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D-Alanine 2, Leucine 5 Enkephaline (DADLE)-mediated DOR activation augments human hUCB-BFs viability subjected to oxidative stress via attenuation of the UPR

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ABSTRACT

Human mesenchymal stem cells (hMSCs) although being potent in repairing injured or ischemic tissues, their success regarding tissue-regenerative approaches are hindered by the paucity in their viability. The elevated levels of reactive oxygen species (ROS) in damaged sites provoke the pernicious effects of donor MSC survival. In the present study, the effect of delta-opioid receptor (DOR) activation on human umbilical cord-blood borne fibroblasts (hUCB-BFs) survival under oxidative stress (H_2O_2) was evaluated. Oxidative stress which is known to trigger pathological conditions of the unfolded protein response (UPR) leads to endoplasmic reticulum stress. Upon its activation by D-Alanine 2, Leucine 5 Enkephaline (DADLE, selective DOR agonist) in hUCB-BFs under oxidative stress, a significant down regulation (~2 folds) of key UPR genes was observed as determined by qPCR, Thioflavin-T protein aggregation assay and western blot analysis. Concomitantly, the oxidative stress-mediated cell-death was ameliorated and the viable-cells' percentage was enhanced following DOR activation. The intracellular ROS production upon H_2O_2 treatment as determined by CM-H_2DCFDA staining was repressed, the anti-apoptotic marker *Bcl-2* was upregulated along with a significant suppression in the expression levels of pro-apoptotic proteins *Bax* and *Bad* upon DOR activation. Upon subsequent treatment with naltrindole, the effects of DADLE-induced cytoprotection were reverted significantly. These results propound the role of DADLE-mediated DOR-activation on improvement of the viability, which might succour successful hUCB-BFs transplants and greatly absolve the inefficacy of tissue-specific engineered transplants.

1. Introduction

Human mesenchymal stem cells are adult multipotent stromal cells which differentiate into multiple cell types (Bianco et al., 2013; Castro-Manrreza and Montesinos, 2015). While in terms with the expeditious advances in the field of mesenchymal stem cell (MSC) research, the ambiguity of its terminology persists as a far cry from being resolved. The characteristics necessary to define these cells alongside its potency and self-renewability remains to be a daunting percept and necessitates further confirmation (Bianco et al., 2008). Therefore the very usage of the term mesenchymal stem cells in this study has been substituted by human umbilical cord blood-borne fibroblasts (hUCB-BFs) pertinent with their source of origin. These cells possess the capacity of robust expansion, and promptly differentiate into cells from a number of mesenchyme-derived tissues, including cartilage, fat, bone and tendon, on apposite external signals (Dominici et al., 2006). The differentiation potential of these cells deviate based on their source and they can be isolated from a wide variety of tissues including bone marrow, muscle, adipose tissue, periosteum and synovial membrane (Hui et al., 2005). Therefore, MSCs are touted to be the most sought after candidates in cellular therapy and acute phase transplantation (Kim et al., 2013; Kim et al., 2016) studies due to their ease of isolation from ubiquitous sources (Hass et al., 2011). The incorporation of these cells into biomaterial scaffolds, coupled with *in vitro* manipulations with regards to the tissue engineering paradigm redirects towards incipient approaches in the restoration and repair function of tissue-specific damaged sites like cartilage (Tan and Hung, 2017), craniofacial bone therapy (Maruyama et al., 2016), etc. In spite of their vantage point, the therapeutic efficacy of the transplanted MSCs remain poor due to their exiguous survival rate and increased cell death after implantation into the ischemic/injured tissues (Singh and Sen, 2016), indicative of an incongruous microenvironment inappropriate for their viability (Toma et al., 2009). Post-transplantation in the inflicted tissues, MSCs encounter an inimical environment, emphasized with multiple adverse factors such as loss of extracellular matrix adhesion i.e. *anoikis*, inflammatory reaction, hypoxia and oxidative stress (Lee et al., 2015). An upsurge of uncurbed production of ROS due to the persistent oxidative stress in injured tissues affects the survival of engrafted MSCs (Souidi et al., 2013). ROS formed as a natural by-product of the normal

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energy metabolism is responsible for oxidative stress induction in the microenvironment and are inclusive of the following: H_2O_2, free radical superoxide anion and hydroxyl radical. Although, ROS have been shown to play a pivotal role in the growth and homeostasis of MSCs through enhancement of cell proliferation, survival and differentiation, uncontrolled ROS leads to mitochondrial dysfunction, cell death, tissue inflammation and aging of MSCs, while significantly compromising their differentiation and regeneration ability (Hou et al., 2013). Moreover, mitochondrial dysfunction has been suggested to be the focal cause of oxidative stress-induced apoptosis during ischemia-reperfusion injury (Iliodromitis et al., 2007). Therefore, oxidative stress portrays an unbalanced condition wherein ROS generation exceeds antioxidant combat mechanisms leading to cellular damage (Atashi et al., 2015). The presence of ROS prompts the induction of ER stress *in vivo* and *in vitro* (Qu et al., 2013; Scheuner and Kaufman, 2008). The fundamental role of ER, an intracellular organelle, is to aid in protein synthesis, folding, assembly and transportation. Any disparity of the ER causes ER stress which activates an unfolded protein response (UPR). Under threshold conditions of ER stress, UPR inhibits protein synthesis via increasing chaperones and degrading proteins (Ron and Walter, 2007). But under prolonged ER-stress the UPR triggers cell-apoptosis(Kim et al., 2008; Rutkowski and Kaufman, 2004). Pre-treatment or pre-conditioning with anti-oxidants although alleviates the deleterious effects of oxidative stress (Singh and Sen, 2016) and enhances cell-viability, but the mechanisms of ROS induced apoptosis on MSCs have not been illustrated till date and poses to be a persistent threat to cell-based therapy (Wei et al., 2010). Opioids are compounds that belong to an endogenous family of polypeptides those elicit opiate effects (Ma et al., 2005; Persson et al., 2003) and [D-Ala^2, D-Leu^5] enkephalin DADLE is a delta-selective synthetic peptide agonist (DADLE) (Su, 2000) and are effective against ischemic injury (Crowley et al., 2017; Fryer et al., 2002; Kaneko et al., 2012). Their effect is evoked via activation of G-protein mediated pathways via opioid receptors. There are 3 opioid receptors, μ (Oprm1)/MOR, κ (Oprk1)/KOR, and δ (Oprd1)/ DOR, each with their own specific agonists and antagonists (Gross, 2003; Staples et al., 2013). Amongst them, DOR-receptor -activation has been shown to be effective for preventing apoptosis of transplanted MSCs in ischemic myocardium (Higuchi et al., 2012), thereby aiding at a pronounced MSC survival in rodents. The present study is therefore aimed at elucidating the role of DOR in the viability of human umbilical blood-borne mesenchymal stem cells (hUCB-BFs) under oxidative stress (H_2O_2) (Gough and Cotter, 2011). We have demonstrated that DOR activation could lead to enhanced hUCB-BFs survival in part via down-regulation of the UPR stress sensors along with modulation of the apoptotic genes, which were activated following the ROS insult.

