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Research article

Keratin as an effective coating material for *in vitro* stem cell culture, induced differentiation and wound healing assays

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ABSTRACT

The utilization of stem cells in tissue engineering holds great promise as efficient tools for tissue regeneration and in treating numerous musculoskeletal diseases. However, several limiting factors, such as precise delivery and control of differentiation of these stem cells as well as mimicking the microenvironment required to modulate stem cell behaviour *in-vivo*, have given rise to an urgent need for the development of new biomaterials which could be tailored to enhance cell renewal and/or direct cell fates. Keratin-rich biological materials offer several advantages, such as biocompatibility, tailorable mechanical properties, huge bioavailability, non-toxicity, non-immunogenic, and intrinsic tissue repair and/or regeneration capabilities, which makes them highly valued. In the present work, we report the preparation of keratin-based biomaterials from goat hair waste and its effectiveness as a coating material for *in vitro* culture and induced differentiation of mesenchymal stem cells (MSC's) and primary goat fibroblast cells. Since no known keratinase enzymes are expressed as such in human and/or animal systems, these keratin biomaterials could be used to slow the rate of degradation and deliver keratin-loaded stem cell scaffolds to induce their directed differentiation *in vivo*. The generated keratin materials have been characterized for surface morphology, protein structures, size and other properties using SDS-PAGE, LC/MS-MS, SEM, FTIR etc. Also, *in vitro* cell culture assays such as cell adhesion, viability using MTT, live dead assays, differentiation assays and *in vitro* scratch/wound healing assays were performed. Our results provide important data supporting tissue engineering applications of these keratinous biomaterials by combining the unique biological characteristics of goat hair-derived keratin material with the regenerative power of stem cells and their combinatorial use in applications such as disease treatment and injury repair as well as their use in the

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preparation of wound healing products, such as dressings and bandages, for management of clinical care in animals.

1. Introduction

Keratins are the principal structural proteins that show high sequence homology across humans as well as animals [1]. The word 'keratin' has been derived from the Greek word "kera" meaning horn since keratins are the major constituents of hard filamentous structures, such as horns, hooves, hair, and nails (hard keratins), as well as the cytoskeleton elements found in epithelial tissues of skin (soft keratins) [2]. The effective utilization of keratin biomaterials generated from low-quality animal fibres, such as goat hair and low-quality wool, otherwise considered municipal waste can be of great significance, particularly in the field of biomedicine and tissue regeneration applications.

The properties such as tissue biocompatibility, biodegradability, non-toxic nature, low immunogenicity, and enhancement of cell proliferation along with appropriate strength and flexibility make keratins a highly promising source for the development of biomaterials [3]. Due to these superior properties, keratins have attracted the attention of the scientific community, especially those working in the field of tissue regeneration and biomedicine, for their suitability for the development of protein-based products for skin regeneration and wound healing [4]. A wide array of keratin-based materials has been developed in the form of films, hydrogels, sponges, and electrospun fibres for their use in drug delivery systems, wound dressings, tissue engineering, and the cosmeceutical industry [5].

Over the years, several protocols have been developed for the extraction of otherwise insoluble keratins. Different extraction conditions, such as reducing conditions as described by the Shindai method or acid-based or enzyme-based oxidative and reductive treatments, have been proposed for the extraction of keratins from different sources [6–8]. The presence of abundant disulfide (-S-S) bonds in addition to the amino (-NH_2) and carboxylic acid (-COOH) groups make it chemically reactive under these conditions. The oxidation of keratin material oxidizes the disulfide linkages and cleaves it into more water-soluble keratoses, which are non-disulfide cross-linkable, hygroscopic, and susceptible to degradation in *in-vivo* and could be used for instant applications, such as preparation of wound dressings, foams, and gels. Under reducing conditions, the disulfide cross-links form free thiol (-SH) groups and protonation of its –NH_2 and/or other functional groups leads to the generation of kerateins. These protonated, unfolded and positive surface charge-carrying kerateins have high chemical reactivity and are more stable at varying pH conditions. Keratein biomaterials have been shown to persist *in-vivo* for a longer time and as such or after further chemical modifications can act as pseudo-natural cationic biopolymers which can find applications in varied areas such as tissue regeneration and cosmeceuticals, biomedical and ceramic industries [9].

Animal-derived tissues, such as nails, hair, feathers, and horn, which largely consist of keratin and keratin-associated proteins [4] are abundantly available as a by-product from animal farms, poultry plants, tanneries, barber shops, abattoirs and fur processing units. These byproducts are not economically important and are considered municipal waste in many countries resulting in the loss of enormous amounts of keratin wealth. To advance the use of keratin-based biomaterials, further studies and analytical methods are required to develop and characterize these biomaterials and explore their applications in different value-added areas including tissue regeneration and wound healing. This study reports the preparation and characterization of keratin composite materials using low-quality animal hairs, thus promoting the concept of "waste to wealth". The developed biomaterials were characterized for protein secondary structures, surface morphology, and mechanical and other properties and evaluated their potential for *in vitro* umbilical cord-derived goat mesenchymal stem cell culture and their induced differentiation. The study also investigated its effect on wound healing function, using *in vitro* wound healing/scratch assays, providing important data in support of tissue engineering applications of keratinous biomaterials. The use of keratin as keratin composite particles for *in vitro* culture and/or induced differentiation *in vivo* for various disease/injury management has an edge over the conventional films and/or hydrogel-based systems as it avoids the issues of oxygen and solubility limitations and doesn't require any cytotoxic crosslinkers which may interfere with cell survival and/or growth properties.

2. Materials and methods

2.1. Raw materials and chemicals

Animal fibres were collected from 08 male goats (04 Bhakarwal and 04 Changthangi coarse hair) maintained at the mountain agricultural research and extension station (MRSS&G) of SKUAST-Kashmir, J&K, India. The chemicals used during the experiments were supplied by Sigma Aldrich, Invitrogen, and/or Hi-Media (unless otherwise mentioned). Cell culture reagents and chemicals, such as FBS, DMEM, trypsin-EDTA and an antimycotic/antibiotic solution, were purchased from Invitrogen and Sigma Aldrich. Dialysis (MWCO 6–8000 Da) membrane was supplied by Spectrum (US, Rancho Dominguez). The plasticware and cell culture plates were purchased from Corning (US). All chemicals used were of cell culture grade. The Ethics Committee for Animal Use at SKUAST-Kashmir (J&K, India) approved the study which complied with all the guidelines for animal ethics in research.

2.2. Keratin extraction, quantification and purification

Animal fibres were washed, cleaned, and de-lipidized using a 1:2 (v/v) solution of methanol and chloroform for 24 h and then washed thoroughly with SDS (0.5%) containing water. This was followed by rinsing the fibers with fresh water and drying them overnight under laminar air flow conditions and thereafter was given a brief UV exposure for sterilization. Keratin was extracted under alkaline conditions following standard protocols with minor modifications [10–13]. Briefly, the processed fibres clipped to 5 mm size were mixed with extraction buffer for 72 h at 50 °C. This was followed with centrifugation and the filtration of supernatant using filter paper (2.5 μm pore size). The filtrates were exhaustively dialyzed against de-ionized water for 24 h at room temperature using a cellulose membrane (MWCO 6–8000 Da) and this procedure was done 4–6 times followed by 40 min of centrifugation (14,000 rpm) [7]. Bradford's method (Bradford Protein Assay, Bio-Rad, India) was employed to estimate the total protein concentration as per the manufacturer's protocol using a multi-plate-reader (Biotek Instruments, Vermont, United States). The dialyzed keratin solution was stored at −20 °C or lyophilized for further particle preparation using ultrasonication and/or downstream processing.

