Regenerative Therapy 22 (2023) 115–127

Contents lists available at ScienceDirect

Regenerative Therapy

journal homepage: http://www.elsevier.com/locate/reth

Original Article

Priming with caffeic acid enhances the potential and survival ability of human adipose-derived stem cells to counteract hypoxia

H.M. Shifa ul Haq [a, 1], Ramla Ashfaq [a, b, 1], Azra Mehmood [a, *], Warda Shahid [a], Ghufran Azam [a], Maryam Azam [a], Saba Tasneem [a], Shehla Javed Akram [d], Kausar Malik [a], Sheikh Riazuddin [a, c, **]

[a] National Centre of Excellence in Molecular Biology, 87-West Canal Bank Road, University of Punjab, Lahore, 53700, Pakistan
[b] Genome Editing Lab, Food Biotechnology Research Center, Pakistan Council of Scientific and Industrial Research (PCSIR) Laboratory Complex, Lahore, 54600, Pakistan
[c] Jinnah Burn & Reconstructive Surgery Centre, Allama Iqbal Medical College, University of Health Sciences, Lahore, Pakistan
[d] DonValley Pharmaceuticals (Pvt) Ltd. Akram Medical Complex, Lahore

ARTICLE INFO

Article history:
Received 22 November 2022
Received in revised form
27 December 2022
Accepted 4 January 2023

Keywords:
Human adipose-derived stem cells
Hypoxia stress
Cobalt chloride
Pre-conditioning
Antioxidants
Caffeic acid

ABSTRACT

The therapeutic effectiveness of stem cells after transplantation is hampered by the hypoxic milieu of chronic wounds. Prior research has established antioxidant priming as a thorough plan to improve stem cell performance. The purpose of this study was to ascertain how caffeic acid (CA) priming affected the ability of human adipose-derived stem cells (hASCs) to function under hypoxic stress. In order to study the cytoprotective properties of CA, hASCs were primed with CA in $CoCl_2$ hypoxic conditions. Microscopy was used to assess cell morphology, while XTT, Trypan Blue, X-gal, LDH, Live Dead, scratch wound healing, and ROS assays were used to analyze viability, senescence, cell death, proliferation, and reactive oxygen species prevalence in the cells. According to our findings, CA priming enhances hASCs' ability to survive and regenerate in a hypoxic microenvironment more effectively than untreated hASCs. Our in-vitro research suggested that pre-treatment with CA of hASCs could be a unique way to enhance their therapeutic efficacy and ability to survive in hypoxic microenvironments.

1. Introduction

Wounds caused by different types of physical (traumatic, accidental, or surgical), chemical (acidic burns), and biological injuries (fungal, pathogenic) result in loss of function and integrity [1,2]. During wound repair, different factors are autonomously up-regulated or down-regulated in the body which ultimately helps in wound healing by involving the synchronized contribution of numerous types of cells including fibroblasts, keratinocytes, macrophages, platelets, and endothelial cells [3]. During the repairing process, reactive oxygen species (ROS) are generated at the wound site by pro-inflammatory cells for defense against invading bacteria. Elevated oxidative stress due to ROS is another fundamental reason behind impaired wound healing [4]. These ROS damage the endothelial cells through oxidative stress (OS) and affect the process of angiogenesis, the ROS also disturb cytokines secretion ultimately causing delayed wound healing [5].

Small traumatic injuries have the self-healing ability by the body's mechanism through a cascade of events but in the case of large and chronic wounds, self-healing is not efficient due to the limited availability of cell production sources. Therefore, the

Abbreviations: Human adipose-derived stem cells, (hASCs); Caffeic acid, (CA); Mesenchymal stem cells, (MSCs); oxidative stress, (OS); Reactive oxygen species, (ROS); Stromal vascular fraction, (SVF).

* Corresponding author.
** Corresponding author. National Centre of Excellence in Molecular Biology, 87-West Canal Bank Road, University of Punjab, Lahore, Pakistan.
E-mail addresses: mshifaulhaq@gmail.com (H.M. Shifa ul Haq), ramlaashfaq@gmail.com (R. Ashfaq), azramehmood@cemb.edu.pk (A. Mehmood), wardashahid.ws18@yahoo.com (W. Shahid), ghufiazam@yahoo.com (G. Azam), maryamazam155@hotmail.com (M. Azam), saba.tasneem@cemb.edu.pk (S. Tasneem), shehlajavedakram@yahoo.com (S.J. Akram), kausarbasit786@yahoo.com (K. Malik), riazuddin@aimrc.org (S. Riazuddin).

Peer review under responsibility of the Japanese Society for Regenerative Medicine.

[1] H.M Shifa ul Haq & Ramla Ashfaq contributed equally to this article.

https://doi.org/10.1016/j.reth.2023.01.001

application of different strategies for quick healing and saving the life of patients is crucially required.

In regenerative cell therapy applications, mesenchymal stem cell (MSCs) transplantation is a promising approach due to certain characteristics possessed by the MSCs, it includes multilineage potential of differentiation, the ability to restrain OS, and the release of many different soluble mediators with valuable trophic and immunomodulatory effects [6]. Stem cells enhance the process of angiogenesis and secrete numerous paracrine factors that efficiently enhance the regeneration process at the wounded area [7,8]. In case of any tissue injury, tissue-resident MSCs participate in tissue regeneration and coordinate with the physiological conditions of the microenvironment of tissue for repairing the damaged tissue. However, in the case of chronic tissue injuries where tissue-resident MSCs are not enough, autologous or allogeneic MSCs can be delivered in a highly targeted manner at the injured area for tissue healing purposes [9,10].

MSCs can be isolated from the stromal fraction of many different types of tissues that include bone marrow (BM-MSCs), umbilical cord, Wharton's jelly (WJMSCs), and adipose tissue (ASCs) [11]. Among different types of stem cells, human adipose-derived stem cells (hASCs) are most commonly used in dermatology [12]. The reason is that isolation of hASCs is simple and highly efficient. Cell yield derived from adipose tissue is 100–1000 times more than that of the bone marrow which makes it an easy target and efficient source for the isolation of stem cells. Combinational therapy of hASCs together with different growth factors could enhance angiogenesis and collagen deposition at the wound site [13,14]. Stem cells and their conditioned medium transplantation at the site of injury have shown excellent regenerative effects in various tissue and organ injuries [15] but, a huge number of cells die due to the pathological environment at the wounded site [16]. This cell death could be due to various factors including hypoxia, excitotoxicity, ROS, inflammatory response, apoptotic cascade activation, excessive autophagy, and so on [17,18].

In-vitro pre-conditioning of stem cells can improve the survival, engraftment, and paracrine properties of stem cells which can ultimately lead to better reparative and regenerative capacity Pre-conditioning of stem cells could be done by supplementation of different tropic factors like cytokines, hormones, growth factors, or the addition of lipopolysaccharides and different pharmacological agents [17]. Different pre-conditioning agents and strategies either activate or suppress different molecular signals and signal transduction cascades in stem cells and affect the expression of multiple factors or proteins [19,20].

Caffeic acid(CA) (3,4-dihydroxycinnamic acid) is a polyphenol that is produced as a secondary metabolite in many edibles such as olives, carrots, coffee beans, and potatoes [21]. CA and its derivatives have been reported to act as an excellent agents against bacteria, viruses, free radicals, and inflammation, and are also known to have antioxidant and immunomodulatory properties [22,23]. In different cell lines like 3T3 fibroblasts, CA stimulated the synthesis of collagen-like polymer and inhibited the generation of ROS [24]. Moreover, CA is also reported to provide cytoprotection against $HgCl_2$ to *Lactuca sativa* seeds which allowed better growth and development of both plant radicles and caulicles [25].

Keeping the anti-inflammatory and antioxidant properties of CA in perspective, it was utilized to pre-condition hASCs and boost their survival and proliferation potential within an in-vitro hypoxic microenvironment. While a chemically induced hypoxic microenvironment was created to replicate chronic stress/injury conditions.

2. Materials and methods

2.1. Lipoaspirate collection

Liposuction samples were collected after informed consent from healthy patients (n=10) undergoing abdominoplasty at Burn and Reconstructive surgery center, Jinnah hospital, Lahore (Pakistan). Patients with diabetes, age above 40yrs, HCV positive, HIV positive or with any viral infection were excluded.

2.1.1. Isolation, culturing, and characterization of human adipose-derived stem cells (hASCs)

2.1.1.1. Isolation. hASCs were isolated from lipoaspirate by the enzymatic digestion method. In brief, liquid adipose tissue was washed with phosphate buffer saline (PBS) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.2 mg/ml amphotericin-B (Sigma–Aldrich). Washing of the sample was followed by enzymatic digestion with the collagenase-I solution (Gibco, USA) in low glucose Dulbecco's modified Eagle's medium (LG-DMEM; Sigma Aldrich, USA). After centrifugation at 1200 rpm for 5–7 min, the resulting stromal vascular fraction (SVF) was passed through a 100 μm cell strainer.

2.1.1.2. Culturing. Isolated hASCs were cultured in LG-DMEM, supplemented with 15% fetal bovine serum (FBS; Sigma–Aldrich, USA), 100 U/ml penicillin, 100 mg/ml streptomycin and 0.2 mg/ml amphotericin-B. Media of the cells was replaced after every 3rd day until cells were 75–85% confluent. All further experiments were carried out at the P3 stage.