2. Materials and methods

2.1. Cell culture

Human umbilical cord blood-derived mesenchymal stem cells (referred to as hUCB-BFs in the study) were obtained from Promo Cell (Heidelberg, Germany). The cells were cultured in low-glucose α-MEM supplemented with 10% FBS and 1% of penicillin/streptomycin in a 5% CO2 incubator with 95% relative humidity at 37 °C. Cells used in the experiments were between passage numbers 2 to 4.

2.2. hUCB-BFs characterization

2.2.1. Osteogenic and adipogenic differentiation of hUCB-BFs

For the induction of osteogenic differentiation, hUCB-BFs were cultured in the osteogenic medium composed of α-MEM supplemented with 10% FBS, 0.1 M dexamethasone (Sigma), 10 mM -glycerol phosphate (Sigma), and 50 M L-ascorbic acid 2-phosphate (Sigma) (Jaiswal et al., 1997). Medium was changed twice a week. For the induction of adipogenic differentiation, cells were treated with adipogenic medium consisting of DMEM (Gibco) supplemented with 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma), 1 M dexamethasone (Sigma), 0.2 mM indomethacin (Sigma), and 10 M h-insulin (Sigma)(Nakamura et al., 2003). After 3 weeks the cells were stained with Alizarin Red and Oil Red O (Sigma) for detection Ca^{2+} and lipid droplet respectively.

2.2.2. hUCB-BFs surface markers by flow cytometry

Flow cytometry (BD FACS Celesta™, New Jersey, USA)based detection of hUCB-BFs markers was carried out using the BD Stemflow™ hHUCB-BFS Analysis Kit according to the manufacturer's protocol (BD Biosciences).

2.3. Immunofluorescence

To observe the presence of DOR on hUCB-BFs, the cells were fixed with 4% paraformaldehyde. Following permeabilization with Triton-X, the cells were stained with polyclonal rabbit-anti human DOR-antibody (1:4 of the recommended dilution; Millipore, USA) at 4 °C overnight with subsequent incubation at room temperature for 30 mins in dark with anti-rabbit IgG (H + L), F(ab′)$_2$ Fragment (Alexa Fluor® 488 Conjugate) (Cell Signaling, USA). The images were recorded using the software; Leica Application Suite Advanced Fluorescence 6000, in Leica DM600B microscope (Leica Microsystems, Germany).

2.4. ROS induction

The oxidative stress conditions were mimicked *in vitro* by the exogenous addition of H_2O_2 (Gough and Cotter, 2011). An optimal dose for H_2O_2 was selected based on ~50% cell death after 48 h of treatment through a dose-dependent study (100–800 μM).

2.5. DOR activation

For the DOR activation studies 100 nM concentration of the agonist DADLE (Sen et al., 2013) and 10 μM of antagonist naltrindole (Sen et al., 2013) were used. The cells were pre-treated with naltrindole (45mins prior to DADLE addition) and D-Alanine 2, Leucine 5 Enkephaline (DADLE) for 24 h before the addition of H_2O_2. The treatment consisted of six different conditions, namely: the untreated control, DADLE treatment, naltrindole treatment, H_2O_2 treatment, H_2O_2 + DADLE treatment and H_2O_2 + DADLE + naltrindole treatment. MOR and KOR agonists [D-Ala2, NMePhe4, Gly-ol]-enkephalin (DAMGO).

(Wen et al., 2013) and BRL-52537(Fang et al., 2013) respectively were also used to elucidate their role in cytoprotection.

2.6. Cell viability assay

2.6.1. MTT assay

Exponentially growing hUCB-BFs were incubated in a 96-well plate along with H_2O_2, with or without DADLE, DAMGO, BRL-52537 and naltrindole. Cell viability was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's protocol (Promega, USA).

2.6.2. Annexin V-FITC/PI double staining

Apoptotic cell death was measured by Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Thermofisher, USA) according to the manufacturer's protocol. The fluorescence intensity of the stained cells was analyzed using BD FACS Diva ver. 8.0.1.1 software from a flow cytometer (BD FACS Celesta™, New Jersey, USA).