2.3. LC-MS/MS analysis

After the in-solution digestion, the samples of extracted keratin were sent for analysis by LC-MS/MS. The proteins were reduced [using Dithiothreitol (DTT), 5 mM] at 60 °C for 45 min and were thereafter alkylated using iodoacetamide (IAA) in the dark for 10 min at room temperature [14]. The alkylated and reduced proteins were digested overnight using sequencing grade modified trypsin (Madison, Promega, WI.) at 37 °C. The peptide extracts were dried under vacuum, subjected to C_{18} clean-up and stored at −80 °C. An ABSCIEX 5600 Triple-TOF (Concord, AB SCIEX, Canada) mass spectrometer connected with an Ekspert-nanoLC 415 system (Dublin, Eksigent, CA) was employed to perform LC-MS/MS analysis (reversed-phase high-pressure liquid chromatography-electrospray ionization tandem mass spectrometry, RP-HPLC-ESI-MS/MS). AB SCIEX, a Protein Pilot software (v. 5.0.1) with the Paragon algorithm for species: *Capra hircus* and quality: 0.05 was used. Only those peptides which showed a confidence score of >0.05 were analyzed.

2.4. SDS-PAGE analysis and particle size analysis

The molecular weight distribution of the extracted protein solutions was analyzed by 1D-SDS-PAGE which was performed following the method of Laemmli as described [15]. Briefly, extracted keratin solutions (~30 μg per well) were mixed with sample loading buffer (2×, 1:1) (Sigma, Laemmli) containing 10% 2-mercaptoethanol, 20% glycerol, 4% SDS, and 0.004% bromophenol blue. The proteins were separated using a 14% separation gel and 5% stacking gel loaded in a vertical slab gel electrophoretic system (Bio-Rad). Electrophoresis was performed at first at 80 V followed at 100 V after the proteins reached the separating gel. The Coomassie brilliant blue R-250 was used for staining [16] and de-staining was performed using 40% methanol and 10% acetic acid solution. Keratin particles were analyzed for particle size using the Zeta sizer instrument and the SEM images (Image J software) before being used for coating cell culture dishes as substrates for *in vitro* cell culture. The surface coating was done by first smearing the cell culture plate with keratin solution and then drying it in incubators and repeating these steps 2–3 times [17]. The coated culture plates were then washed using cold PBS (phosphate buffer saline), dried, and then again washed 2–3 times with the culture medium (DMEM) and dried again.

2.4.1. Scanning electron microscopy (SEM) and FT-IR spectroscopy

The scanning electron microscope (JEOL, JSM-6360, USA) was employed to analyze the micro-architecture of the keratin samples which were gold-sputtered (18 mA for 10 s) before observation. FT-IR spectra were analyzed (400–4000 cm^{-1}, a resolution of 8 cm^{-1}) using an FT-IR spectrophotometer (Nicolet 20 DXB).

2.5. Cell culture studies

2.5.1. Goat fibroblast and MSC primary culture and identification

Primary goat fibroblast cells and mesenchymal stem cells (isolated from the ear lobe and umbilical cord of goats maintained at MRCSG at SKUAST-Kashmir) following all ethical procedures as approved by the institutional ethical committee, were maintained in T-75 culture flasks in DMEM incorporated with 300 U/ml of streptomycin/penicillin and 15% FBS. The T-25 cell culture flasks were used for seeding the cells and the CO_2 incubator (5% CO_2) for culturing at 37 °C. For MSC culture, plastic-adherent cells similar to fibroblasts were found after 4–5 days of growth. After a 5-day gap, the media was changed to avert any mechanical stress which was followed by a 3-day gap till a confluent cell monolayer was found. The characterization of MSC's was done by analyzing their potential to differentiate into chondrocytes, osteoblasts, and adipocytes (tri-lineage) and through the expression of standard marker profile for MSCs by semi-quantitative PCR analysis i.e. positive for CD105 and CD90, but negative for CD45 and CD34.

2.5.2. Seeding efficiency

To assess the seeding efficiency, primary goat fibroblasts derived from goat ear were cultured in T-75 culture flasks and used for the seeding of culture plates coated with the keratin and control dishes. The cells were seeded in wells (5×10^4 per well) and maintained for 10 h at 5% CO_2 and 37 °C. The cells were detached using trypsin-EDTA after rinsing with PBS, were counted and seeding efficiency was determined (attached cells/seeded cells). All experiments were replicated at least thrice (n = 3).

2.5.3. MTT assay/live dead assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay was performed by culturing goat primary fibroblast cells, cultured as mentioned earlier.To determine the biocompatibility of the extracted materials, Live-dead assays (according to manufacturers' protocol) and MTT assays were performed [18]. The cells were seeded in triplicates in a 96-well plate without and with keratin coating under 5% CO_2 in air at 37 °C. Uncoated culture surfaces were used to compare the cell viability whereas the wells treated with standard fibronectin coating served as a positive control. After washing with 1× PBS, the cells were incubated at 37 °C for 4 h in MTT solution (0.05%, w/v) within the wells which was followed with the addition of DMSO. The absorbance was measured at 570 nm using a spectrophotometer (Amersham Life Sciences, USA).

2.5.4. In vitro wound healing assay

This assay was done using primary goat fibroblast cells cultured as described earlier. The 12 well plates with and without keratin and keratin coating and seeded with cells were maintained at 5% CO_2 in the air and 37 °C to 80–90% confluency. After removing the medium, cells were washed with serum-free medium once and maintained in it for 24 h till a monolayer was formed. A 10 μl pipette tip was used to induce artificial injury to this monolayer by scratching across the plate and the cell debris or detached cells were removed by washing the wells twice. From this point onward, cells were maintained in a serum-free medium containing mitomycin C (Mumbai, Sigma, India, 10 μg/ml) to prevent cell proliferation. An inverted phase-contrast microscope (Olympus Corporation) was used to take high-resolution images of the scratched areas after regular intervals to determine the cell migration rate using ImageJ software.

2.5.5. Differentiation studies

Multi-lineage differentiation assays into the chondro-, adipo- and osteogenic directions were done using umbilical cord-derived goat mesenchymal stem cells at passage 4. Briefly, for adipo- and osteogenesis, 6-well plates seeded with cells were maintained in a culture medium at 5% CO_2 and 37 °C. The specific differentiation medium available commercially (Sigma-Aldrich) was used to replace the medium. For osteogenic differentiation, Keratin-BMP2 composite was used to coat the culture dishes and calcium in the matrix of osteoblasts was stained using alizarin red S (Sigma-Aldrich). ALP activity that indicates osteogenic differentiation was performed using a ALP assay kit (Abcam) according to the manufacturer's protocol as described by Choi et al. [19]. Alcian Blue was used to stain the chondrogenesis pellets to examine cartilage glycosaminoglycans. The degree of osteogenic and chondrogenic differentiation was determined by calculating the ratio of stained cell area to total cell area in the image. The ratio of stained cell area to total cell area was measured in the image to determine the degree of differentiation (osteogenic and chondrogenic).