2.1.1.3. Characterization. For the characterization of hASCs, immunocytochemistry was performed for CD71, CD73, CD90, CD105, CD14, and CD45. hASCs were trypsinized and plated in 6 well plates over autoclaved coverslips at a density of 1×10^5 hASCs/well. After overnight incubation at 37 °C and 5% CO_2, cells were washed with PBS and fixed using 2% paraformaldehyde (PFA; Sigma–Aldrich, USA) and blocked with 5% bovine serum albumin (BSA; Sigma–Aldrich, USA). Cells were treated with primary antibodies diluted at 1:150 for 24 h at 4 °C and then 500 μl of secondary antibody (anti-mouse) diluted at 1:350 for 2 h at 37 °C. Afterward, cells were washed thrice with PBS followed by the addition of 4′,6-diamidino-2-phenylindole (DAPI) diluted at 1:1000 for 20 min at room temperature. hASCs were analyzed using an Olympus BX61 microscope (Olympus, Japan). The details of antibody markers used in the experiment are given in Table 1.

2.1.1.4. Trilineage. Additionally, by using StemPro® Differentiation Kits, the differentiation potential of naïve hASCs into adipocytes, chondrocytes and osteocytes were examined by staining with oil

Table 1
MSCs markers used for hASCs characterization.

MSCs marker	Catalog number	Manufacturer Company
Primary antibodies		
CD71 (mouse monoclonal IgG)	Sc- 65882	Santa Cruz, USA
CD73 (mouse monoclonal IgG)	Sc-398260	Santa Cruz, USA
CD90(mouse monoclonal IgG)	Sc-53456	Santa Cruz, USA
CD105(mouse monoclonal IgG)	Sc −18838	Santa Cruz, USA
CD14 (mouse monoclonal IgG)	Sc-1182	Santa Cruz, USA
CD45 (mouse monoclonal IgG)	Sc-1178	Santa Cruz, USA
Secondary antibody		
Anti-mouse	A21202	Invitrogen

red O, Alcian blue 8GX, and Alizarin red S stain respectively by using the protocol as mentioned previously by Azam et al. [26].

2.2. In vitro stress optimization and confirmation of injury

For mimicking chronic in-vitro wound conditions $CoCl_2$ (Sigma–Aldrich, USA) was used to create a hypoxia stress microenvironment [27]. Cells were exposed to different dilutions (100 μM, 150 μM, 250 μM, 350 μM, 400 μM, 500 μM) of $CoCl_2$ for 24 h and the stress dose was optimized by performing the following assays.

2.2.1. Microscopic analysis

To evaluate the effect of hypoxia stress on cellular morphology, 1×10^5 hASCs/well were seeded in 6 well plates. Cells were exposed to hypoxia stress and after 24 h detailed microscopic analysis was performed and 20–25 images per group were taken by using an Olympus IX51 microscope (Olympus, Japan).

2.2.2. Cells viability assays

To evaluate the effect of hypoxia stress on cell injury following two assays were performed.

2.2.2.1. XTT assay. For evaluation of cellular viability microculture tetrazolium (XTT) assay was performed. Cells were seeded at a density of 1×10^4 hASCs/well in a 96-well tissue culture microplate. Cells were exposed to $CoCl_2$ for 24 h in LG-DMEM with 5% FBS. After treatment, 50 μl/well XTT reagent (Roche, Switzerland) was added and the experiment was performed as per the manufacturer's protocol. The percentage viability was calculated by the following formula.

Cell viability (% of control) = Experimental group OD/Control group OD × 100.

2.2.2.2. Trypan blue assay. Trypan blue assay was performed for live and dead cell exclusion. Cells were seeded at a density of 5×10^4 hASCs/well in 24-well tissue culture plates. After treatment completion with stress doses, cells were washed, trypsinized and then cells pellet was dissolved in 1 ml LG-DMEM media with 15% FBS. Live/Dead Cells were counted using a hemocytometer using the following formula.

Death Rate (%) = Dead cells/total no of cells (live + dead) × 100.

2.3. CA priming dose optimization

Before evaluating the priming effects of CA on hASCs under hypoxia stress, CA cytotoxic and cytoprotective doses were analyzed and optimized. For this purpose, hASCs were incubated with a range of CA concentrations (10 μM, 15 μM, 25 μM, 50 μM, 75 μM, and 100 μM) for 24 h and different assays (XTT, trypan blue, and microscopic analysis) were performed to pick a suitable priming dose of CA.

2.4. Cytoprotective dose selection

After a careful selection of a cytoprotective range of CA doses on hASCs, the priming dose of CA was further optimized in combination with a selected dose of hypoxia stress (500 μM) by employing XTT (as aforementioned method) and LDH assay.

2.4.1. LDH assay

Lactate dehydrogenase (LDH) was performed with selected priming doses of CA to pick a final preconditioning dose. LDH-based toxicology assay kit (Sigma–Aldrich, USA) was used and the experiment was performed as per the manufacturer's protocol. After completion of the experiment absorbance values were taken at a wavelength of 490 nm along with the background absorbance at 690 nm

2.5. Cytoprotective effects in injury condition

To study the cytoprotective effects of the optimum dose of CA on hASCs following experimental groups of cells were made:

Control (hASCs without pretreatment or hypoxia stress for 24 h in media with 5% FBS).

Pre.hASCs (hASCs pretreated with 75 μM CA for 24 h in media with 5% FBS & without any stress).

Hypoxia stress (Cells incubated for 24 h in media with 5% FBS + hypoxia stress for 24 h).

Pre.hASCs + Stress (Pretreated hASCs with 75 μM CA for 24 h in media with 5% FBS + hypoxia stress for 24 h).

2.6. Cellular apoptosis: annexin V assay

Cells were seeded at a density of 1×10^5 hASCs/well over autoclaved coverslips placed in 6 well plates. After completion of described treatments, cells were washed and fixed and blocking was done using PBS, 2% PFA, and 5% BSA respectively. After blocking, the primary antibody (annexin V) diluted as 1:200 was added and cells were incubated overnight at 4 °C. After 24 h, cells were washed and a secondary antibody was added and followed by incubation and cell staining with DAPI using the same procedure as explained above (section 2.2), and 20–25 images per group were taken using an Olympus BX61 microscope.

2.7. Live dead assay

A live/dead assay was performed using a live/dead double staining kit (Sigma–Aldrich, USA). Cells were seeded at a density of 1×10^5 hASCs/well over autoclaved coverslips placed in 6 well plates. After the completion of treatment, the experiment was performed as per the manufacturer's protocol and 20–25 images per group were taken by using an Olympus BX61 microscope.

2.8. Cellular senescence: β-gal staining assay

Cellular senescence was analyzed using a senescence detection kit (Abcam) in all four experimental groups. hASCs were seeded in a 24-well plate while ensuring the cellular density of 1×10^5 cells per well. After completion of the treatments, the assay was performed as per the manufacturer's protocol and the β-Galactosidase (β-gal) positive cells were observed under the Olympus IX51 microscope (Olympus, Japan). Images were taken (20 images per group), stained cells were counted, and results were analyzed.

2.9. Cellular migration: scratch wound assay

To check the migration potential of CA-primed hASCs, a scratch wound healing assay was performed. Cells were seeded with a density of 0.8×10^5 hASCs/well in 6-well plates. After completion of treatments, two sharp perpendicular scratches were made using 200 μl micropipette tips. Cells were allowed to grow in serum-free media for 24 h in standard conditions. After 24 h cells were washed, fixed, and stained with crystal violet. After staining the healing effect of cells was observed by analyzing the filling of scratched empty spaces and images were taken. The same procedure was repeated to check the filling of the scratched empty spaces after 48 h incubation in another experiment.

2.10. Reactive oxygen species assay (ROS)

For measurement of reactive oxygen species (ROS) generation in the cells, ROS assay was performed using the DCFDA/H2DCFDA - Cellular ROS Assay Kit (ab113851). Briefly, hASCs were trypsinized and plated in 6 well plates over autoclaved coverslips at a density of 1×10^5 hASCs/well. After completion of treatment in all 4 experimental groups, the assay was performed as per the manufacturer's instruction, and 20–25 images per group were taken by using an Olympus BX61 microscope. The fluorescent intensity was measured using Image J software and the intensity fold was computed.

2.11. Gene expression analysis: real time-polymerase chain reaction (qRT-PCR)

To examine the effect of CA preconditioning at the molecular level, expression of mRNA of different pro-apoptotic, angiogenesis, cell survival, and inflammation associated (Table 2) markers were evaluated by qRT-PCR. Briefly, RNA was extracted from all experimental groups using TRIzol reagent (Sigma–Aldrich, USA). cDNA was synthesized by using 1 μg RNA and Revert Aid H-Minus first strand cDNA synthesis kit (Invitrogen, USA). qRT-PCR was done by using a master mix (Maxima Syber Green, Fermentas, USA) and reactions were carried out on PikoReal 96 (Thermo Scientific, USA). GAPDH was used as an internal control and the relative expression of each marker was examined using quantification cycle values.

2.12. Statistical analysis

Statistical analysis of data was done by using GraphPad Prism 7 software (USA). The data were represented as mean ± standard deviation. One-way ANOVA with Bonferroni's post-test was used for the comparison of various experimental groups, while the value of $p<0.05$ was considered statistically significant. All the experiments were performed thrice and each group was independently divided into triplicates.

Table 2
Primer sequences.