2.7. Thioflavin-T staining and fluorescence detection

The treated cells were stained with Thioflavin-T (ThT) for the detection of protein aggregates in the cells, resulting from ROS-induced ER stress as described previously (Baumann et al., 2016) using a fluorescence microscope (Leica) at an excitation and emission maxima of 350 and 438 nm, respectively.

2.8. Measurement of intracellular ROS

Intracellular ROS were measured using a fluorescent dye technique-2′,7′-dichlorofluorescin diacetate (H_2DCFDA) (Invitrogen, USA) as described previously(Signoretto et al., 2016). The images were collected using a microscope (Leica) by excitation at 488 nm and emission 534 nm with a blue filter.

2.9. Quantification of DNA damage

The number of AP sites was measured using OxiSelect Oxidative DNA Damage Quantitation Kit (Cell Biolabs, San Diego, CA, USA) following DOR-activation on hUCB-BFs under ROS induced oxidative stress.

2.10. Quantitative (q) and semi quantitative PCR analysis

Total RNA was prepared using the Trizol reagent (Takara, Japan). Reverse transcription was carried out using Primescript c-DNA synthesis kit (Takara, Japan), according to the manufacturer's protocol. qPCR was carried out using SYBR Taq-II supermix (Takara, Japan) using specific primers (Table 1). Relative mRNA levels of the gene of interest were either normalized to the housekeeping gene-RPL-19 (for UPR) or to β-actin (for *Bcl-2*, *Bax* and *Bad*) using ΔΔCt method. Semi-quantitative PCR was performed using the EmeraldAmp GT mastermix (Takara, Japan) for *Bcl-2*, *Bax* and *Bad* (25 cycles) and *DOR* (30 cycles) with β-actin as the housekeeping gene (18 cycles). A 2% agarose gel electrophoresis was run and stained with Ethidium Bromide. Bands were visualized in a gel documentation system (UVP, USA).

2.11. Protein extraction and western blot analysis

Cells were harvested post H_2O_2 treatment and protein was extracted using the lysis buffer comprising of 20% glycerol, 0.125 M Tris-HCl (pH 6.8), 1% Bromophenol blue, 46% SDS and 10% β mercaptoethanol. The proteins were quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific) (Fischer et al., 1999). The expression levels of the ER-stress proteins *PERK*, *IRE-1α*, *BiP* along with the anti-apoptotic and apoptotic proteins *Bcl-2* and *Bad* respectively were determined using standard western blot technique (Wei et al., 2010) and the bands were observed using enhanced chemiluminescence (UVP, USA). All antibodies were purchased from Cell Signaling (MA, USA). Densitometric analysis of the protein bands was carried out using the Quantity one basic software, version 4.6.6 (Biorad).

2.12. Statistical analysis

Statistical analysis was performed using ANOVA (analysis of variance). Level of significance was set at $^*p < 0.05$.

3. Results

3.1. Characterization of hUCB-BFs via surface markers and differentiation

The cultured hUCB-BFs from umbilical cord blood were able to differentiate into osteogenic and adipogenic lineages as seen from the Ca^{2+} and lipid droplet deposition respectively in the figures: 1a, b & c. These differentiation studies were performed to demonstrate the characteristic differentiation traits of hUCB-BFs (Sarugaser et al., 2009)(Li et al., 2005). Consistent with previous reports (Chang et al., 2009)(Baksh et al., 2007) and (Singh and Sen, 2016) the cultured hUCB-BFs expressed CD73, CD90 and CD105, but not the negative cocktail markers, CD34, CD45, CD11b, CD19 and HLA DR as determined by flow cytometry (Fig.1d).

3.2. Expression of DOR on hUCB-BFs

DOR expression was detected and confirmed in the plasma membrane and cytoplasm of hUCB-BFs through immunofluoresecence and semi-quantitative PCR (Fig.1 e and h).

3.3. DOR activation via DADLE conferred cytoprotection to the hUCB-BFs under H_2O_2-induced oxidative stress

As a ROS inducing agent, H_2O_2 has already been well established to cause the pathological conditions of oxidative stress (Jaimes et al., 2001) resulting in cell death. To demonstrate the detrimental effects of H_2O_2, a dose-dependent study ranging from 0 to 800 μM of H_2O_2 was performed. After an incubation period of 48 h, the optimal dose was considered to be 600 μM of H_2O_2 (Fig.2a) at which ~40% cell viability was observed when compared with the control. To ensure the protective effect of DOR activation against H_2O_2 induced cell death, pre-treatment with DOR agonist DADLE (added 24 h prior to H_2O_2 without any media change), significantly increased the viability of hUCB-BFs to ~85% as shown in Fig. 2b, when compared to the H_2O_2 alone treated cells (~40%) which was significantly inhibited (~50%) by naltrindole indicating that DADLE might be utilizing the DOR to induce its protective effect (Fig. 2b). However neither the MOR agonist, DAMGO nor the KOR agonist, BRL-52535 could show such survival effect (Fig. 2b). DADLE-mediated improved survivability was further validated with the Annexin-V-binding assay where a similar protective pattern of the agonist DADLE was observed compared to H_2O_2 treated cells (51.9% vs 33.6%) which was reduced to 40.5% following naltrindole pre-treatment (Fig. 2c). These results indicate that the significant increase ($^*p < 0.05$) in the hUCB-BFs viability upon ROS-induced ER stress could be as a result of DADLE-mediated DOR activation.

Table 1
Sequence of the primers used in PCR.