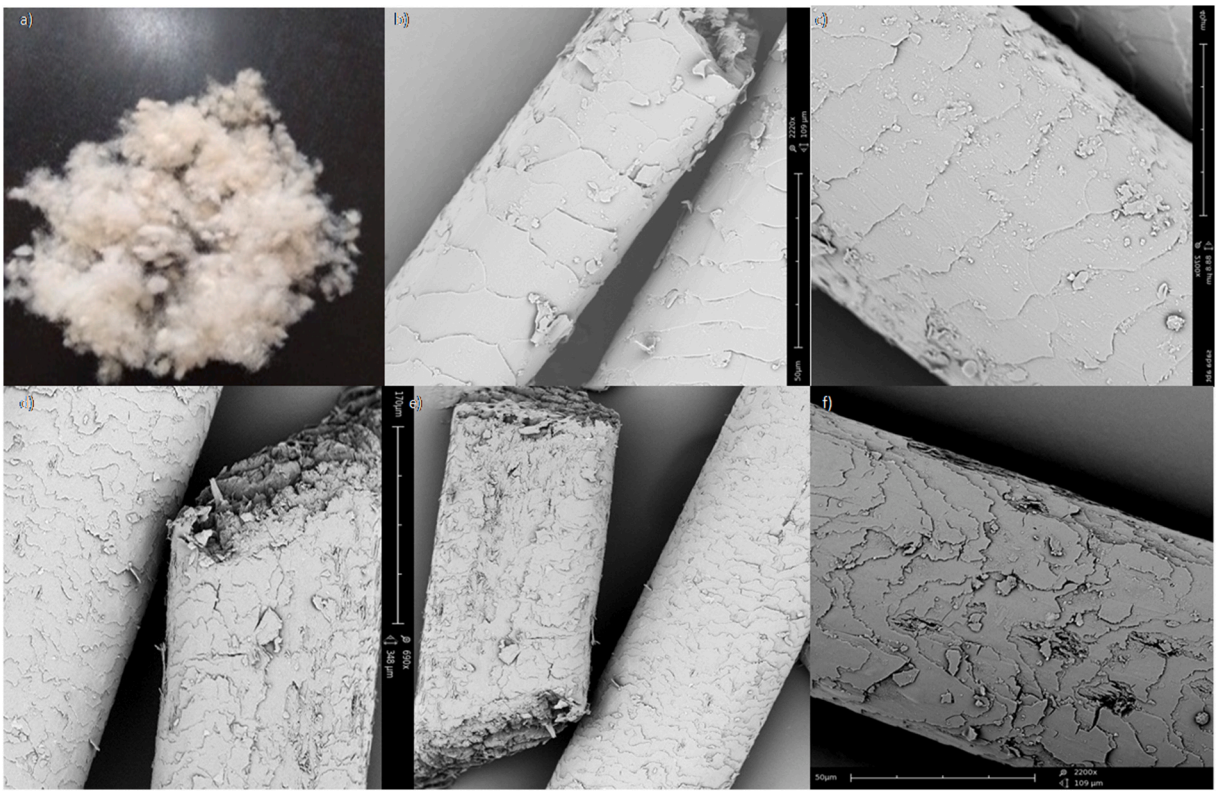

Fig. 1. Images of Intact cleaned goat hair (a), Scaning Electrom Microscopy (SEM) images of processed goat hair (b, c) and post- delipidation (d,e,f).

2.6. Statistical analysis

All data was generated by replicating the experiments at least thrice (n = 3) for quantitative parameters and after analysis, the results were expressed as mean ± SD in the figures. The images were evaluated by using ImageJ software. Unpaired student's t-test at a significance level of ≤0.05 was used to reveal the significance between the pair of means.

3. Results and discussion

3.1. Keratin extraction from goat hair waste and purification

Following the processing of fibres and their microscopic analysis (Fig. 1), extraction protocols reported earlier (such as Shindai, conventional, and modified) at different pH (pH 8.0–9.5) and reducing conditions for standardizing the extraction process were used for the maximum amount of good quality keratin. SDS-PAGE fractionation and Bradford's protein quantification assay (Fig. 2A) results indicate that nearly 70–75% of the protein was extracted using the Shindai method in agreement with the results of Nakamura et al. [8]. Also, use of thiourea removed proteins from the cortex part of the animal fibre and our extraction buffer with both thiourea and urea (2.6 M thiourea, 25 mM Tris-HCl, 5% 2-mercaptoethanol, and 5 M urea) resulted in a greater number of bands on SDS-PAGE gel compared to urea alone (Fig. 1) indicating that Shindai method that uses both thiourea and urea produces a higher yield of solubilized proteins from animal hairs [8]. Although the use of 2-mercaptoethanol resulted in a slightly higher protein yield, buffer having DTT was preferred because of the foul smell and toxicity associated with 2-mercaptoethanol which is not suitable for downstream applications and also because DTT produced sharper and clearer bands. SDS-PAGE analysis (Fig. 2) showed the characteristic bands of Keratin Intermediate filaments (IF) with minor high molecular weight proteins (125–135 and 110–115 kDa), microfibril keratins (40–60 kDa) and matrix proteins (20-10 kDa).

Following SDS-PAGE analysis and in-solution digestion, extracted samples were examined by LC-MS/MS. The MS spectra generated against the UniProt *Capra hircus* database recognized nearly 128 confident proteins (removing redundancy) from animal fibre extraction (Fig. 2B). LC-MS/MS results showed that the majority of these proteins belonged to KRT (keratin) and KAP (keratin-associated proteins) groups which constituted more than 60% of total identified proteins. These results agreed with previous findings on wool and human hair keratins [16]. Most of these KRTs were type I (molecular weight ranged between 40 and 50 kDa) and type II (50–70 kDa) proteins whereas the matrix consisted of mostly keratin-associated proteins (KAPs) which are usually lower molecular weight proteins (10–25 kDa) as was confirmed by the results of our SDS-PAGE analysis. This MS-based proteomics served as a novel approach for the annotation and characterization of the proteins in goat hair necessary for establishing a prospective relationship between its constituent proteins and the quality and/or quantity of extracted keratin biomaterial. The present work majorly emphasized the generation of an efficient and modified method for keratin biomaterial extraction and purification from goat hair waste with an emphasis on dialysis to remove all impurities including salts for its applications for *in vitro* cell culture assays.

3.2. Keratin and keratin-composite characterization

The SEM of the keratin and keratin composite materials used to coat the culture dishes are presented in Fig. 3A. The SEM visualizes the surface physical properties of these keratin materials as porous with open interconnectivity and architectural arrangements. The images show large porous and interconnected architectures consisting of comparably less porous morphology. The surface shows even roughness, indicative of polymerization and cross-linking properties of these biomaterials. This kind of morphology is essential for the fabrication of films and hydrogels for their use in tissue regeneration and/or wound healing. The FT-IR spectrum of extracted keratins (Fig. 3B) showed characteristic absorptions around 3300 and 3050 cm^{-1} indicating the primary amine (N–H) group. The bands present around 1533 and 1650 correspond to the amide II and I, respectively. The average size of the particles of keratin was observed in the

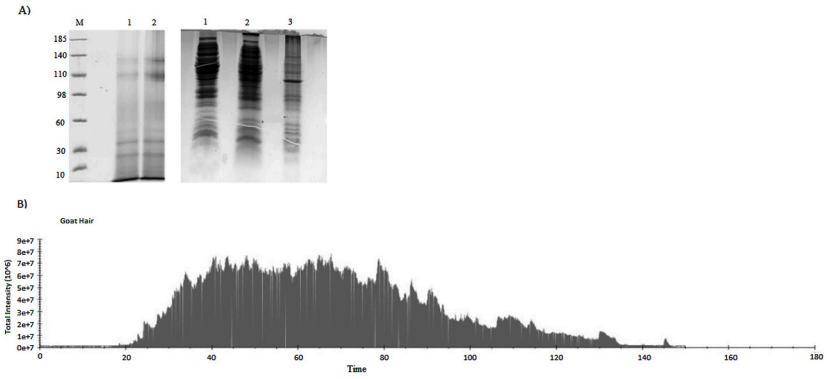

Fig. 2. Representative SDS-PAGE gel pictures for fractionation pattern of total protein extracted from goat hair waste (**A**), Representative chromatogram for LC/MS-MS analysis of extracted proteins from goat hair (**B**).

