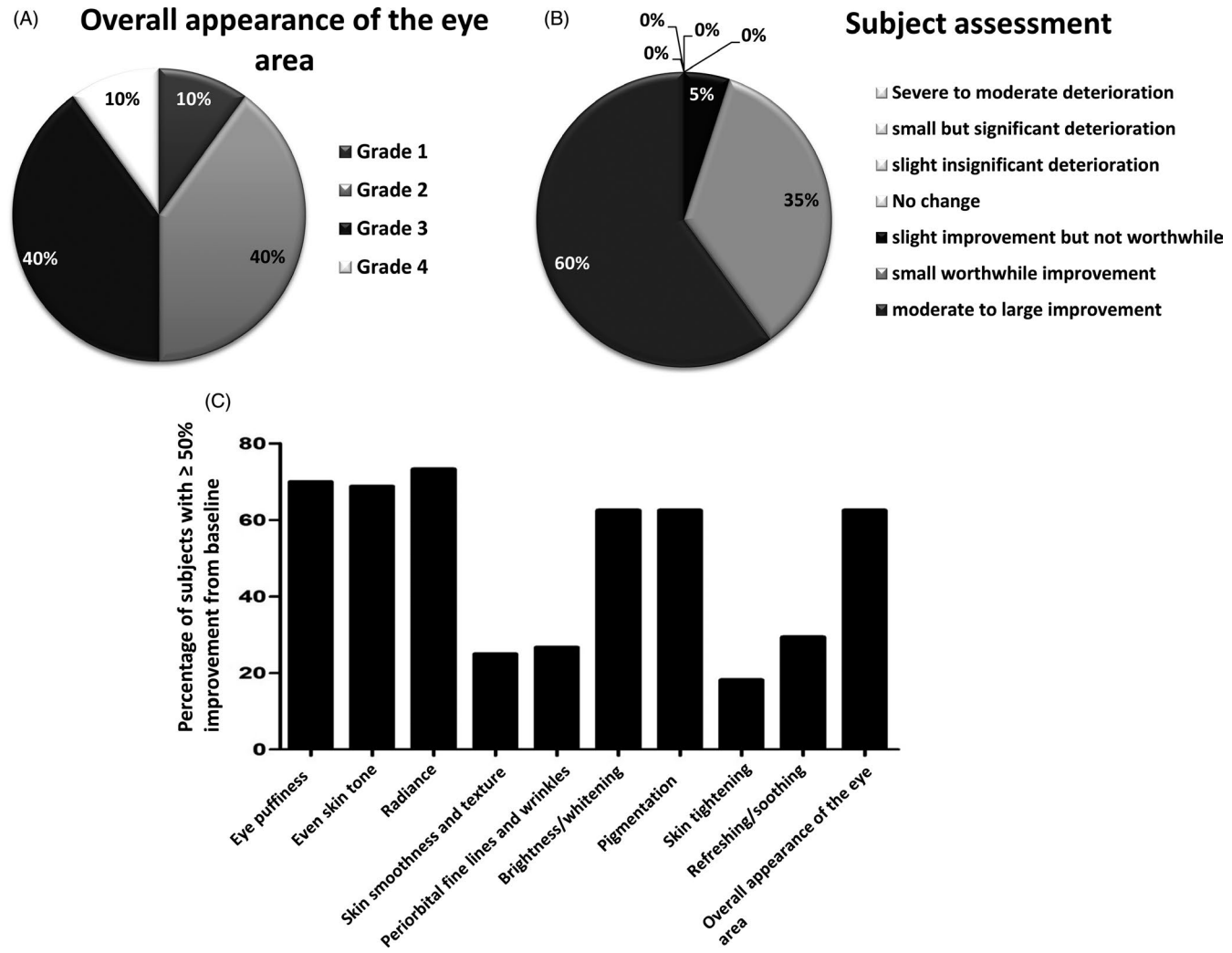

FIGURE 4 (A) Dermatological assessment of the overall appearance of the eye area. 40% of the subjects felt Grade 3 and Grade 4 improvement, followed by Grade 2 and Grade 1 improvement by 10% of the subjects. (B) Assessment of improvement by subjects—60% of the subjects felt moderate to large improvement upon usage of the formulation, whereas 35% of the subjects felt small worthwhile improvement and 5% of the subjects felt slight improvement. None of the subjects felt deterioration in the skin conditions upon usage of the product. (C) The proportion of subjects with ≥50% improvement (Grade 3 and above) for each of the parameters tested at the end of 12 weeks post- application of the formulation. Grade 0: Baseline, Grade 1: 1%–24% improvement, Grade 2: 25%–49% improvement, Grade 3: 50%–75% improvement, Grade 4: 75%–100% improvement. The subjects who could not complete the 2nd and/or 3rd follow-up were excluded from calculating the percentage showing improvement ≥50% from the baseline at the end of 12 weeks