Name	Forward primer	Reverse primer	Amplicon length (bp)
PERK	TCATCCAGCCTTAGCAAACC	ATGCTTTCACGGTCTTGGTC	98
IRE1α	CTCTGTCCGTACCGCC	GAAGCGTCACTGTGCTGGT	127
ATF4	GTCCCTCCAACAACGCAAG	CTATACCCTCCAACAACAGCAAG	111
ATF6	TTGACATTTTTGGTCTTGTGG	GCAGAAGGGGAGACACATTT	99
BiP	CACAGTGGTGCCTACCAAGA	TGTCTTTTGTCAGGGGTCTTT	107
CHOP	AGCCAAAATCAGAGCTGGAA	TGGATCAGTCTGGAAAAGCA	90
RPL19	ATGTATCACAGCCTGTACCTG	TTCTGGTCTCTTCCTCCTTG	133
Bcl-2	GGTCATGTGTGTGGAGAGAGCG	CCGTACAGTTCCACAAAGGC	144
Bax	TCATCCAGGATCGAGCAGG	GGCAATCATCCTCTGCAGC	155
Bad	GCAAGCATCATCGCCAGG	ATCCCTTCGTCGTCCTCC	154
DOR/Oprd1	CCAGCTGGTACTGGGACA	CACAACCACCAGCACCATG	184
β-actin	ATGTACCCTGGCATTGCC	CTGCTGGAAGGTGGACAG	150

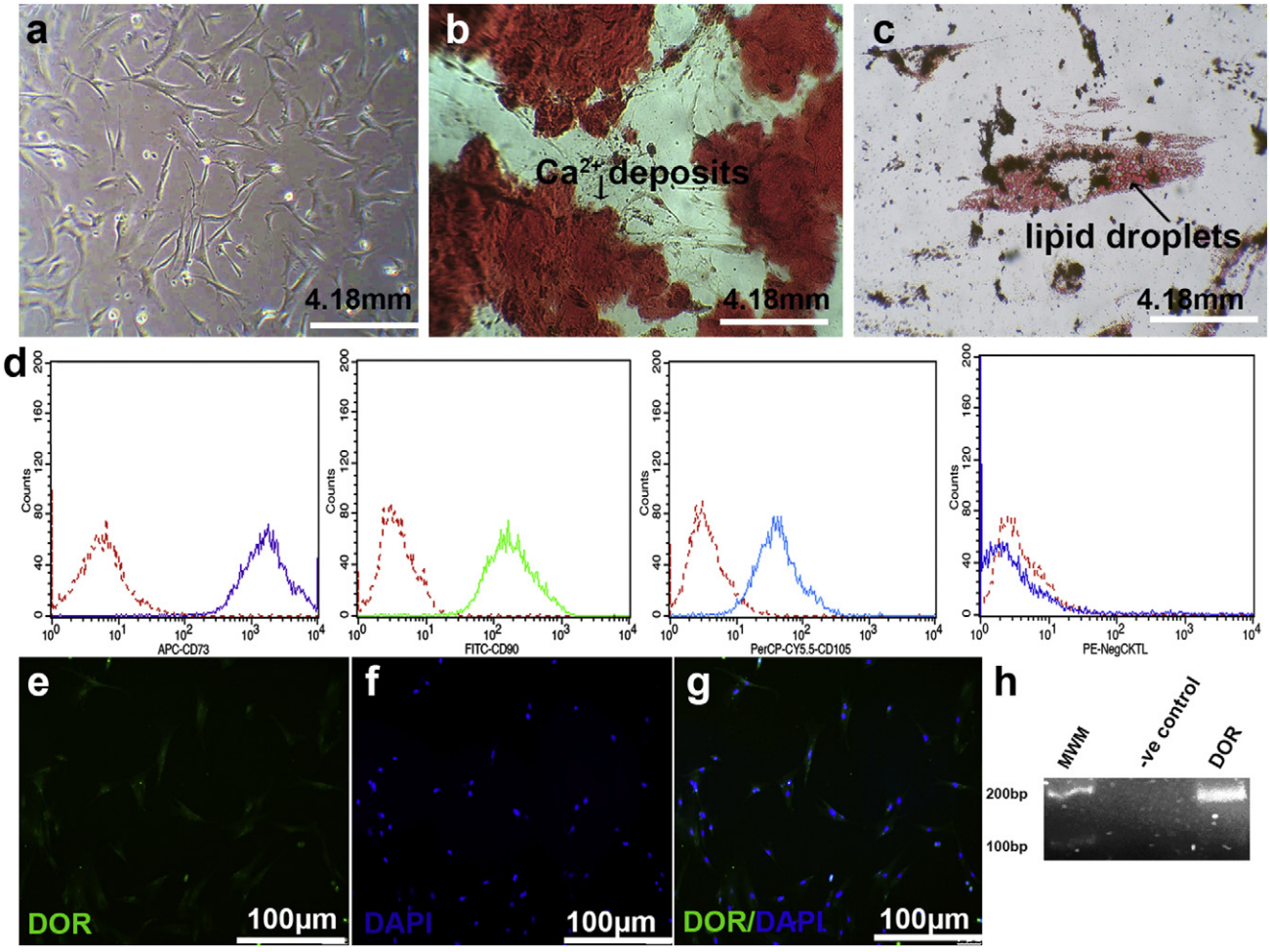

Fig. 1. Characterization of HUCB-BFs. a. Confluent culture of hUCB-BFs, (b and c). Phase contrast images of differentiated adipogenic and osteogenic cultures from HUCB-BFss showing phenotypic changes as lipid droplet accumulation and mineralisation of the cultures, d. Flow cytometric analysis of hUCB-BFs characterized following the guidelines of International Society of Cellular Therapy (ISCT)- Positive markers CD90, CD 105 and CD73; Negative marker cocktail CD45, CD34, CD11b, CD19 and HLA-DR, (e–g). Expression of DOR as determined by immunofluorescence, green-Alexa Fluor 488, Blue-DAPI, h. Semi quantitative PCR analysis of DOR expression in hUCB-BFs, MWM: molecular weight marker.