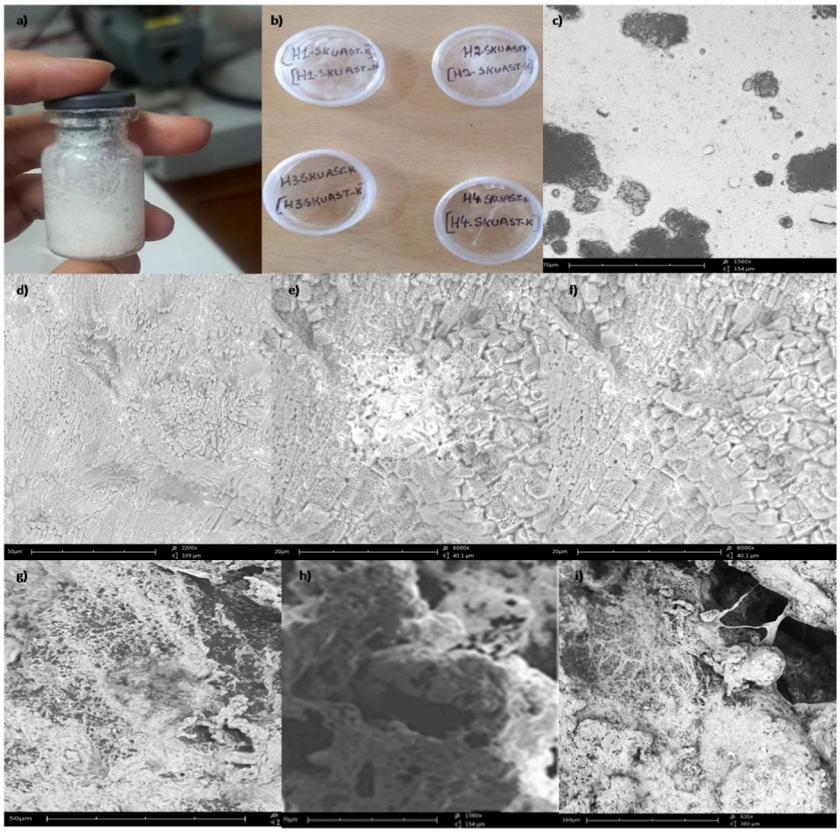

Fig. 3A. Representative optical images of extracted goat hair keratin particles before and after lyophilization (a, b), SEM images of keratin and keratin composite particles (c, d, e), keratin coated culture surfaces (f, g, h, i).

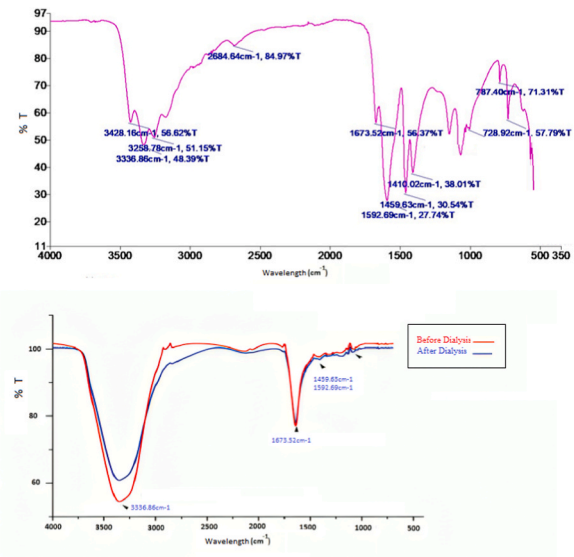

Fig. 3B. FT-IR analysis of extracted keratin lysate before and after dialysis and keratin BMP2 composite particles.

micrometer range which exhibited morphological and surface properties consistent with their mechanical properties and protein content as revealed by the results of scanning electron microscopy (SEM) and FT-IR [20,21].

3.3. Cell culture studies

3.3.1. Biocompatibility and differentiation assays

The evaluation of biocompatibility and cell viability of these keratin materials is crucial to demonstrate their application as biomaterials [22]. The *in vitro* culture and characterization of goat primary fibroblast cells and umbilical cord-derived mesenchymal stem cells were done complying with the established guidelines and ethical clearance from the institutional committee for ethics (Fig. 4). The multi-lineage differentiation of umbilical cord-derived MSCs was apparent by our differentiation assays. The semi-quantitative PCR-based expression profile of the markers on the cell surface (CD-105, CD-45, CD-34 and CD-90) was used for their characterization (Fig. 4).

The MTT and live-dead assays were employed for measuring the cell metabolic activity as a measure of cell viability. The MSCs derived from the goat umbilical cord were used for live-dead assays. Post 07 days of seeding of the MSCs, live-dead assays showed more than 80% cell viability in stem cells showing no significant cytotoxicity in the keratin material (Fig. 5). MTT results for goat primary fibroblast cells showed cell viability of more than 90% with reference to control (fibronectin-coated culture for each time point viz. post 48 h and 72 h). The seeding efficiency results also point towards good seeding efficiency, normal adhesion as well as growth and/or gross morphology of primary goat fibroblast cells when grown on keratin-coated culture plates. The cells were found to adhere on keratin-coated surfaces and proliferated progressively as much as compared to the fibronectin-coated control culture plates. Although the viability of cells seeded on keratin-BMP2 composite coated surfaces was found lower as compared to the fibronectin control, it was found to be effective in inducing differentiation (osteo-induction) of mesenchymal stem cells compared to the uncoated surfaces and didn't point towards any significant anti-proliferative and/or cytotoxic properties (Fig. 4, Fig. 5).

In our differentiation experiments, we also used bone morphogenesis protein-2 (BMP2) for directed differentiation of stem cells towards osteogenic lineage along with keratin as a keratin-BMP2 composite. The keratin-BMP-2 coating showed a significantly stronger ability to induce MSC osteogenic differentiation than the control groups. Our preliminary experiments have shown promising results for the use of this keratin-BMP2 composite which is capable of inducing directed osteogenic differentiation in mesenchymal stem cells *in-vitro* (Fig. 4).