the major cosmetic concerns with no standard treatment. Thus, cosmetic formulation that inhibits hyperpigmentation may be useful to improve the periorbital skin conditions. The most prominent change during aging is the wrinkling of the face skin. Periorbital skin is one of the major sites of wrinkles in the face due to its frequent movement and skin thinning. The wrinkles can arise due to natural aging leading to decreased thickening of the epidermis or dermis or via environmental stress. These stress inducers have been reported to target collagen and elastin fibers leading to skin thinning and wrinkle formation.[24] The topical application of our bioactive formulation has reduced the eye puffiness, periorbital fine lines, wrinkles, and crow's feet, hyperpigmentation and improved the skin smoothness and texture, radiance, skin brightness, skin tone suggesting the periorbital skin rejuvenation in clinical study.

Another condition linked to periorbital hyperpigmentation is oxidative stress and inflammation leading to excessive deposition of the pigments.[24,25] The formulation developed and used in this study contains ascorbic acid and kojic acid. Secreted factors from MSC-CM, ascorbic acid, and kojic acid have shown to possess anti-oxidant and anti-inflammation properties, which might have contributed to the improved periorbital skin conditions.[26,27] The ingredients used in some of the cosmetic formulation such as hydroquinone, kojic acid, vitamin C, growth factors, and others have shown to trigger irritation or worsen the skin condition.[26–29] However, the application of our in-house developed cosmetic formulation neither induced skin irritation nor worsened the skin condition. Thus taken together, the novel cosmetic formulation emerged as a safe and effective product and significantly promoted the rejuvenation of the periorbital skin.

The present study has few limitations: Firstly, use of small sample size for the clinical study. Secondly, this is a single-arm, open label, and non-comparative study design lacking the placebo group. Further, the comparative study with larger sample size covering wider population range adds more value to our current clinical study.

5 | CONCLUSION

The results of in vitro and clinical studies suggest that the novel bioactive formulation containing GF/CKs promote skin rejuvenation thereby improving the periorbital skin condition. The current study established the safety and efficacy of the test product without major side effects. Such topical products can be used as an alternative to surgical rejuvenation procedures or in combination, which may provide a synergistic effect beneficial for skincare.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION

S.B. performed the experiments, collected, analyzed, and interpreted the data and wrote the manuscript. M.A. designed the study and performed the experiments, and analyzed data. S.P. supported and coordinated the clinical study and collected the data. M.B.V. supported and coordinated the clinical study and collected the data. P.G. edited the manuscript, coordinated the clinical study. B.S.P. performed the experiments and compiled the data. S.M. designed the experiments and analyzed the data. R.N.S. designed and supervised the study, reviewed and approved the manuscript. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