3.4. DOR activation significantly diminished the intracellular ROS production

To confirm the intracellular production of ROS on exogenous addition of H_2O_2 and the subsequent effect of DOR activation, fluorescence images were taken following treatment with H_2DCFDA (Fig.2d). DADLE pre-treated hUCB-BFs showed significantly attenuated production of intracellular ROS substantiated by an extenuated production of the fluorescent product DCF (di-chlorofluorescein), when compared to only H_2O_2 treated cells.

3.5. DOR activation diminished the formation of AP sites under oxidative stress

DADLE-mediated DOR activation under oxidative stress showed a significant down regulation in the formation of 7.6 AP sites/10^5 bases as compared to the cells under H_2O_2 induced oxidative stress which formed 22.66 AP sites/10^5 bases(the control showed a baseline figure of 2.1 AP sites/10^5 bases) (Fig.2e). On inhibition of the DOR activation via the antagonist naltrindole, no significant repression was observed in the loss of AP sites with respect to the DADLE-treated cells, as indicated by the increase in the number of AP sites formation 17.4 AP sites/10^5 bases. This implies the cytoprotective role of DADLE mediated DOR activation on the hUCB-BFs under oxidative stress.

3.6. DOR activation showed a marked down regulation in the expression levels of UPR genes

ER stress induces UPR, which signals the nucleus to regulate the transcription of genes involved in protein folding and processing to increase the ER protein folding capacity, thus reducing the ER workload. UPR initially aims to alleviate the stress by inducing several UPR stress sensors. If homeostasis cannot be achieved, the UPR leads to programmed cell (Oslowski and Urano, 2010). *IRE1α*, *PERK* and *ATF6α* are the three major sensors that are involved in regulating transcription during ER stress (Halperin et al., 2014). Incongruous protein folding aggravates the dissociation of *BiP* from the lumenal domains of *PERK* and *IRE-1α* (Bertolotti et al., 2000). The transcription factor C/EBP homologous protein (CHOP) functions as a downstream component of ER-stress pathways, concurrent with the IRE-1α, PERK and ATF6 pathways, prompting apoptosis under the ER stress conditions curbing the expression of anti-apoptotic B-cell lymphoma (*Bcl-2*) (McCullough et al., 2001; Puthalakath et al., 2007). In the present study, the effect of ROS-induced ER stress on hUCB-BFs and the effect of DOR activation on expression levels of the UPR genes were analyzed by qPCR. Under H_2O_2 induced oxidative stress, *IRE-1α* showed the highest fold upregulation (~3.5 fold), followed by *BiP*, *PERK*, *ATF-4* and *CHOP*. No significant change was observed in *ATF-6* expression levels under oxidative stress. The high expression levels of the UPR genes were significantly down-regulated on DADLE induced DOR activation (Fig.3a). Whereas, on naltrindole