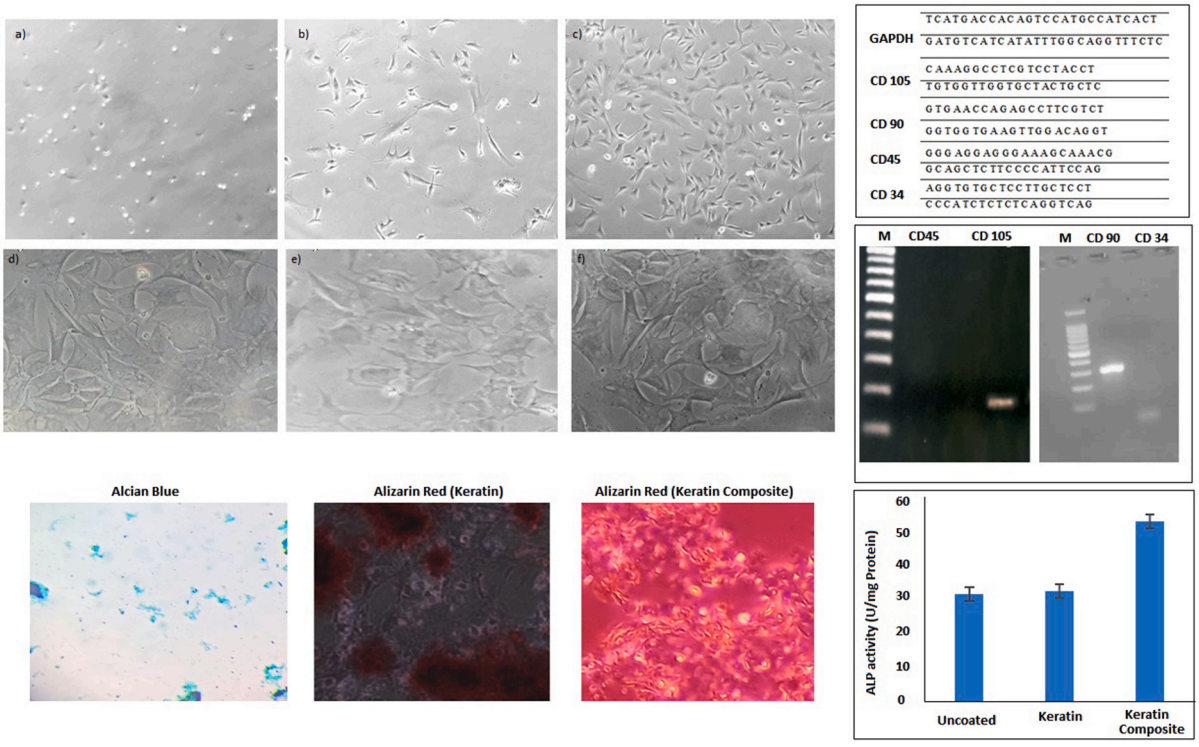

Fig. 4. Morphological features of MSC derived from goat umbilical cord and its marker-based characterization. Primary culture after 4–5 days and spindle form, with uniform fibroblast-like morphology with 80–85% confluence, Lower Panel: Differentiation of MSC's after 21 days *in vitro* into chodrogenic **(**Alcian Blue staining**)** and osteogenic (alizarin red staining) lineage. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

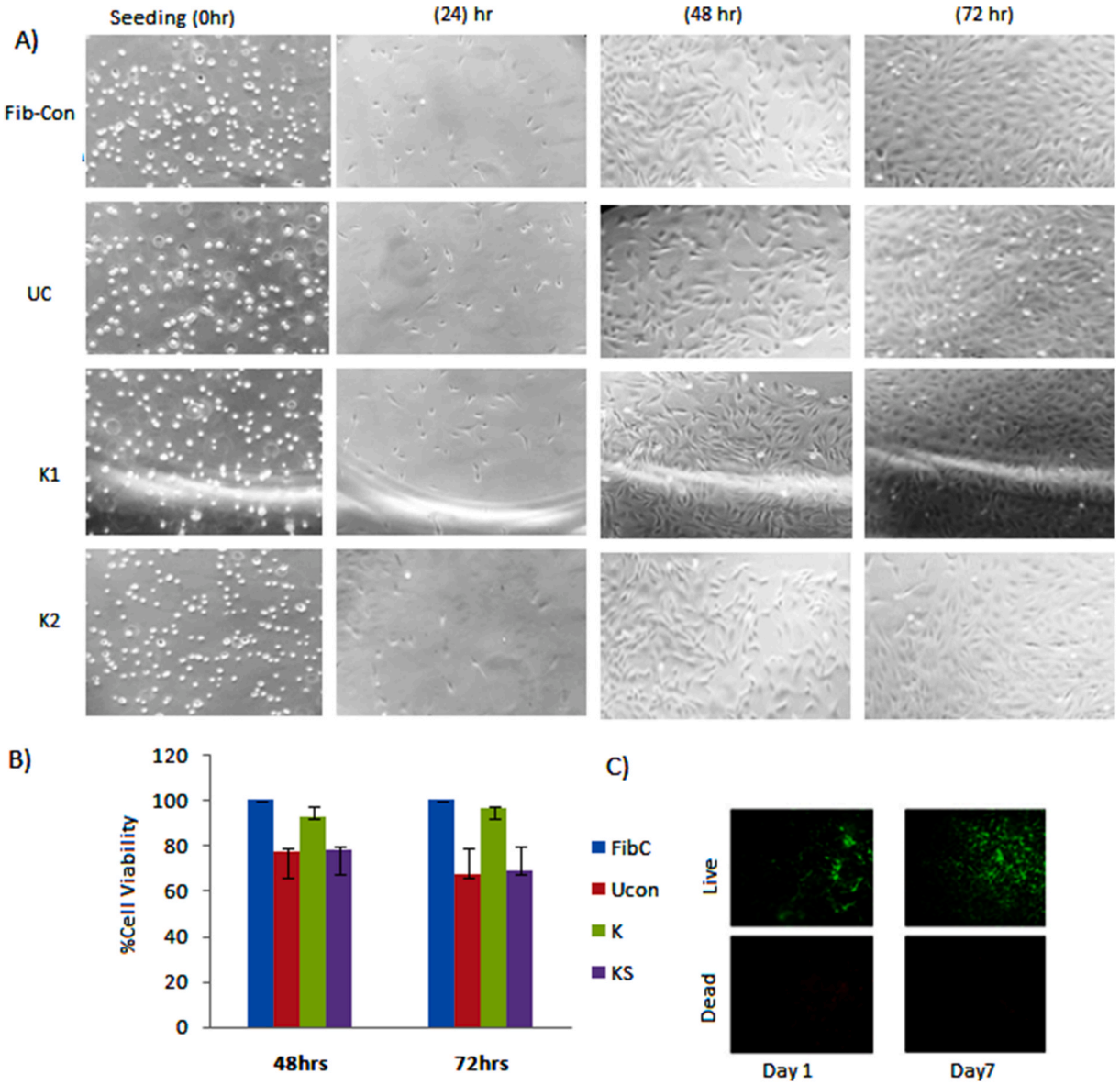

Fig. 5. Seeding efficiency and morphology/biocompatibility of cells post 24, 48 and 72 h of cell culture (A), Percent cell viability (MTT) and Live Dead assay results of goat fibroblast cells post 48 and 72 h of cell culture (B, C), Fib-Con represents fibronectin control, UC is uncoated control, **K** and **KS** represent Keratin and Keratin BMP2 coated culture surfaces.