Genes	Primer Sequence (5′-3′)	Product size (bp)	Tm (°C)
GAPDH	F: AACGTGTCAGTGGTGGACCT R: TGCTGTAGCCAAATTCGTTG	250	58
BAX	F: AAGTCCAATGTCCAGCCCAT R: AAGTCCAATGTCCAGCCCAT	163	58
BCL-XL	F: GGAGCTGGTGGTTGACTTTCT R: GGGCCTCAGTCCTGTTCTCT	95	60
FADD	F: CTGCGGGAGTAGTTGGAAAGT R: GGAAATGGGACAAAACATCCT	241	60
TGFβ1	F: ACTGCAAGTGGACATCAACG R: TGCGGAAGTCAATGTACAGC	218	58
IL-6	F: GGCTGAAAAAGATGGATGCT R: GTACTCATCTGCACAGCTCT	156	57
PI3–K	F: ACAGGTCAATGGCTGCATCA R: CAATCGGTGACTGTGTGGGA	171	57
PCNA	F: AGGCACTCAAGGACCTCATC R: GTATCCGCGTTATCTTCGGC	244	58
IGF-1	F: GCTCTTCAGTTCGTGTGTGG R: AGCCTCCTTAGATCACAGCTC	134	58
CASP-3	F: AGCAAACCTCAGGGAAACATT R: CTCAATGCCACAGTCCAGTTC	307	58
AKT	F:CGGGGTAGGGAAGAAAACTATC R:TGACAGAGTGAGGGGACACAT	185	60

3. Results

3.1. Confirmation of mesenchymal origin of hASCs

For MSCs characterization, an immunofluorescent assay for both positive and negative cell surface markers was performed. High expression of positive cell surface markers of hASCS including $CD71^+$, $CD73^+$, $CD90^+$, and $CD105^+$ while negligible expression was found for negative markers including $CD14^-$ and $CD45^-$ (Fig. 1). Moreover, the trilineage differentiation potential of hASCs into adipocytes, chondrocytes, and osteocytes on staining with respective dyes showed strong positive results.

3.2. Effect of $CoCl_2$ doses on hASCs viability and morphology

Morphological analysis to inspect the cytotoxic effect of different doses of hypoxia on hASCs, was carried out by detailed microscopy which showed that by increasing concentration of hypoxia, cells shape appeared more pinched and distorted while in the control group cells were found to be healthy, elongated and of spindle-like shape (Fig. 2a).

XTT assay was performed to analyze the cell proliferation rate of hASCs that were subjected to different concentrations of hypoxia ($CoCl_2$) stress. The results demonstrated that as compared to the Control, there was a gradual decrease in the viability of cells with the increasing concentration of hypoxia stress dose. The 500 μM concentration of $CoCl_2$ showed 40.0 ± 2.86% cell viability as compared to the Control (100 ± 3.93%) (Fig. 2b).

Results of the trypan blue assay showed a similar trend of cellular death. With the increasing concentration of hypoxia stress, there was a significant gradual decrease in the number of viable cells as compared to the control. The lowest percentage viability (65 ± 9%) was found with a 500 μM concentration of $CoCl_2$ (Fig. 2C).

Overall, the results of these experimentations suggested that cellular death is directly proportional to the increasing concentration of hypoxia stress and thus that dose (500 μM) was selected where almost 50% of cells were found to be dead.

3.3. Analysis of hASCs sensitivity against various doses of CA

Microscopic analysis of hASCs treated with different doses of CA showed that with increasing concentration of CA, cells started to attain a more rounded shape, especially at 100 μM concentrations despite showing maximum viability at this concentration (Fig. 3a).

To analyze the cell proliferation rate under treatment with different pre-conditioning doses of CA, XTT assay was performed. 75 μM concentrations showed a cell proliferation rate of 118 ± 4% as compared to Control (100 ± 2%) (Fig. 3b). For further assessment of the percentage viability of cells trypan blue exclusion assay was performed. Similar to XTT results, the percentage viability of the Control and 75 μM CA pre-treated groups was found to be comparable (Fig. 3c).

Overall, based on these results and for keeping the cells in the natural morphological shape, a 75 μM CA priming dose was selected for further experiment.

3.4. Cytoprotective effect of caffeic acid priming of hASCs against hypoxia stress

To analyze the effect of different priming doses of CA in combination to stress on cellular proliferation and cytotoxicity, XTT and LDH assays were performed. There was a significant decrease in the proliferation rate of untreated hASCs (55 ± 14%) as compared to the Control group (100 ± 16%) (Fig. 4a). Moreover, LDH release values

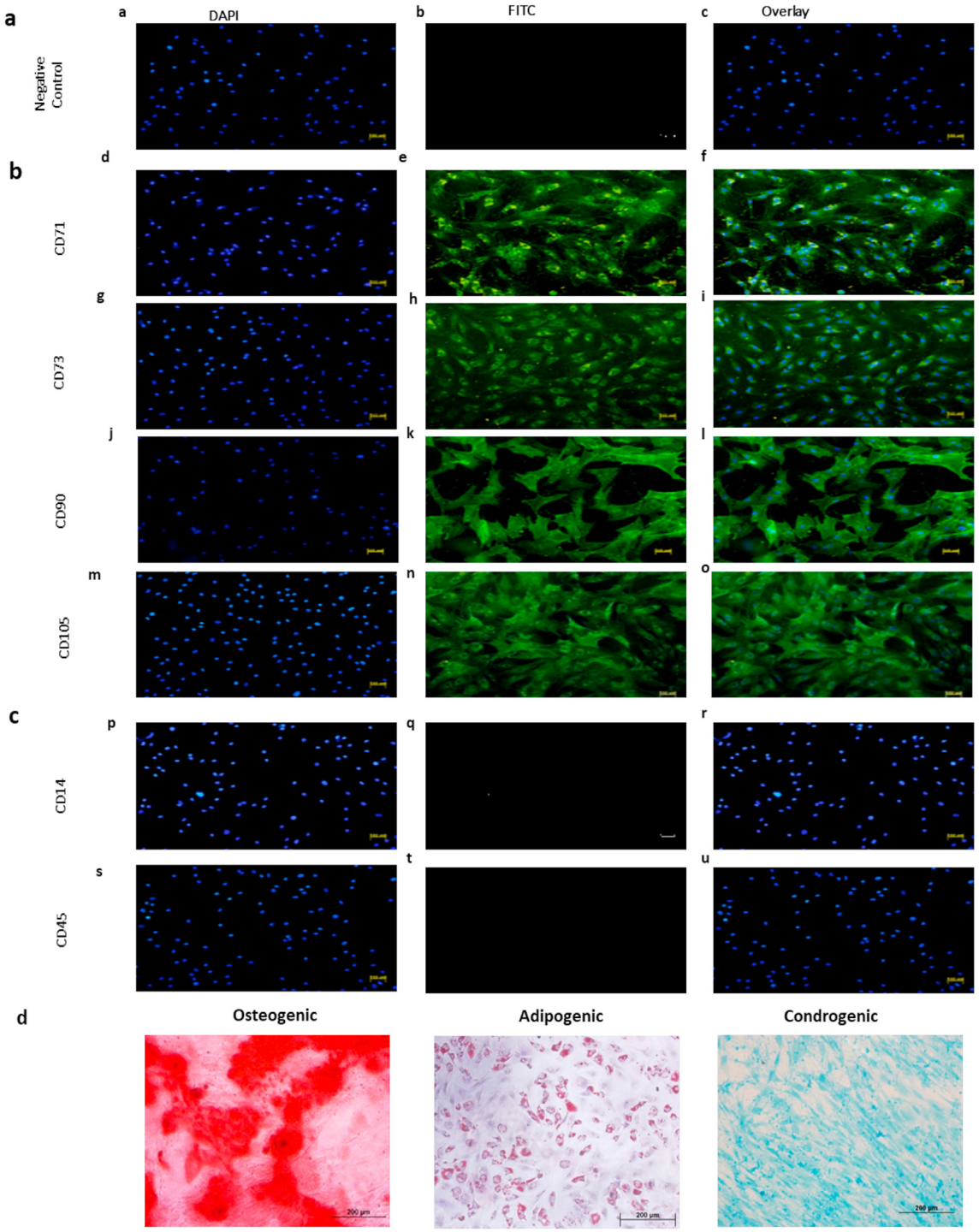

Fig. 1. Immunocytochemical expression and trilineage differentiation potential of hASCs. (a) Expression in Sec. Control. (b) Expression of CD71, CD73, CD90, and CD105. (c) Expression of CD14 and CD45 of MSCs in cultured hASCs (Magnification: 100×). (d) Trilineage differentiation potential of hASCs into adipogenic, chondrogenic, osteogenic cells. Osteogenic differentiation can be observed by Alizarin red staining of calcium phosphate produced by osteocytes; adipogenic induction can be observed by oil red O staining of lipid droplets; and chondrogenic differentiation can be observed by Alcian blue staining of proteoglycans deposition (Magnification: 200 ×, scale bar: 200 μm).

were found to be highest in the untreated hASCs (100 ± 102%) as compared to the Control (315 ± 104%) (Fig. 4b). This indicated a positive effect of CA priming against hypoxia.

Analyzing all the optimization results of hypoxia and CA pre-conditioning doses, 500 μM of hypoxia ($CoCl_2$) dose for 24 h and 75 μM pre-conditioning dose of CA for 24 h were chosen and experimental groups of cells were made.