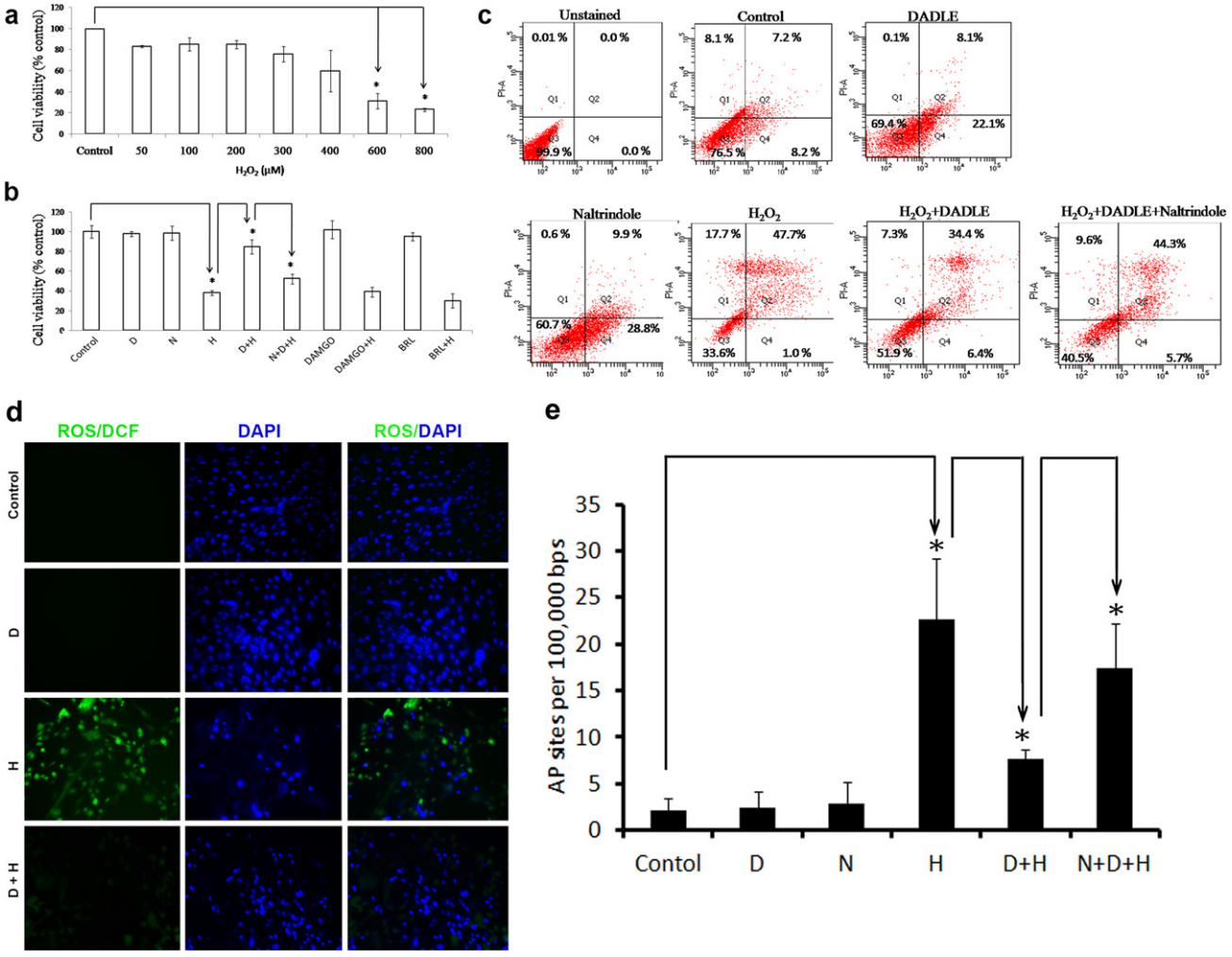

Fig. 2. Effect of DOR activation on ROS-induced cell death. a. Dose-dependent study of H_2O_2. The values are expressed as a mean of three independent runs, b. Assessment of cell viability following DOR, MOR and KOR activation under the treatment conditions abbreviated as C: Control, D: DADLE, H: H_2O_2 – treated, N: naltrindole. Values expressed as the mean ± SD of 2 independent runs. *$p < 0.05$, c. Annexin-V binding assay: Representative ($n = 2$) flow cytometricconfirmation of cell viability, d. Representative fluorescent images ($n = 2$) ofintracellular ROS as measured using CM-H_2DCFDA, e. Estimated number of AP sites/10^5 nucleotides following DOR activation under H_2O_2 induced oxidative stress.

treatment no significant repression in the fold change of the aforesaid genes were observed. Hence, DADLE mediates the significant down regulation in expression of the UPR genes, thereby reducing the ER stress, which subsequently might lead to rise in the hUCB-BFs viability (Fig. 3a). This cytoprotection is indicative of the plausible mechanism of DADLE-mediated DOR activation. Neither MOR nor KOR was found to have any effect on UPR gene expression following oxidative stress.

3.7. Formation of protein aggregates were mitigated on DOR activation

The UPR imbalance resulting in ER stress, elicited via the ROS-induced oxidative stress results in the accumulation of protein aggregates. On DOR activation via DADLE the hUCB-BFs demonstrated a marked reduction of ThT-protein aggregates as shown by the significant reduction of fluorescence intensity (Fig.3b). On the contrary, the naltrindole treated cells showed a marked rise in the fluorescence intensity when compared to the DADLE-treated cells under oxidative stress, signifying that inhibition of DOR results in accumulation of misfolded proteins. This indicates that DADLE may alleviate the accumulation of the protein aggregates, thereby abating the ER stress evoked due to ROS.

3.8. DOR activation significantly repressed the expression levels of UPR proteins

The effect of ROS-induced ER stress under DOR activated hUCB-BFs were studied for analysing the expression levels of the UPR proteins by western blot. Under H_2O_2 induced oxidative stress, the UPR proteins *BiP*, *PERK* and *IRE1α* were significantly upregulated (2.3–2.8 fold) while on DADLE-mediated DOR activation significant downregulation in the band pattern (similar to the control) was observed. In addition in the cells treated with naltrindole, levels of the UPR proteins showed an enhanced expression (similar to H_2O_2 alone treatment) (Fig.3c and d). This further indicates that the potential DOR activation via DADLE can significantly diminish the expression of UPR proteins, thereby reducing the ER stress, which might lead to rise in the hUCB-BFs viability.

3.9. DOR activation upregulated the expression levels of anti-apoptotic Bcl-2 and represses apoptotic Bad and Bax both at the transcript and protein levels

Activation of DOR via DADLE regulated the expression levels of anti-apoptotic *Bcl-2* and pro-apoptotic markers, *Bax* and *Bad* (Fig.4a). Under H_2O_2 induced oxidative stress, the expression levels of apoptotic genes *Bax* and *Bad* were upregulated by ~4 folds and ~11folds respectively.