3.3.2. Wound healing and scratch assays

In vitro wound healing assays were performed using goat-derived primary fibroblast cells (Fig. 6) and umbilical cord-derived goat MSCs (Fig. 7). The scratch with the cell-free area was examined by the program ImageJ and the percentage of *in vitro* wound healing was measured (Figs. 6 and 7). The migration capacity of cells grown on plates coated with the keratin particles was found to be significant compared to the cells grown in control plates after 24 h. After 52 h, we found almost complete closure of scratch/wound in keratin-coated dishes compared to the control dishes indicating an increase in the migration capacity and wound healing potential of cells grown on keratin-coated dishes thus pointing towards a prospective role of keratin biomaterials in wound healing. A similar pattern of cell migration in response to induced scratch was observed (Fig. 7) in the case of MSCs derived from the umbilical cord of a goat *in vitro*.

4. Discussion

Keratins extracted from animal fibres are rich in cysteine content and have been found to possess suitable mechanical and biological characteristics for binding and cell attachment [23] required in a suitable biological material for *in vitro* cell culture. Keratin

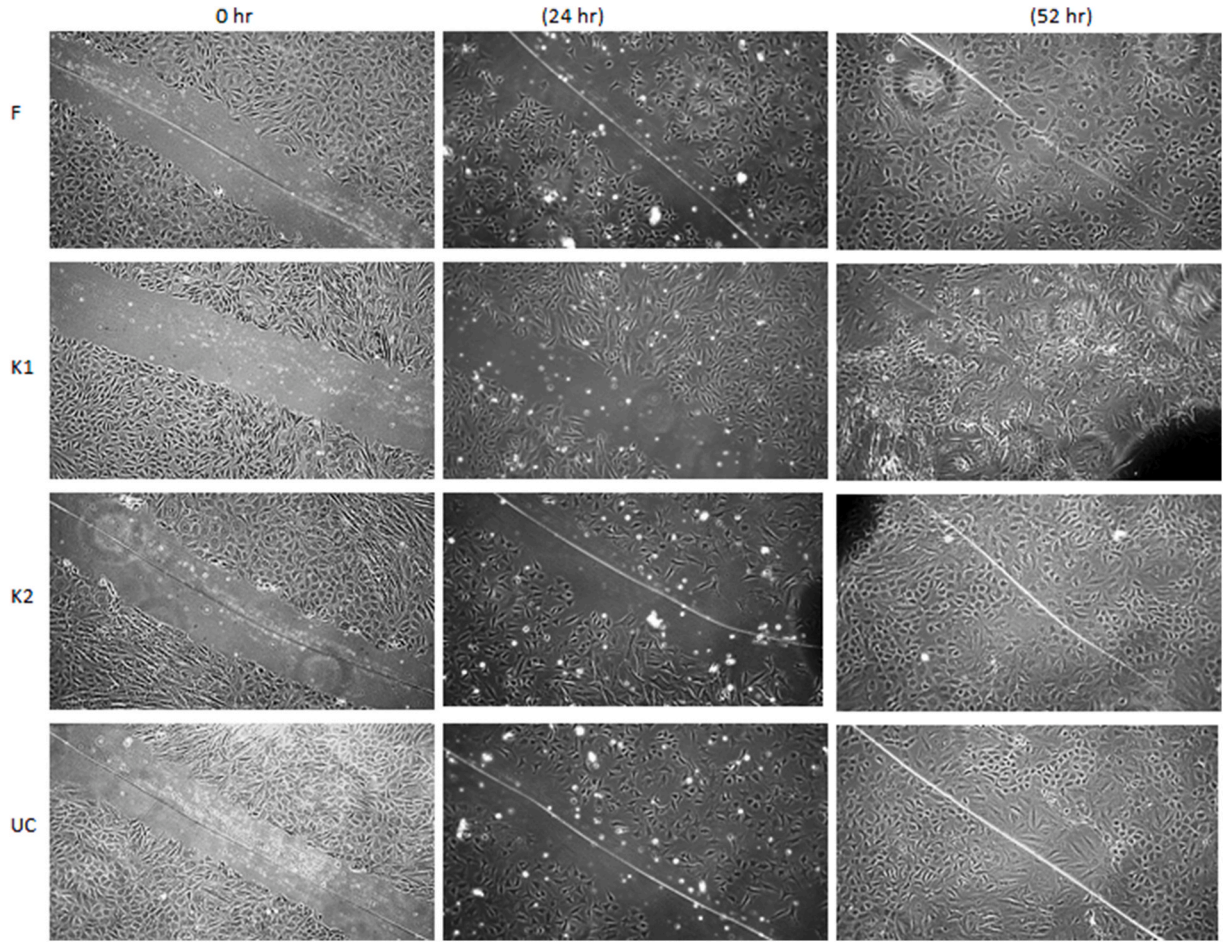

Fig. 6. Representative images of wound/scratch healing assay with goat fibroblast cells cultured on standard fibronectin (F), K1, K2: Keratin and Keratin composite coated culture dishes. Uncoated (UC) served as negative control.

composites have been used to substitute soft tissue areas or to aid tissue enhancement by filling the spaces such as during breast augmentation [24] as well as fabricated into flexible and biocompatible constructs implicated in promoting tissue growth and nerve regeneration [25]. Studies have indicated the nerve regeneration potential of keratin biomaterials. For example, Nakaji-Hirabayashi et al. [26] and Ranjit et al. [27] reported significant improvements in Schwann cell migratory and proliferative ability using keratin hydrogels *in vivo*. The presence of certain motifs, such as LDS (Leu-Asp-Ser), LDV (Leu-Asp-Ser), and RGD (Arg-Gly-Asp), in keratin and their resistance to proteolytic degradation warrant their use in tissue engineering and wound healing. Several keratin-derived materials, such as hydrogels, gels and films, have been successfully investigated for a range of wound types with positive healing outcomes. Also, owing to the presence of certain binding amino acid motifs (REF), closely related to the ones present in extracellular matrix (ECM), the keratin-based biomaterials have the capability to offer cellular attachment and proliferation *in vitro*. This property of fibre-derived keratin particles was exploited in this study to evaluate their role as prospective cell culture coating material for *in vitro* culture and proliferation of goat primary fibroblast cells as well as MSCs derived from goat umbilical cord for prospective *in vitro* cell culture applications. The advantages of using keratin particles and their composites extracted from low-quality animal fibres over other conventional materials, films and hydrogel-based systems are that it is easily available, cost-effective and easy to use and also avoid the issues of oxygen and solubility limitations and there is no need for adding any cytotoxic cross-linkers that interfere with cell survival and growth properties.