3.5. Annexin V apoptosis analysis

Annexin V assay was done for analyzing the effect of CA pre-conditioning on the survival ability of hASCs upon exposure to a hypoxic stressed environment. Microscopic visualization of apoptosis showed that annexin-V positive cells in Pre. hASCs + Stress group (31 ± 0.02%) were significantly low as

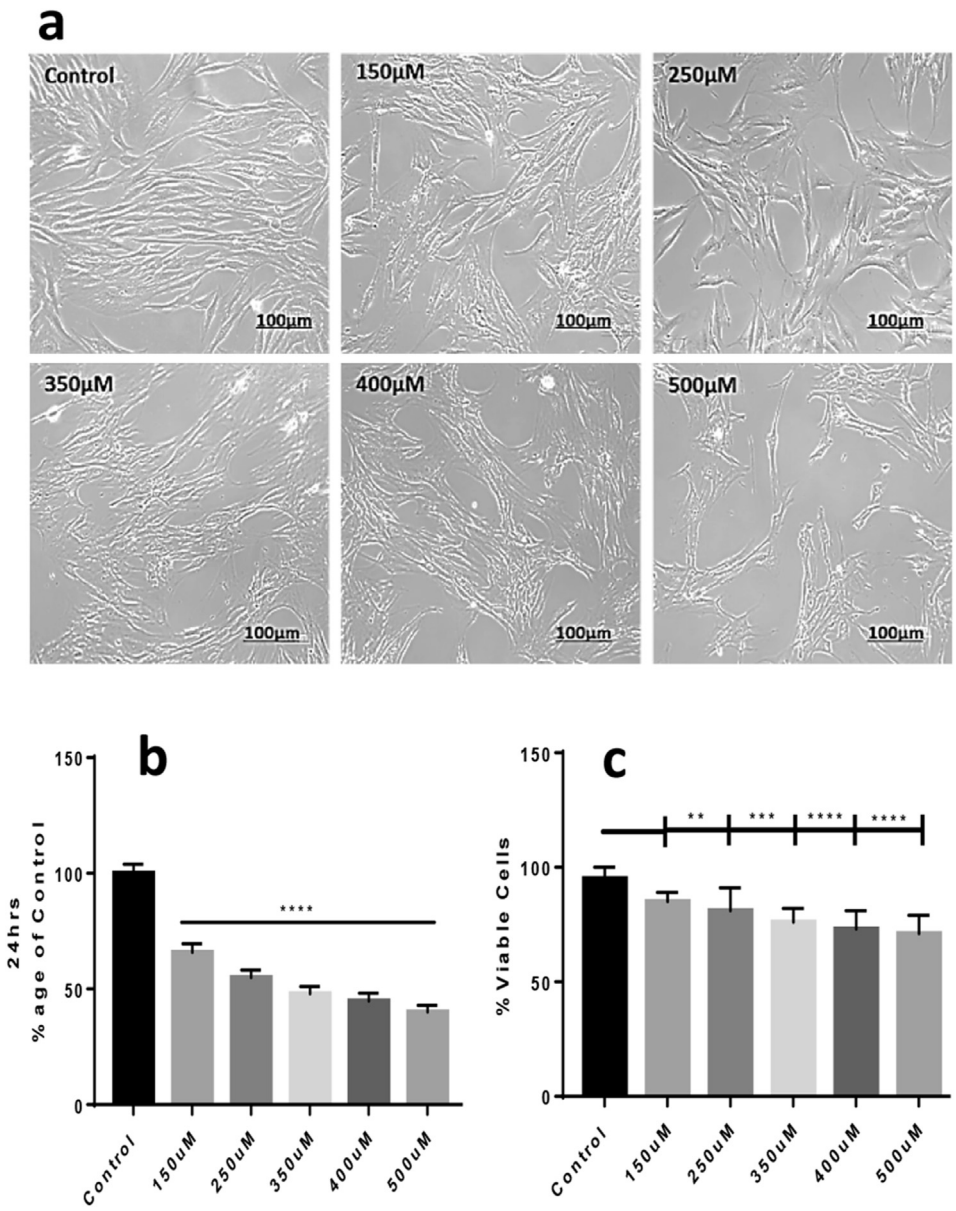

Fig. 2. Hypoxia stress optimization. (a) Microscopic analysis of diverse hypoxia doses over the morphology of hASCs (Magnification: 200× scale bar: 100 µm). (b) XTT results show the effect of hypoxia stress on cellular viability. (c) Trypan blue exclusion assay. All graphical values are represented as mean ± SD while $^{*}P = 0.0264$, $^{**}P \leq 0.0043$, $^{***}P = 0.0002$, $^{****}P < 0.0001$ vs. Control group.

compared to the Hypoxia Stress group (94 ± 0.02%) indicating a cytoprotective effect of CA priming (Fig. 5a).

3.6. Live/dead cellular analysis

A live dead assay was done for visualizing the percentage of live cells in untreated and pre-treated hASCs when exposed to hypoxic conditions. The CaAM (green fluorescent dye) was retained by live cells while EthD1 (red fluorescent dye) is retained by dead cells. The percentage of live cells in the Pre. hASCs + Stress group was found to be 97 ± 12%, showing a marked cell survival increase with more stained live cells as compared to the Hypoxia Stress group (72 ± 8%) (Fig. 5c).

3.7. Assessment of senescence-associated (β-gal) activity

Priming with CA helped hASCs in reducing cellular senescence when exposed to hypoxia stress, showing its positive attributes as an antioxidant (Fig. 6a). Maximum uptake of β-Gal was observed in the hypoxia stress group. The percentage of senescent cells in the Hypoxia Stress group was found to be 6.2 ± 1.6% while in that of the Pre. hASCs + Stress group was 2.2 ± 1.1%, suggesting the protective role of CA against senescence (see Fig. 6a).

3.8. Assessment of in-vitro wound healing ability

In-vitro cellular wound, healing potential was measured by observing cells' migratory rate towards the empty streak. Results of the assay displayed the migratory and wound healing potential of CA primed hASCs after exposure to hypoxia stress. Pre. hASCs + Stress group of hASCs exhibited enhanced migration of cells towards the empty streak area as compared to the Hypoxia Stress group (Fig. 6c). After 24 h of scratch, wound healing/scratch filling percentage shown by Control (62 ± 5%) and Pre. hASCs + Stress (61 ± 5%) was similar. Analogous results to 24 h

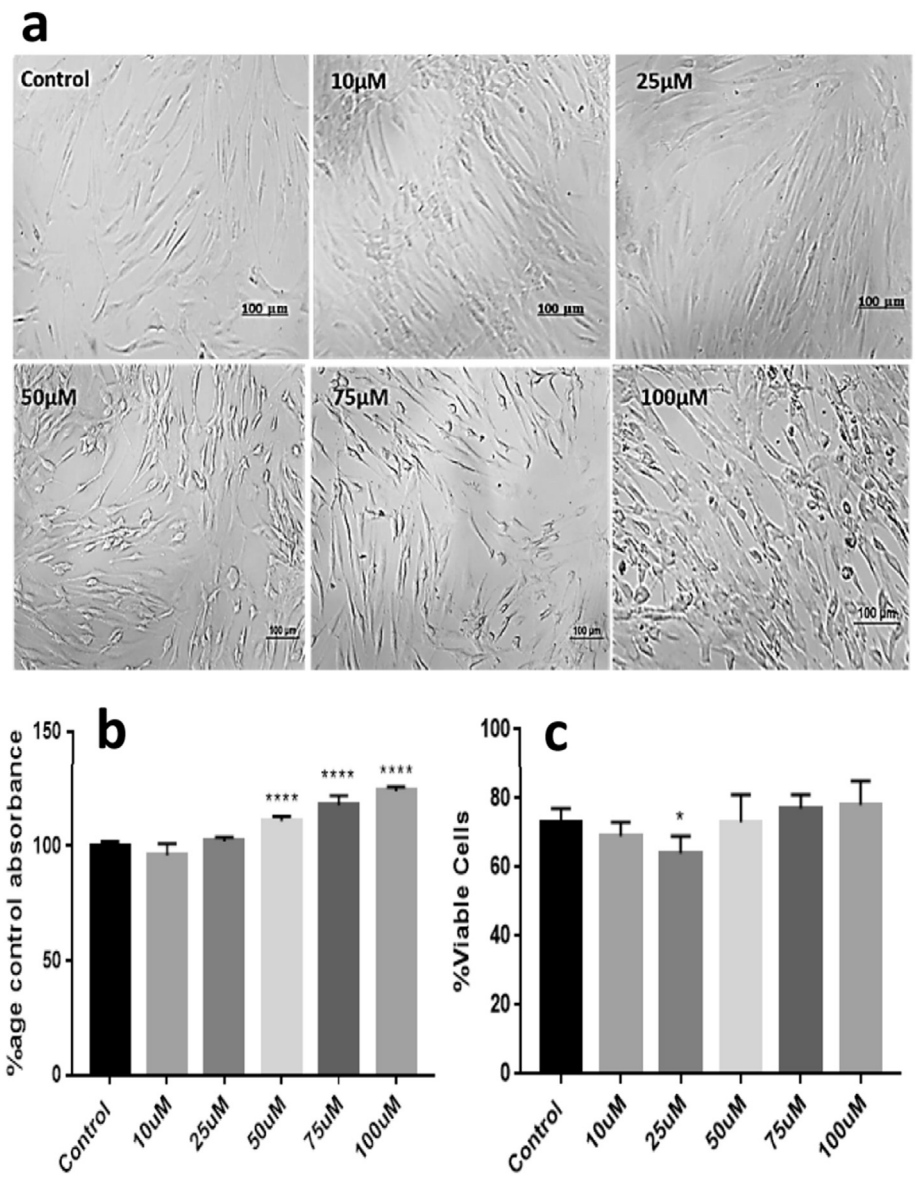

Fig. 3. Priming dose optimization. (a) Microscopic analysis of CA pre-conditioning (24 h) on the morphology of hASCs (Magnification: 200× scale bar: 100 μm). (b) XTT assay. (c) Trypan blue exclusion assay. All graphical values are represented as mean ± SD while $*P = 0.0443$ and $****P < 0.0001$ vs. the Control group.