1. Freitag FM, Cestari TF. What causes dark circles under the eyes? *J Cosmet Dermatol*. 2007;6(3):211-215.
2. Roh MR, Chung KY. Infraorbital dark circles: definition, causes, and treatment options. *Dermatol Surg*. 2009;35(8):1163-1171.
3. Balkrishnan R, McMichael AJ, Camacho FT, et al. Development and validation of a health-related quality of life instrument for women with melasma. *Br J Dermatol*. 2003;149(3):572-577.
4. Malakar S, Lahiri K, Banerjee U, Mondal S, Sarangi S. Periorbital melanosis is an extension of pigmentary demarcation line-F on face. *Indian J Dermatol Venereol Leprol*. 2007;73(5):323-325.
5. Sarkar R, Ranjan R, Garg S, Garg VK, Sonthalia S, Bansal S. Periorbital hyperpigmentation: a comprehensive review. *J Clin Aesthet Dermatol*. 2016;9(1):49-55.
6. Costin GE, Hearing VJ. Human skin pigmentation: melanocytes modulate skin color in response to stress. *FASEB J*. 2007;21(4):976-994.
7. Murakami M, Matsuzaki F, Funaba M. Regulation of melanin synthesis by the TGF-beta family in B16 melanoma cells. *Mol Biol Rep*. 2009;36(6):1247-1250.
8. Kim ES, Jeon HB, Lim H, et al. Conditioned media from human umbilical cord blood-derived mesenchymal stem cells inhibits melanogenesis by promoting proteasomal degradation of MITF. *PLoS One*. 2015;10(5):e0128078.
9. Kim WS, Park SH, Ahn SJ, et al. Whitening effect of adipose-derived stem cells: a critical role of TGF-beta 1. *Biol Pharm Bull*. 2008;31(4):606-610.
10. Muthukumar A, Seetharam RN. Stem cell derived cosmetic products: an overview. *Manipal J Med Sci*. 2016;1(2):46-52.
11. Pawitan JA. Prospect of stem cell conditioned medium in regenerative medicine. *Biomed Res Int*. 2014;2014:965849.
12. Sachdev M, Chullikana A, Seetharam RN, et al. A single arm, monocentric, prospective, open label study to evaluate anti-ageing efficacy of human growth factors and cytokines containing cosmetic skin serum in indian female population. *J Cosmet Dermatol Sci Appl*. 2017;7:118-132.
13. Balasubramanian S, Thej C, Walvekar A, et al. Evaluation of the secretome profile and functional characteristics of human bone marrow mesenchymal stromal cells-derived conditioned medium suggest potential for skin rejuvenation. *J Cosmet Dermatol Sci Appl*. 2017;7(1):99-117.
14. Park BS, Jang KA, Sung JH, et al. Adipose-derived stem cells and their secretory factors as a promising therapy for skin aging. *Dermatol Surg*. 2008;34(10):1323-1326.
15. Pal R, Hanwate M, Jan M, Totey S. Phenotypic and functional comparison of optimum culture conditions for upscaling of bone marrow-derived mesenchymal stem cells. *J Tissue Eng Regen Med*. 2009;3(3):163-174.
16. Goncalez ML, Correa MA, Chorilli M. Skin delivery of kojic acid-loaded nanotechnology-based drug delivery systems for the treatment of skin aging. *Biomed Res Int*. 2013;2013:271276.
17. Bai J, Yang C, Du S-Y, et al. Penetration and pharmacokinetics of ferulic acid after dermal administration. *Trop J Pharm Res*. 2019;18(4):843-849.
18. Baglio SR, Pegtel DM, Baldini N. Mesenchymal stem cell secreted vesicles provide novel opportunities in (stem) cell-free therapy. *Front Physiol*. 2012;3:359.
19. Chen L, Tredget EE, Wu PY, Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS One*. 2008;3(4):e1886.
20. Gaur M, Dobke M, Lunyak VV. Mesenchymal stem cells from adipose tissue in clinical applications for dermatological indications and skin aging. *Int J Mol Sci*. 2017;18(1):208.
21. Das S, Wong ABH. Stabilization of ferulic acid in topical gel formulation via nanoencapsulation and pH optimization. *Sci Rep*. 2020;10(1):12288.
22. Monti D, Tampucci S, Chetoni P, et al. Permeation and distribution of ferulic acid and its alpha-cyclodextrin complex from different formulations in hairless rat skin. *AAPS PharmSciTech*. 2011;12(2):514-520.
23. Aldag C, Nogueira Teixeira D, Leventhal PS. Skin rejuvenation using cosmetic products containing growth factors, cytokines, and matrikines: a review of the literature. *Clin Cosmet Investig Dermatol*. 2016;9:411-419.

24. Colvan L, Fleck T, Vega VL. Global periorbital skin rejuvenation by a topical eye cream containing low molecular weight heparan sulfate (LMW-HS) and a blend of naturally derived extracts. *J Cosmet Dermatol*. 2019;18(2):530-538.
25. Diehl C. Melanocytes and oxidative stress. *J Pigment Disord*. 2014;1(4):127.
26. Bandyopadhyay D. Topical treatment of melasma. *Indian J Dermatol*. 2009;54(4):303-309.
27. Saeedi M, Eslamifar M, Khezri K. Kojic acid applications in cosmetic and pharmaceutical preparations. *Biomed Pharmacother*. 2018;110:582-593.
28. Couteau C, Coiffard L. Overview of skin whitening agents: drugs and cosmetic products. *Cosmetics*. 2016;3:27.
29. Vashi NA, Kundu RV. Facial hyperpigmentation: causes and treatment. *Br J Dermatol*. 2013;169(Suppl 3):41-56.

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