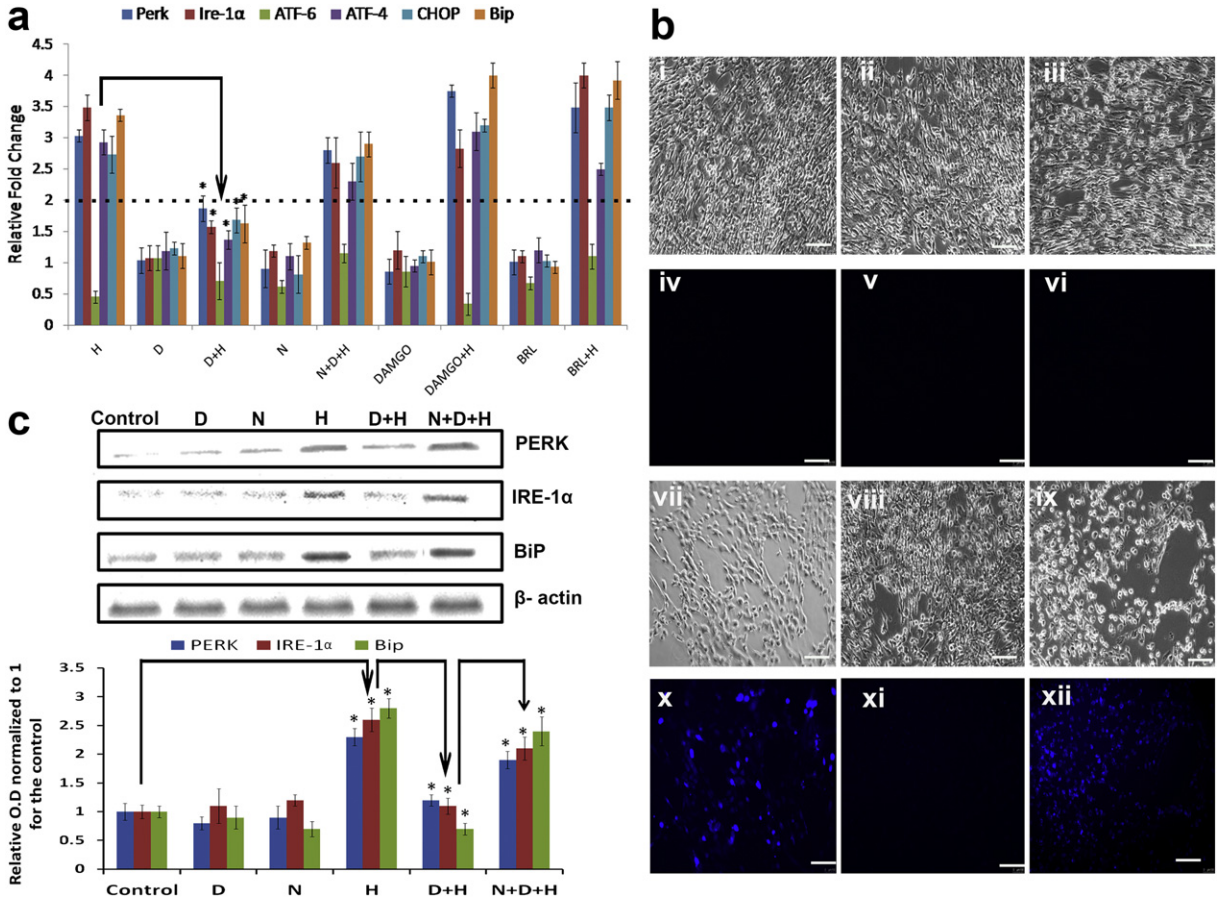

Fig. 3. DOR activation represses the UPR and corresponding protein aggregation. a. Relative fold change (normalized to 1 for the untreated control) of key UPR genes *PERK*, *IRE-1α*, *ATF-4*, *CHOP* and *BiP* upon DOR, MOR and KOR activation (under different treatment conditions), *$p < 0.05$ vs control (only H treated), Data +/− SD of 2 independent runs; dotted line represents 2 fold change in gene expression b. Thioflavin-T protein aggregates under the different treatment conditions. Phase contrast images of (i) untreated control, (ii) only D, (iii) only N, (vii) only H, (viii) D + H and (ix) N + D + H treated cells; (iv–vi) corresponding fluorescent images of i, ii and iii respectively; (x–xii) corresponding fluorescent images of vii, viii and ix respectively, c. Representative western blot image of DOR-mediated down regulation in the ER-stress proteins- PERK, IRE-1α and *BiP* under ROS-induced oxidative stress (loading control used-β actin), d. Densitometric analysis of the western blot images. Data +/− SD of 2 independent runs done in duplicate dishes for individual treatment.

On treatment with DADLE, the expression levels of these apoptotic genes were repressed to <2 for *Bax* and ~2 for *Bad* (Fig.4b). As for the anti-apoptotic marker *Bcl-2*, upon oxidative stress its expression was at a basal level, while on DOR activation the fold change expression increased to ~4 (Fig.4b). However, in cells pre-treated with the DOR antagonist naltrindole, DADLE did not show any such significant effect on the apoptotic modulators under oxidative stress (Fig. 4a and b). These results were in correspondence at the protein levels, as indicated by the band pattern and densitometric analysis obtained via western blotting of the aforementioned genes (Fig. 4c and d). For example, the anti-apoptotic protein Bcl2 was upregulated ~3.5 times on DADLE treatment in cells grown under oxidative stress when compared to the control (H_2O_2 treatment alone). This upregulation was significantly inhibited by naltrindole. Similarly H_2O_2 treatment up-regulated pro-apoptotic *Bad* by ~5 folds which came down to the basal level following pre-treatment with DADLE. However, blocking the DOR with its antagonist naltrindole significantly ameliorated this effect of DADLE on *Bad* protein expression (Fig. 4c and d). Overall these findings implemented that the DADLE-mediated DOR activation might sustain hUCB-BFs viability potentially via controlling the regulation of the anti-apoptotic and apoptotic markers, alongside the reduction of intracellular ROS and ER-stress.