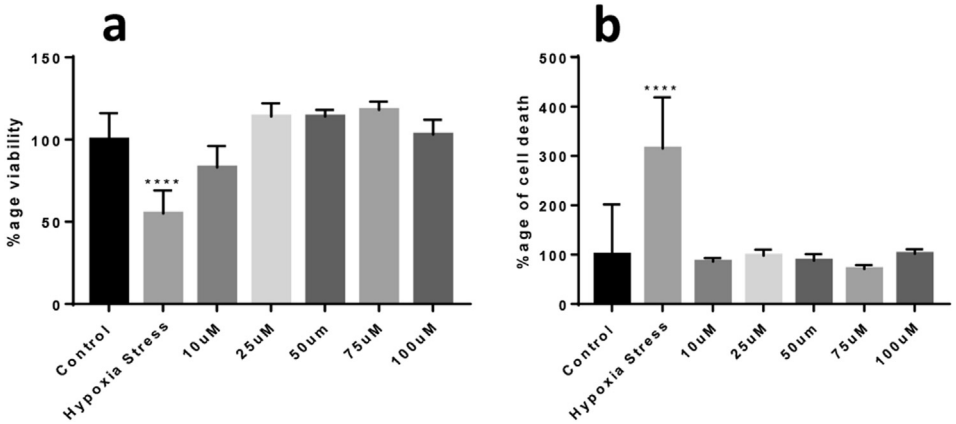

Fig. 4. Efficacy of different priming doses against hypoxia stress. (a) XTT assay showing percentage viability of cells treated with different priming doses of CA under selected doses of hypoxia stress. (b) Quantitative analysis of LDH release values of cells treated with different priming doses of CA under selected doses of hypoxia stress. Values are represented as mean ± SD while $****P < 0.0001$ vs. Control group.

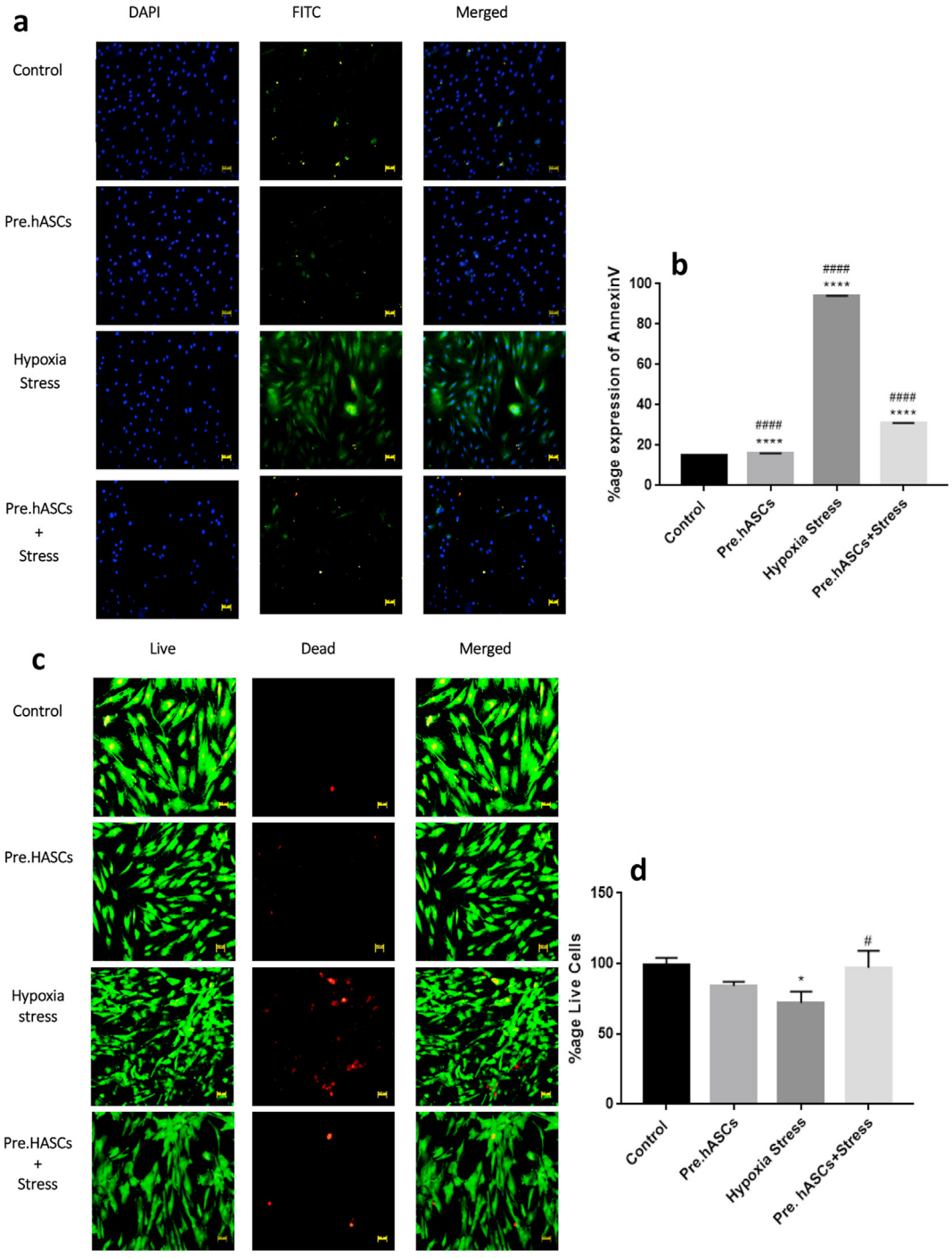

Fig. 5. Cytoprotective effect of a selected priming dose of CA against hypoxia. (a) Annexin-V immunocytochemical staining to observe apoptosis among different groups. In merged images, blue fluorescence shows staining of cells nuclei by DAPI while green fluorescence is emitted by the Annexin-V antibody stained cells (Magnification: 100×, scale bar: 105 μm) (b) Graphical representation of immunocytochemical analysis results. (c) Live/dead microscopic analysis of different experimental groups. Green and red fluorescence are emitted by live and dead cells, respectively (Magnification: 100×, scale bar: 105 μm). (d) Graphical representation of the quantitative analysis of the live/dead results. Values represent mean ± standard deviation whereas $^{*}P = 0.0168$, $^{****}P < 0.0001$vs. Control group while$P < 0.0001$ vs. Hypoxia stress group.

were observed after 48 h, indicating that CA priming had made hASCs capable to retain their proliferation and migration potential even after being exposed to hypoxia stress. Overall results of these experiments indicated that antioxidant treatment while using CA has significantly enhanced the survival and regenerative potential of hASCs to survive in a hypoxic environment.

3.9. Caffeic acid treatment reduces the generation of ROS

ROS generated in cells as a result of numerous chemical reactions or in the case of a stressed environment and are deleterious for the cells. ROS level was measured in all four experimental groups of cells to check the efficacy of CA priming (Fig. 7).