Although stem cell-based tissue engineering provides a promising substitute for tissue regeneration and repair, the major lacunae to be addressed in this field are precise delivery and control of differentiation of stem cells *in-vivo* as well as mimicking the microenvironment required to modulate stem cell behaviour within the living systems which necessitates the development of new biomaterials. In the present work, we suggest the use of keratin and keratin-based composites for *in vitro* culture and inducing directed differentiation in stem cells. Our results indicate the biocompatibility of keratin-based biomaterials as a coating material for MSC culture whereas keratin-composites were found to be very effective in directed differentiation of these mesenchymal stem cells [27]. We observed a significant increase towards osteogenic lineage differentiation in mesenchymal stem cells grown on keratin-BMP2 coated dishes as compared to the control. BMP2 is a member of the transforming growth factor-β superfamily and promotes bone

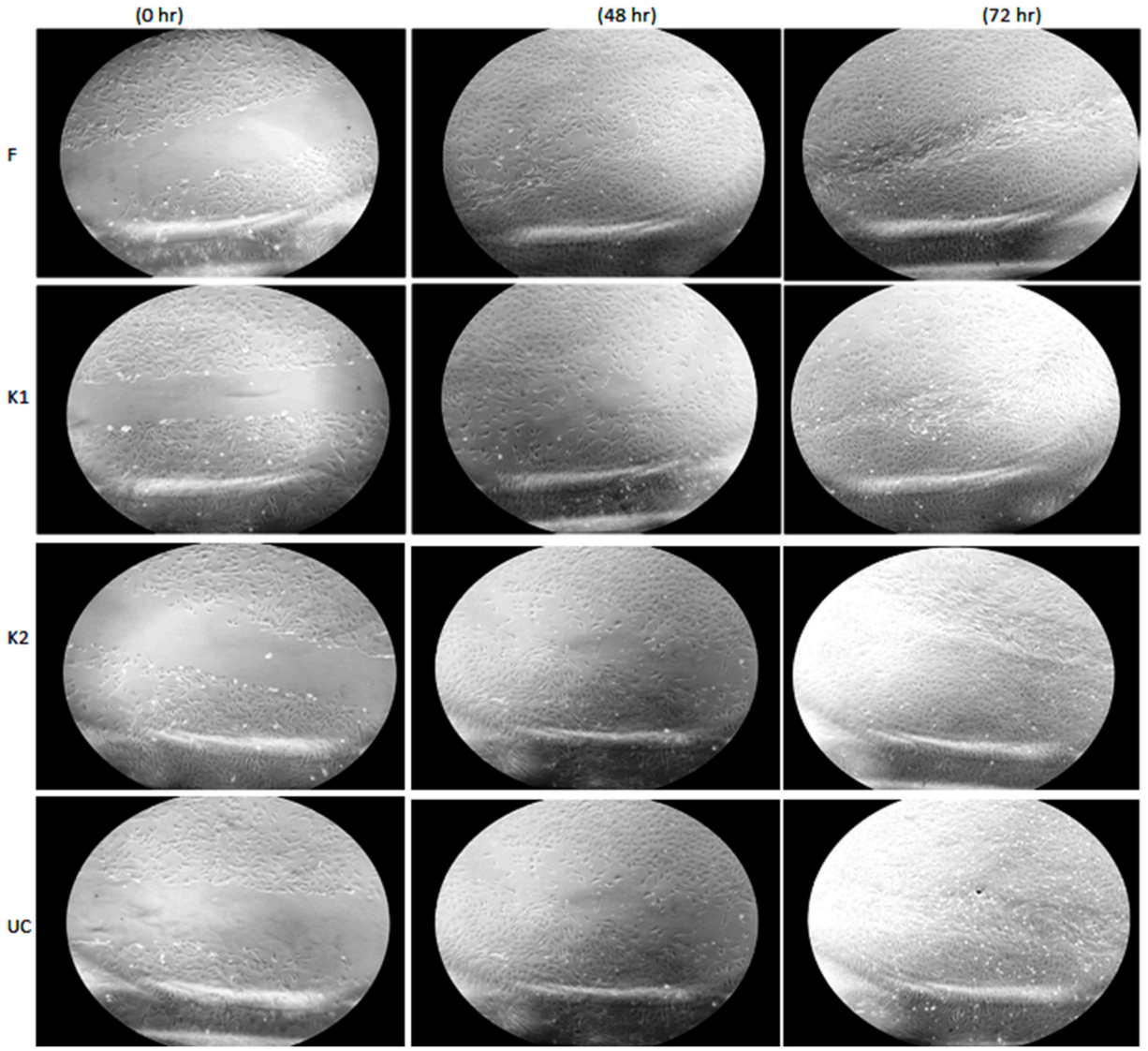

Fig. 7. Representative optical images of wound/scratch healing assay with umbilical cord-derived MSC's cultured on standard fibronectin (F), K1, K2: Keratin and Keratin BMP2 coated culture dishes. Uncoated (UC) served as negative control.

formation by directing MSC differentiation into osteoblasts/osteocytes (Einhorn et al., 2003). The recombinant BMP-2 (rhBMP-2) is currently approved by the US Food and Drug Administration (FDA) for clinical uses. Being low in immunogenicity and having intrinsic anti-inflammatory properties further advocates their prospective applications in the area of regenerative medicine [28]. These results point towards the use of keratin-based materials as carriers for payloads *in vivo* and as substrates for the seeding of cells in *in vitro*. Previous studies have established the use of keratin-based biomaterials in inducing cell differentiation, e.g. hCSCs (human cardiac stromal cells) during an *in vitro* study have been shown to shift to a smooth muscle cell lineage [29]. Since for tissue regeneration, a stem cell population with high differentiation potential is required, these keratin composite particles could be used for promoting the bone formation potential of MSCs and thus hold great potential for their use in stem cell therapies for musculoskeletal diseases. Also, our *in vitro* wound healing assay results showed an increase in migration and/or wound closure in the case of primary goat fibroblast monolayer cultured on keratin-coated culture dishes *in vitro.* Keratins have also been demonstrated to improve the wound healing and tissue regeneration environment by modulating macrophage function, thereby increasing the production of cytokines with anti-inflammatory activities and reducing pro-inflammatory cytokines [30,31]. Their capability to instigate macrophage differentiation and potential wound-healing capacity [31] have also been documented. Taken together, our results point towards the application of keratin-based materials and their suitability in the area of stem cell-based tissue engineering and wound healing.

5. Conclusions

The study examined some general properties of kerateins which support their use as a biomaterial. The results expand our understanding of the suitability of keratins extracted from low-quality animal fibres for therapeutic applications which are otherwise considered municipal waste, hence promoting value addition and waste-to-wealth concepts. The results reveal the potential of keratin-based materials as an option in the area of tissue regeneration and wound healing due to their superior physical and chemical characteristics and high biocompatibility and pave the way for future studies to explore the use of these animal fibre-derived keratins for the development of controlled delivery systems for a variety of payloads for tissue engineering applications. The present study will open up new ways of understanding the genesis of MSC's osteogenic differentiation at the molecular level which could be useful in possible therapeutic implications of the same. This becomes particularly important given the fact that keratin is the only natural polymer which goes unaffected by specific tissue turnover-related enzymes.

Data availability statement

Data will be made available on request.

Ethics approval

The Ethics Committee for Animal Use at SKUAST-Kashmir (J&K, India) approved the study which complied with all the guidelines for animal ethics in research.