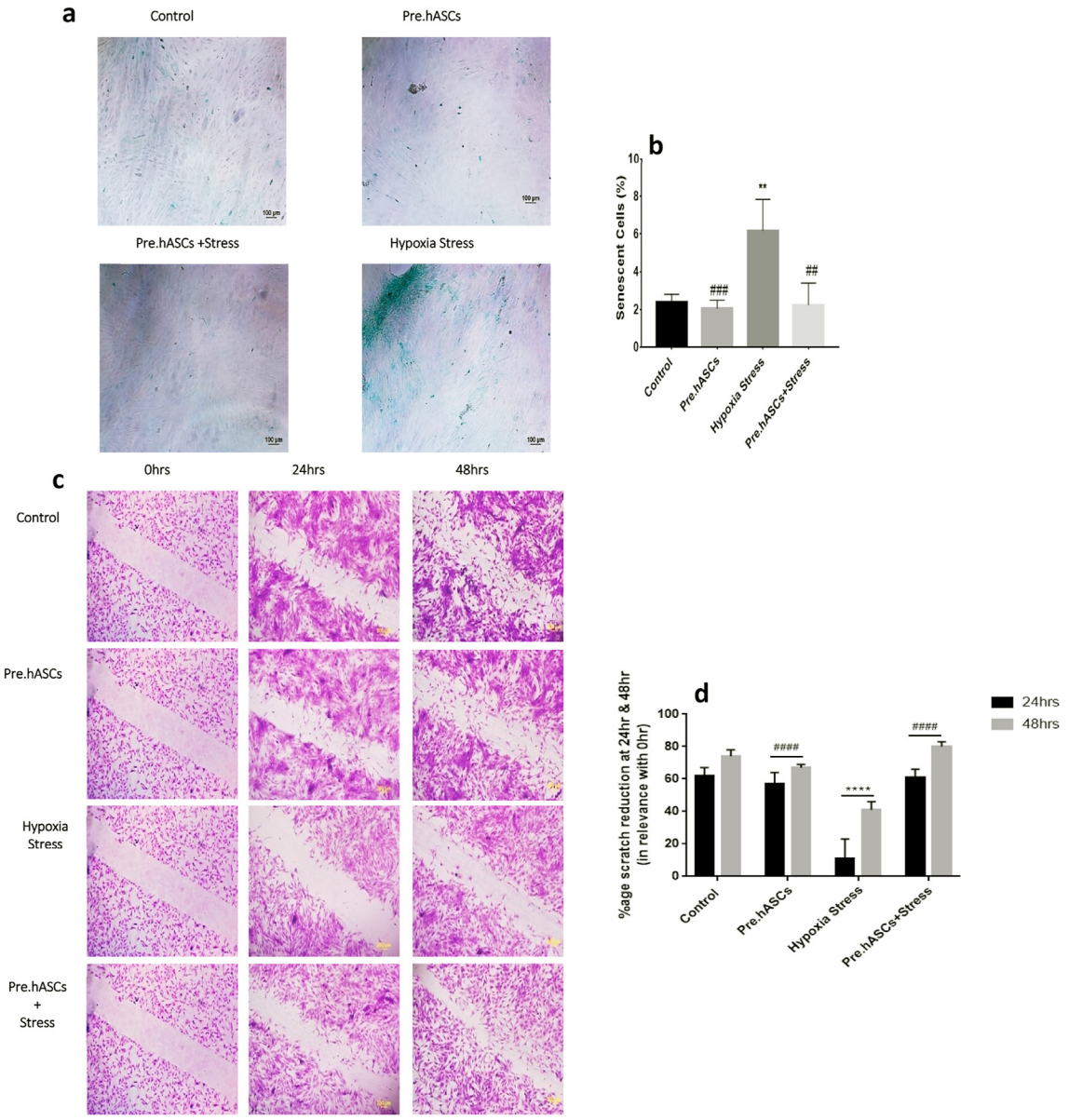

Fig. 6. Cellular migration and senescence analysis. (a) Crystal violet stained hASCs, subjected to the scratch wound assay. Single streak images were taken at 40x magnification for all the experimental groups at a scale bar of 200 μm. (b) Graphical representation of *in vitro* scratch wound assay. (c) Microscopic images of cellular senescence assay, β-gal stained cells retained blue-green dye (Magnification: 100×, scale bar: 100 μm). (d) Quantitative analysis of cellular senescence assay. All graphical values are represented as mean ± standard deviation whereas $^{**}P = 0.001$, $^{****}P < 0.0001$ versus the Control group while $^{\#\#}P = 0.001$, $^{\#\#\#}P < 0.001$ and $^{\#\#\#\#}P < 0.0001$ versus Hypoxia stress group.

Experimental results depicted that CA significantly reduces the level of ROS generation in the Pre. hASCs + Stress group (98 ± 53) as compared to the stress group (218 ± 13). Hence, cells pretreated with CA are more prone to ROS generation as compared to untreated cells.

3.10. CA reduces apoptosis gene expressions and upturns cell survival, angiogenic and inflammation-associated gene markers

Semi-quantitative real-time PCR analysis portrayed significantly enhanced expression of pro-apoptotic markers including BAX, FADD, and Casp-3 (Fig. 8a) in the Hypoxia Stress group as compared to the Pre. hASCs + Stress group that indicated the positive effect of CA priming over hASCs to withstand in the hypoxic stressed conditions. Similarly, significantly enhanced expression of cell survival (IGF-1, FGF-7, AKT, PCNA, PI3–K, BCL-XL) (Fig. 8b), angiogenic (VEGF, SDF-1) (Fig. 8c) and inflammation associated markers (IL-6 and TGFβ1) (Fig. 8d) was found in CA primed hASCs as compared to the untreated cells. Statistical individual values for each graph are on the graph's peaks.

4. Discussion

Healing of chronic wounds is a serious medical issue and the transplantation of hASCS has emerged as a favorable modality [28]. hASCs support the healing process by releasing essential paracrine factors that stimulate the production of stem cells at the site of injury [29]. Moreover, applications of hASCs in different experimental models proved their efficacy in healing due to their regenerative, immunomodulatory, pro-angiogenic, and anti-apoptotic properties [30,31]. However, often the application of hASCs alone does not show satisfactory therapeutic results due to

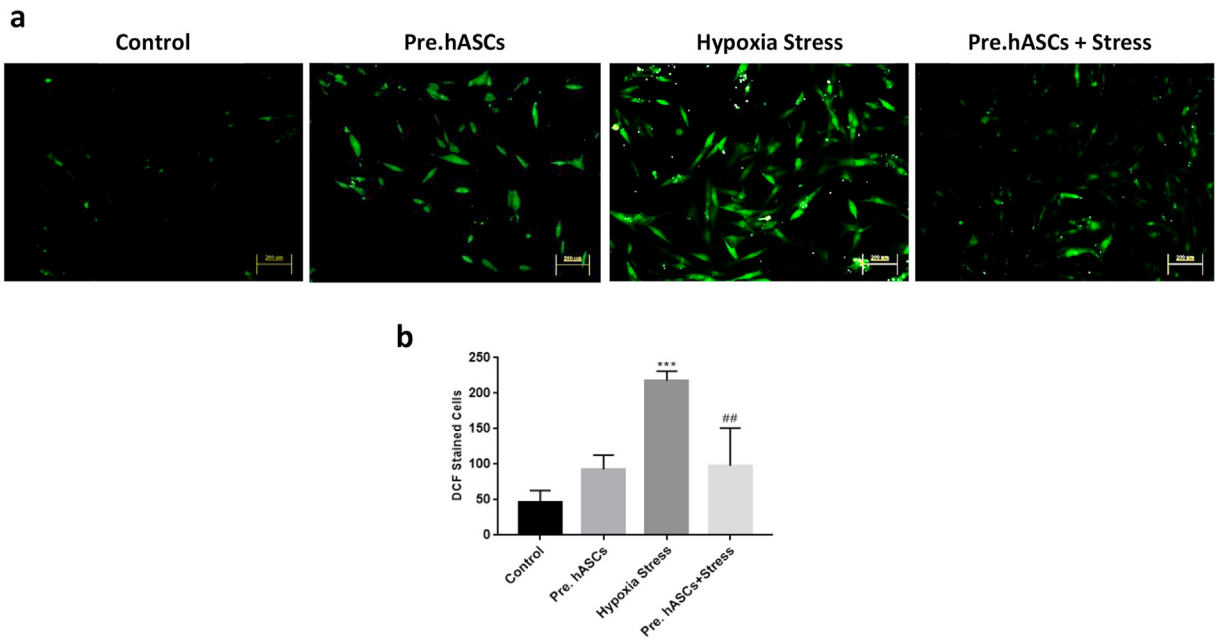

Fig. 7. ROS activity analysis. (a) DCF-fluorescence (green) images of different experimental groups of hASCs (Magnification: 100x, scale bar: 200 μm). (b) Assessment of intensity folds in different experimental groups of hASCs calculated by image J software. Data values are represented as mean ± standard deviation whereas $^{***}P = 0.0007$ versus the Control group while $^{\#\#}P = 0.007$ vs. the Stress group.

the pathological and hypoxic stressed microenvironment of the wounded area [16–18]. This problem could be overcome by pre-conditioning of hASCs with different chemicals and pharmacological agents that not only strengthen cell survival rate but also enhance their regenerative potential for quick wound healing [17,32]. In consideration of this strategy, the present study was performed to determine the positive attributes of CA pre-conditioning over hASCs to maintain their efficacy in presence of a hypoxic microenvironment similar to a chronic wound.

In this study, hASCs were characterized to fulfill the minimal criteria set for MSCs by the International Society for Cellular Therapy. For the confirmation of mesenchymal lineage of hASCs, cells were examined for their property of plastic adherence, expression of cell surface markers including CD71+, CD73+, CD90+, and CD105+ without the expression of $CD14^-$ and $CD45^-$ and for their trilineage differentiation potential into adipocytes, chondrocytes, osteocytes.

For creating an in-vitro chemical hypoxic (chronic wound) model, $CoCl_2$ was used as it's a well-known hypoxia-inducing agent. In normoxic conditions, enzymes including prolyl hydroxylase domain (PHDs) and HIF prolyl hydroxylase (HPH) mediate ubiquitin protease pathway degrades hypoxia-inducible factor- α (HIF1-α) but cobalt (Co) in $CoCl_2$ replaces iron (Fe) in PHDs as a chelating agent and makes these unable to combine with HIF1-α for degradation and thus stabilize HIF1-α and in this way acts as a hypoxia-mimicking agent [33]. $CoCl_2$ has been used both as a pre-conditioning agent as well as a hypoxia-inducing agent. The effect of $CoCl_2$ doses ranging from 50 to 400 μM concentrations has been reported for simulating hypoxia to induce chondrogenic differentiation of hASCs [27].

Hypoxia stress was optimized by performing XTT, trypan blue assay, and morphological assessments. Similar to previous studies [34,35], with the increasing concentration of $CoCl_2$ number of viable cells was found to be decreased in a dose-dependent manner. In our experiment, with increasing hypoxia stress the shape of the cells started to become distorted (Fig. 2a). Cell viability was reduced to 50% at 500 μM $CoCl_2$ concentration (fig. 2b). So, this concentration was selected for creating the in-vitro hypoxia model. A similar approach was used for analyzing the sensitivity of hASCs for different doses of CA pre-conditioning. The final selection of the CA priming dose was made after performing XTT and LDH assays in combination with the selected stress dose. 75 μM priming dose of CA was selected against 500 μM $CoCl_2$ stress after analyzing cellular morphology, proliferation, and cytotoxicity (fig. 3).

CA and its derivatives have been demonstrated to play a significant role in protecting cells and plants from bacteria, viruses, free radicals, and inflammation in different in-vitro and in-vivo studies [36,37]. There is not much data available related to the use of CA as a pre-conditioning agent for stem cells while this is a potent antioxidant agent. Therefore, the restorative and antioxidant effects of CA pre-conditioning on hASCs have been evaluated against hypoxia stress in the present study by performing annexin V, live dead, β-gal, scratch wound assays and at the end gene expression analysis.

Annexin V assay revealed hASCs pre-treated with CA retained significant cell viability after exposure to hypoxia stress while the untreated group had nearly 80% apoptotic cells as revealed by Annexin V assay (fig. 4a). Furthermore, live/dead assay and cellular senescence assay indicated, a higher number of dead and senescent cells in the untreated hASCs group as compared to the pre-treated hASCs group. Cellular viability of CA pre-treated hASCs depicted that CA has increased the survival ability of hASCs to withstand the hypoxic microenvironment. Moreover, CA priming impact over hASCs migration showed better potential to migrate towards the scratch area to heal the wound as compared to the untreated hASCs depicting its prominence for use in the treatment of chronic wounds (fig. 6c). Moreover, ROS assay further supported the argument of CA primed cells are prone to survive better in a hypoxic stressed environment as compared to untreated cells.

Cellular apoptosis can transpire via a death receptor-dependent (extrinsic) pathway like Fas-associated death domain (FADD) or intrinsic or mitochondrial pathway through the action of BAX protein [38]. BAX is an important pro-apoptotic member of the Bcl-2 protein family that promotes cellular apoptosis by promoting the

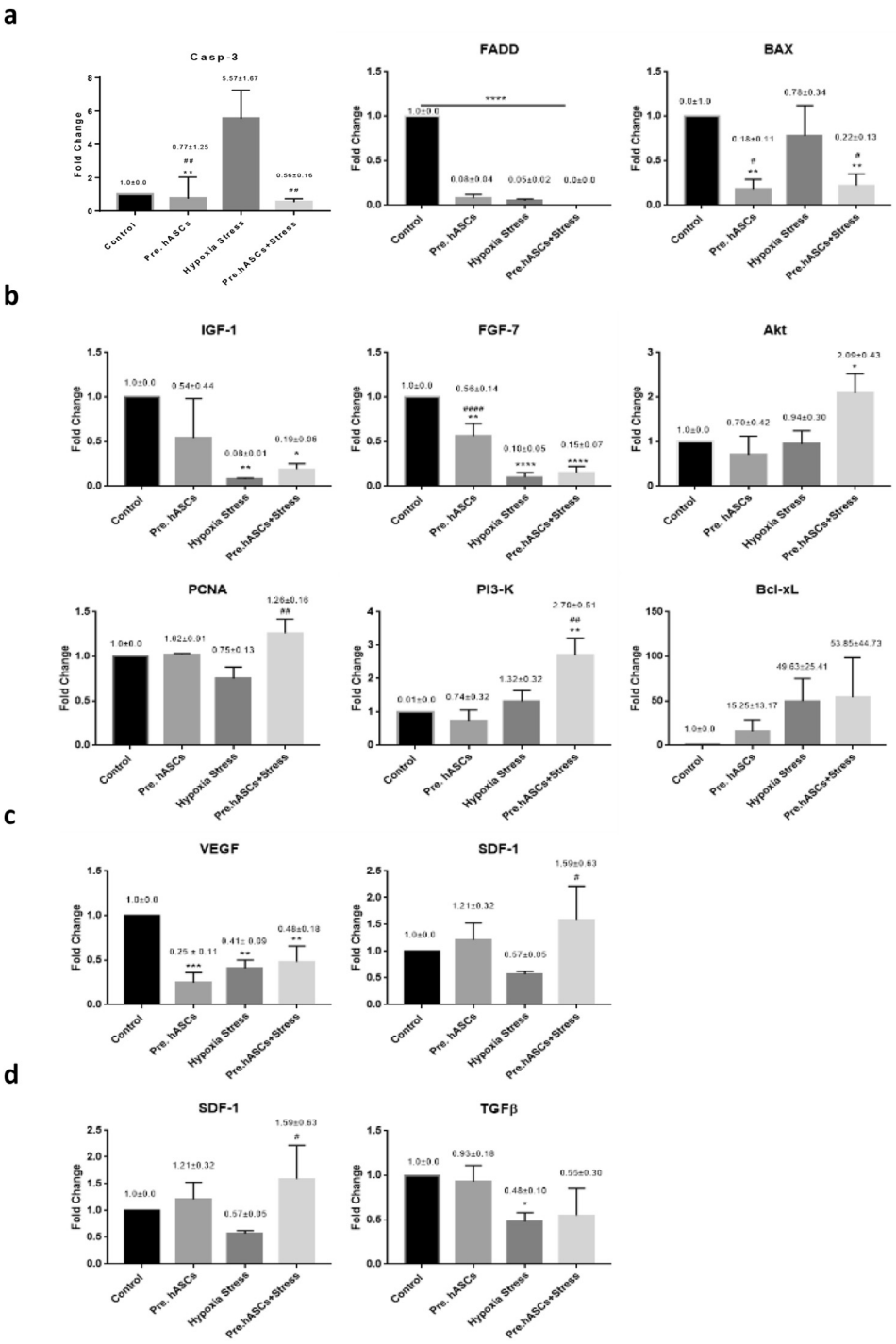

Fig. 8. Gene expression analysis. (a) Pro-apoptotic markers (BAX, FADD, Casp-3). (b) Cell survival markers (IGF-1, FGF-7, Akt, PCNA, PI3−K, Bcl-xL). (c) Angiogenic markers (VEGF, SDF-1). (d) Inflammatory associated markers (IL-6, TGF-β). Data values represent mean ± standard deviation whereas $^{*}P \leq 0.0246$, $^{**}P \leq 0.0061$, $^{****}P < 0.0001$ vs. Control group and $P \leq 0.0415$, $P \leq 0.0030$, $^{\#\#\#\#}P < 0.0001$ vs. Stress group.

release of cytochrome-c in mitochondria [39]. In our study, the expression of various pro-apoptotic markers (BAX, FADD, CASP-3) showed increased expression in untreated cells as compared to CA pre-treated cells when exposed to hypoxia stress. MSCs express multiple paracrine factors including VEGF, FGF-7, IL-6, and SDF-1 [40]. The proliferation and angiogenesis of MSCs are regulated by these paracrine factors whereas TGF-β is involved in cellular differentiation [41,42]. In response to some other cell growth factors like IGF-1 and FGF-7, the PI3-kinase/Akt pathway becomes activated which then plays key roles in cellular metabolism, inflammation, cell survival, and motility [43,44]. Similarly increased expression of BCL-XL which is an anti-apoptotic protein enhances cell survival and expansion of embryonic stem cells [45]. Different cytokines released by immune cells like IL-6, and TNF-α play an important role in the regulation of inflammation [46] and thus can provide beneficial aspects in the wounded area. Gene expression

analysis of cell survival (IGF-1, FGF-7, AKT, PCNA, PI3–K, BCL-XL), angiogenic (VEGF, SDF-1), and inflammation associated markers (IL-6 and TGFβ1) exemplified the robustness/shielding effects of antioxidant pretreatment of hASCs.

Increased expression of cell survival markers and decreased expression of pro-apoptotic markers confirmed diminished damaging effects of hypoxia stress by the protective effect of CA pre-conditioning.

5. Conclusion

This study reveals, CA pre-conditioning of hASCs increases their survival ability and enhances the capability to withstand a stressed environment such as of a chronic wound as compared to untreated cells. Moreover, increased expression of cell survival, angiogenic and inflammation-associated genes affirmed that CA renders hASCs more capable of countering the hypoxia as prevailing in a chronic wound environment compared to untreated cells.

Author contributions

H.M. Shifa ul Haq & **Ramla Ashfaq:** Conceptualization, Methodology, Data organization, Software, Data Curation, Writing-Original draft, Writing – Review & Editing. **Azra Mehmood:** Supervision and validation. **Warda Shahid:** Assisted in experimentation. **Hafiz Ghufran:** Characterization data, Reviewing, and editing. **Maryam Azam, Saba Tasneem:** Reviewing and editing. **Shehla Javed Akram** & **Kausar Malik:** Provided resources. **Sheikh Riazuddin:** Reviewing and editing.

Funding

This work was financially supported by research grants from the Higher Education Commission of Pakistan (NRPU 14181, TDF02-163).

Declaration of competing interest

The authors of this article declare no conflict of interest. The funders had no role in designing the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