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CRediT authorship contribution statement

Hina F. Bhat: Writing – original draft, Validation, Supervision, Methodology, Data curation, Conceptualization. **Nuzhat Amin:** Methodology, Investigation. **Zarka Nasir:** Methodology, Investigation. **Saba Nazir:** Methodology, Investigation. **Zuhaib F. Bhat:** Writing – original draft, Validation, Methodology, Data curation. **Abrar A. Malik:** Methodology, Investigation. **Nazir A. Ganai:** Writing – review & editing, Visualization, Data curation. **S. Mudasir Andrabi:** Writing – review & editing, Visualization, Data curation. **Riaz A. Shah:** Writing – review & editing, Visualization, Data curation. **Rana Muhammad Aadil:** Writing – review & editing, Visualization, Software. **Asif H. Sofi:** Resources, Investigation. **Gholamreza Abdi:** Writing – review & editing, Visualization, Resources, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] R. Tevlin, H. desJardins-Park, J. Huber, S.E. DiIorio, M.T. Longaker, D.C. Wan, Musculoskeletal tissue engineering: adipose derived stromal cell implementation for the treatment of osteoarthritis, Biomaterials 286 (2022) 121544.
[2] B.S. Lazarus, C. Chadha, A. Velasco-Hogan, J.D.V. Barbosa, I. Jasiuk, M.A. Meyers, Engineering with keratin: a functional material and a source of bioinspiration, iScience 24 (2021) 102798.
[3] M. Konop, M. Rybka, A. Drapała, Keratin biomaterials in skin wound healing, an old player in modern medicine: a mini review, Pharmaceutics 13 (2021) 2029, https://doi.org/10.3390/pharmaceutics13122029.
[4] M.S. Bala, K.W. Chin, B.Y. Alashwal, A. Gupta, Development of wound healing spray from keratin protein, Maejo Int. J. Energy Environ. Commun. 2 (2020) 43–49.
[5] M.A. Khosa, A.A. Ullah, Sustainable role of keratin biopolymer in green chemistry: a review, J. Food Process. Beverages 1 (2013) 4.
[6] S. Feroz, N. Muhammad, J. Ranayake, G. Dias, Keratin-based materials for biomedical applications, Bioact. Mater. 5 (2020) 496–509.
[7] N. Senthilkumar, S. Chowdhury, P. Sanpui, Extraction of keratin from keratinous wastes: current status and future directions, J. Mater. Cycles Waste Manag. 25 (2022) 1–16, https://doi.org/10.1007/s10163-022-01492-9.
[8] A. Nakamura, M. Arimoto, K. Takeuchi, T. Fujii, A rapid extraction procedure of human hair proteins and identification of phosphorylated species, Biol. Pharm. Bull. 25 (2002) 569–572.
[9] R. Karthikeyan, S. Balaji, P.K. Sehgal, Industrial applications of keratins-A review, J. Sci. Ind. Res. 66 (2007) 710–715.
[10] S. Reichl, M. Borrelli, G. Geerling, Keratin films for ocular surface reconstruction, Biomaterials 32 (2011) 3375–3386.
[11] I.B. Khalifa, N. Ladhari, M. Touay, Application of sericin to modify textile supports, J. Text. Inst. 103 (2012) 370–377.
[12] M. Borrelli, N. Joepen, S. Reichl, D. Finis, M. Schoppe, G. Geerling, S. Schrader, Keratin films for ocular surface reconstruction: evaluation of biocompatibility in an in-vivo model, Biomaterials 42 (2015) 112–120.
[13] V.D. Maria, B. Celina, J.F. Nora, Development of biodegradable films based on chitosin/glycerol blends suitable for biomedical applications, J. Tissue Sci. Eng. 7 (2016) 187.
[14] T. Muller, D. Winter, Systematic evaluation of protein reduction and alkylation reveals massive unspecific side effects by iodine-containing reagents, Mol. Cell. Proteomics 16 (7) (2017) 1173–1187.

[15] Z.F. Bhat, J.D. Morton, S.L. Mason, A.E.A. Bekhit, T. Mungure, Pulsed electric field improved protein digestion of beef during in vitro gastrointestinal simulation, LWT–Food Sci. Technol. 102 (2019) 45–51.
[16] V. Neuhoff, R. Stamm, H. Eibl, Clear background and highly sensitive protein staining with Coomassie Blue dyes in polyacrylamide gels: a systematic analysis, Electrophoresis (Weinheim, Fed. Repub. Ger.) 6 (1985) 427–448.
[17] J.A. Tuly, H. Ma, H.J. Lee, J.W. Song, A. Parvez, N. Saqib, W. Yaseen, Z. Xinyan, Insights of keratin geometry from agro-industrial wastes: a comparative computational and experimental assessment, Food Chem. 418 (2023) 135854.
[18] S.L. Garcia, F. Rodriguez-Lozano, J.L. Sanz, L. Forner, M.P. Pecci-Lloret, A. Lozano, L. Murcia, S.S. Bautista, R.E. Onate-Sanchez, Biological properties of Ceraputty as a retrograde filling material: an in vitro study on hPDLSCs, Clin. Oral Invest. 27 (2023) 4233–4243.
[19] Y. Jung, S.S. Kim, Y.H. Kim, S.H. Kim, B.S. Kim, S. Kim, C.Y. Choi, S.H. Kim, A poly (lactic acid)/calcium metaphosphate composite for bone tissue engineering, Biomaterials 32 (2005) 6314–6322.
[20] E. Wojciechowska, A. Włochowicz, A. Wesełucha-Birczyńska, Application of Fourier-transform infrared and Raman spectroscopy to study degradation of the wool fibre keratin, J. Mol. Struct. 511 (1999) 307–318.
[21] D.J. Lyman, J. Murray-Wijelath, M. Feughelman, Effect of temperature on the conformation of extended α-keratin, Appl. Spectrosc. 55 (2001) 552–554.
[22] H. Lee, K. Noh, S.C. Lee, I.-K. Kwon, D.-W. Han, I.-S. Lee, Y.-S. Hwang, Human hair keratin and its-based biomaterials for biomedical applications, Tissue Eng. Regen. Med. 11 (2014) 255–265.
[23] M.E. Furth, A. Atala, M.E. Van Dyke, Smart biomaterials design for tissue engineering and regenerative medicine, Biomaterials 28 (2007) 5068–5073.
[24] M. Konop, M. Rybka, A. Drapała, Keratin biomaterials in skin wound healing, an old player in modern medicine: a mini review, Pharmaceutics 13 (2021) 2029.
[25] P. Sierpinski, J. Garrett, J. Ma, P. Apel, D. Klorig, T. Smith, L.A. Koman, A. Atala, M. Van Dyke, The use of keratin biomaterials derived from human hair for the promotion of rapid regeneration of peripheral nerves, Biomaterials 29 (2008) 118–128.
[26] T. Nakaji-Hirabayashi, K. Kato, H. Iwata, Essential role of structural integrity and firm attachment of surface-anchored epidermal growth factor in adherent culture of neural stem cells, Biomaterials 29 (2008) 4403–4408.
[27] E. Ranjit, S. Hamlet, R. George, A. Sharma, R.M. Love, Biofunctional approaches of wool-based keratin for tissue engineering, J. Sci. Adv. Mater. Dev. 7 (2022) 100398.
[28] J.G. Rouse, M.E. Van Dyke, A review of keratin-based biomaterials for biomedical applications, Materials 3 (2010) 999–1014.
[29] B.T. Ledford, J. Simmons, M. Chen, H. Fan, C. Barron, Z. Liu, M. Van Dyke, J.Q. He, Keratose hydrogels promote vascular smooth muscle differentiation from c-kit-positive human cardiac stem cells, Stem Cell. Dev. 26 (2017) 888–900.
[30] B.V. Fearing, M.E. Van Dyke, In vitro response of macrophage polarization to a keratin biomaterial, Acta Biomater. 10 (2014) 3136–3144.
[31] M. Waters, P. Vande Vord, M. Van Dyke, Keratin biomaterials augment anti-inflammatory macrophage phenotype *in vitro*, Acta Biomater. 66 (2018) 213–223.