[1] Goorani S, Zangeneh MM, Koohi MK, Seydi N, Zangeneh A, Souri N, et al. Assessment of antioxidant and cutaneous wound healing effects of Falcaria vulgaris aqueous extract in Wistar male rats. Comp Clin Pathol 2019;28(2):435–45.
[2] Tabari S, Carpi S, Polini B, Nieri P, Esfahani M, Moghadamnia A, et al. Topical application of silymarin enhances cutaneous wound healing in rats. South Afr J Bot 2019;124:494–8.
[3] Xu M, Hua Y, Qi Y, Meng G, Yang S. Exogenous hydrogen sulphide supplement accelerates skin wound healing via oxidative stress inhibition and vascular endothelial growth factor enhancement. Exp Dermatol 2019;28(7):776–85.
[4] Kim JH, Yang B, Tedesco A, Lebig EGD, Ruegger PM, Xu K, et al. High levels of oxidative stress and skin microbiome are critical for initiation and development of chronic wounds in diabetic mice. Sci Rep 2019;9(1):1–16.
[5] Lin J, Shi Y, Liao J, Wu Y, Lin H, Wu J, et al. Gastrodin alleviates oxidative stress-induced apoptosis and cellular dysfunction in human umbilical vein endothelial cells via the Nrf2/HO-1 pathway and accelerates wound healing in vivo. Front Pharmacol 2019;10:1273.
[6] Strioga M, Viswanathan S, Darinskas A, Slaby O, Michalek J. Same or not the same? Comparison of adipose tissue-derived versus bone marrow-derived mesenchymal stem and stromal cells. Stem Cell Dev 2012;21(14):2724–52.
[7] Tan J, Luo G. Advanced techniques in burn wound repair. In: Severe Trauma and Sepsis. Springer; 2019. p. 345–55.
[8] Ye L, Sun X, Zhang Y, Zhang Y. Hypoxic condition up-regulates the expression of angiopoietin-2 in ADSCs. J Biomed Sci Eng 2019;12(2):77–84.
[9] Beane OS, Fonseca VC, Cooper LL, Koren G, Darling EM. Impact of aging on the regenerative properties of bone marrow-, muscle-, and adipose-derived mesenchymal stem/stromal cells. PLoS One 2014;9(12).
[10] Madonna R, Renna FV, Cellini C, Cotellese R, Picardi N, Francomano F, et al. Age-dependent impairment of number and angiogenic potential of adipose tissue-derived progenitor cells. Eur J Clin Invest 2011;41(2):126–33.
[11] Lindner U, Kramer J, Rohwedel J, Schlenke P. Mesenchymal stem or stromal cells: toward a better understanding of their biology? Transfus Med Hemotherapy 2010;37(2):75–83.
[12] Naderi N, Combellack EJ, Griffin M, Sedaghati T, Javed M, Findlay MW, et al. The regenerative role of adipose-derived stem cells (ADSC) in plastic and reconstructive surgery. Int Wound J 2017;14(1):112–24.
[13] Zhang L, Zhang B, Liao B, Yuan S, Liu Y, Liao Z, et al. Platelet-rich plasma in combination with adipose-derived stem cells promotes skin wound healing through activating Rho GTPase-mediated signaling pathway. Am J Tourism Res 2019;11(7):4100.
[14] Hong SJ, Jia S-X, Xie P, Xu W, Leung KP, Mustoe TA, et al. Topically delivered adipose derived stem cells show an activated-fibroblast phenotype and enhance granulation tissue formation in skin wounds. PLoS One 2013;8(1):e55640.
[15] Schaefer R, Spohn G, Baer PC. Mesenchymal stem/stromal cells in regenerative medicine: can preconditioning strategies improve therapeutic efficacy. Transfus Med Hemotherapy 2016;43(4):256–67.
[16] Yu SP, Wei Z, Wei L. Preconditioning strategy in stem cell transplantation therapy. Translational stroke research 2013;4(1):76–88.
[17] Doorn J, Moll G, Le Blanc K, van Blitterswijk C, de Boer J. Therapeutic applications of mesenchymal stromal cells: paracrine effects and potential improvements. Tissue Eng B Rev 2012;18(2):101–15.
[18] Hausenloy DJ, Yellon DM. Preconditioning and postconditioning: underlying mechanisms and clinical application. Atherosclerosis 2009;204(2):334–41.
[19] Yang J-X, Zhang N, Wang H-W, Gao P, Yang Q-P, Wen Q-P. CXCR4 receptor overexpression in mesenchymal stem cells facilitates treatment of acute lung injury in rats. J Biol Chem 2015;290(4):1994–2006.
[20] Zhao S-L, Zhang Y-J, Li M-H, Zhang X-L, Chen S-L. Mesenchymal stem cells with overexpression of midkine enhance cell survival and attenuate cardiac dysfunction in a rat model of myocardial infarction. Stem Cell Res Ther 2014;5(2):37.
[21] Tošović J. Spectroscopic features of caffeic acid: theoretical study. Kragujevac Journal of Science 2017;(39):99–108.
[22] Kinra M, Arora D, Mudgal J, Pai K, Rao CM, Nampoothiri M. Effect of caffeic acidon ischemia-reperfusion-induced acute renal failure in rats. Pharmacology 2019;103(5–6):315–9.
[23] Yang L-C, Pei-Cheng Lin D, Chang H-H, Tung K-C. Caffeic acidPhenethyl ester reduces hypoxia-induced reactive oxygen species and mitigatates inflammation in corneal endothelial BCE C/D-1b cells. Curr Top Nutraceutical Res 2019;17(4).
[24] Armutcu F, Akyol S, Ustunsoy S, Turan FF. Therapeutic potential of caffeic acidphenethyl ester and its anti-inflammatory and immunomodulatory effects. Exp Ther Med 2015;9(5):1582–8.
[25] Rocha JE, Guedes TA, Bezerra CF, Costa MdS, Campina FF, de Freitas TS, et al. Mercury chloride phytotoxicity reduction using antioxidative mechanisms evidenced by caffeic acidFTIR. Appl Geochem 2019;104:109–15.
[26] Azam M, Ghufran H, Butt H, Mehmood A, Ashfaq R, Ilyas AM, et al. Curcumin preconditioning enhances the efficacy of adipose-derived mesenchymal stem cells to accelerate healing of burn wounds. Burns & Trauma 2021;9.
[27] Teti G, Stefano F, Viviana S, Eleonora M. Laura I, Antonio M, et al. The hypoxia-mimetic agent cobalt chloride differently affects human mesenchymal stem cells in their chondrogenic potential. Stem cells international 2018;2018:3237253.
[28] Bura A, Planat-Benard V, Bourin P, Silvestre J-S, Gross F, Grolleau J-L, et al. Phase I trial: the use of autologous cultured adipose-derived stroma/stem cells to treat patients with non-revascularizable critical limb ischemia. Cytotherapy 2014;16(2):245–57.
[29] Nie C, Yang D, Morris SF. Local delivery of adipose-derived stem cells via acellular dermal matrix as a scaffold: a new promising strategy to accelerate wound healing. Med Hypotheses 2009;72(6):679–82.
[30] Mattar P, Bieback K. Comparing the immunomodulatory properties of bone marrow, adipose tissue, and birth-associated tissue mesenchymal stromal cells. Front Immunol 2015;6:560.
[31] Rasmussen JG, Frøbert O, Holst-Hansen C, Kastrup J, Baandrup U, Zachar V, et al. Comparison of human adipose-derived stem cells and bone marrow-derived stem cells in a myocardial infarction model. Cell Transplant 2014;23(2):195–206.
[32] Ashfaq R, Mehmood A, Ramzan A, Hussain I, Tarar MN, Riazuddin S. Antioxidant pretreatment enhances umbilical cord derived stem cells survival in response to thermal stress in vitro. Regen Med 2020;15(3):1441–53.
[33] Yoo HI, Moon YH, Kim MS. Effects of CoCl2 on multi-lineage differentiation of C3H/10T1/2 mesenchymal stem cells. KOREAN J PHYSIOL PHARMACOL 2016;20(1):53–62.
[34] Ciafrè SA, Niola F, Giorda E, Farace MG, Caporossi D. CoCl(2)-simulated hypoxia in skeletal muscle cell lines: role of free radicals in gene up-regulation and induction of apoptosis. Free Radic Res 2007;41(4):391–401.
[35] Yang C, Ling H, Zhang M, Yang Z, Wang X, Zeng F, et al. Oxidative stress mediates chemical hypoxia-induced injury and inflammation by activating NF-κb-COX-2 pathway in HaCaT cells. Mol Cell 2011;31(6):531–8.

[36] Hao R, Li F, Song X, Tan X, Sun-Waterhouse D, Li D. Caffeic acidphenethyl ester against cadmium induced toxicity mediated by CircRNA modulates autophagy in HepG2 cells. Ecotoxicol Environ Saf 2020;197:110610.
[37] Espíndola KMM, Ferreira RG, Narvaez LEM, Rosario ACRS, da Silva AHM, Silva AGB, et al. Chemical and pharmacological aspects of caffeic acidand its activity in hepatocarcinoma. Front Oncol 2019;9.
[38] Thomas LR, Henson A, Reed JC, Salsbury FR, Thorburn A. Direct binding of Fas-associated death domain (FADD) to the tumor necrosis factor-related apoptosis-inducing ligand receptor DR5 is regulated by the death effector domain of FADD. J Biol Chem 2004;279(31):32780–5.
[39] Lee D-H, Szczepanski M, Lee YJ. Role of Bax in quercetin-induced apoptosis in human prostate cancer cells. Biochem Pharmacol 2008;75(12):2345–55.
[40] Park BS, Jang KA, Sung JH, Park JS, Kwon YH, Kim KJ, et al. Adipose-derived stem cells and their secretory factors as a promising therapy for skin aging. Dermatol Surg 2008;34(10):1323–6.
[41] Eom YW, Oh J-E, Lee JI, Baik SK, Rhee K-J, Shin HC, et al. The role of growth factors in maintenance of stemness in bone marrow-derived mesenchymal stem cells. Biochemical and biophysical research communications 2014;445(1):16–22.
[42] Fatimah SS, Tan GC, Chua K, Fariha MMN, Tan AE, Hayati AR. Stemness and angiogenic gene expression changes of serial-passage human amnion mesenchymal cells. Microvascular research 2013;86:21–9.
[43] Martini M, De Santis MC, Braccini L, Gulluni F, Hirsch E. PI3K/AKT signaling pathway and cancer: an updated review. Ann Med 2014;46(6):372–83.
[44] Vanhaesebroeck B, Guillermet-Guibert J, Graupera M, Bilanges B. The emerging mechanisms of isoform-specific PI3K signalling. Nat Rev Mol Cell Biol 2010;11(5):329–41.
[45] Bai H, Chen K, Gao Y-X, Arzigian M, Xie Y-L, Malcosky C, et al. Bcl-xL enhances single-cell survival and expansion of human embryonic stem cells without affecting self-renewal. Stem Cell Res 2012;8(1):26–37.
[46] Mehrzadi S, Safa M, Kamrava SK, Darabi R, Hayat P, Motevalian M. Protective mechanisms of melatonin against hydrogen-peroxide-induced toxicity in human bone-marrow-derived mesenchymal stem cells. Can J Physiol Pharmacol 2017;95(7):773–86.