4. Discussion

Implementation of MSCs in the aspect of tissue-engineering has been shown to be apt in the synergism of cell biological and biomaterial technologies (Maruyama et al., 2016; Tan and Hung, 2017). The MSCs on integration with the current bio-scaffolds indeed prove to be reconstructive (Dehghanifard et al., 2013) in acute phase stroke injuries (Toma et al., 2009). This potency of the MSCs is mainly bestowed to their stark immune-modulatory, expansion and differentiation capabilities. Yet their efficacy remains thwarted by the low survival rates at the injury sites in which the severe microenvironment plays a pivotal role (Lee et al., 2015). Although, the synchronisation of bio-scaffold-based tissue engineering have manifested its success and appears to be one of the most promising tools in cellular therapy (Wei et al., 2010), it is inflicted with poor cellular survival which considerably limits its therapeutic efficacy (Zhu et al., 2006). MSCs die out from the injured sites within a few days due to a combination of harsh environmental conditions as evidenced by elevated levels of reactive oxygen species, hypoxia/serum-deprivation, *anoikis* and inflammation (Lee et al., 2015). ROS prominently leads to loss of adhesion of MSCs via the integrin-related focal adhesion pathway and pronounced inflammation (Rodrigues et al., 2012). High levels of ROS are associated with significantly lower MSC counts as the exogenous ROS burst in the microenvironment of damaged area serves as a critical factor for cellular apoptosis (Wei et al., 2010). MSCs exposed to prolonged oxidative stress may also be functionally impaired along with persistent DNA Damage (Jin et al., 2010). The over-accumulation of oxidative stress that leads to ER stress, causing an imbalance in the UPR, are all contemplated to be amalgamated events (Malhotra and Kaufman, 2007). UPR which acts as a binary switch between cell survival and death decisions (Oslowski and Urano, 2010) depending upon the degree of ER stress, gets activated

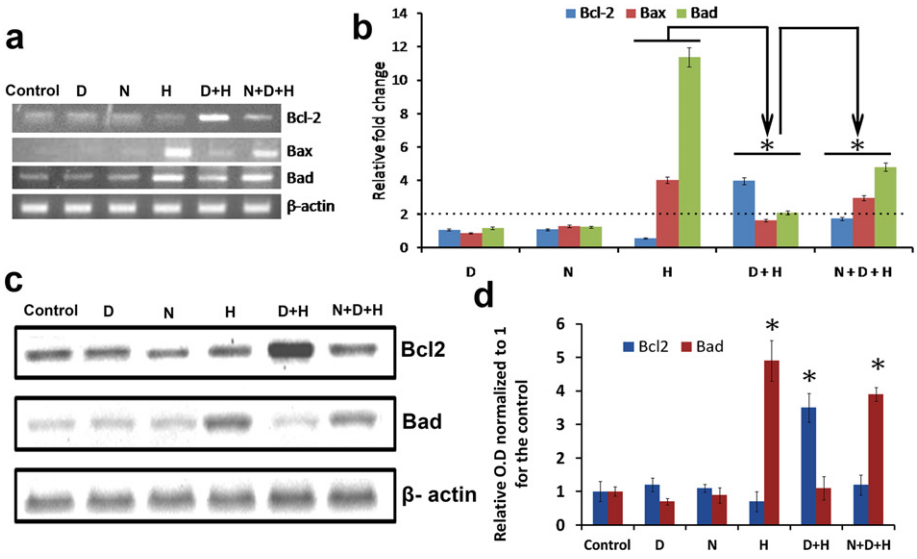

Fig. 4. DOR activation regulates the expression levels of *Bcl-2*, *Bax* and *Bad*. a. Semi quantitative PCR analysis of *Bcl-2*, *Bax* and *Bad* genes, b. qPCR data for the same under the various treatment conditions. *$p < 0.05$ vs control (only H treated), data +/− SD of two independent runs; dotted line represents 2 fold change in gene expression, c. Western blot analysis of *Bcl-2*, *Bax* and *Bad* (loading control used-β actin) under various treatment conditions (representative image), d. Densitometric analysis of the western blot images. Data +/− SD of 2 independent runs done in duplicate dishes for individual treatment.

under ROS-mediated oxidative stress. Apoptosis plays a significant role in the pathophysiology of various diseases (Galluzzi et al., 2009), that occurs through a cascade of various cellular events involving several apoptotic-regulatory genes and it gets enhanced in the presence of ROS. The *Bcl-2* family of proteins represents a major hallmark in the *anti-apoptotic* signal transduction cascades by acting upstream of irreversible damage to cellular constituents (Weyhenmeyer et al., 2012; Yang et al., 2009). The regulation of apoptotic cell death is greatly determined by *Bcl-2/Bax* ratio (Bar-Am et al., 2005). Although, previous studies have proved that, co-injection of MSCs with antioxidant like *N*-acetyl cysteine (NAC) increases the viability and integration potential of MSCs post transplantation (Sun et al., 2014), the survivability inflicted MSC transplantation failure still persists. Therefore, it is of paramount concern for the ROS levels to be waned out at the damaged site for the successful transplantation of hUCB-BFs. The current study is therefore focused on the loss of hUCB-BFs viability upon H_2O_2 induced oxidative stress and its concomitant recuperation on DOR activation (Fig. 2a–e). Even though DADLE has been shown to evoke non-DOR specific responses (Baldelli et al., 2006), the feasibility of such an instance was repudiated in this study as determined by lack of uplifted survival due MOR and KOR activation of the cells under oxidative stress. This redirected to the conjecture that the cyto-protection conferred by DADLE might be due to activation of DOR (Borlongan et al., 2000, 2001). The present study has shown that hUCB-BFs death under oxidative stress could be induced in part by an up-regulation of the UPR target genes *PERK*, *IRE1*, *ATF-4*, *CHOP* and *BiP*, as a result of surplus ER stress condition. The type-1 transmembrane protein kinases: PERK and IRE1, positioned in the endoplasmic reticulum (ER) transmit stress signals in response to dismayed protein folding. The functionally interchangeable luminal domains of these two proteins impart an ER stress response and in unstressed cells, both lumenal domains form a stable complex with the ER chaperone *BiP*. Any discrepancy in the protein folding instigates the reversible dissociation of *BiP* from the lumenal domains of PERK and IRE1, the loss of which correlates with the formation of high-molecular-mass complexes of activated PERK or IRE1. Therefore, the overexpression of *BiP* impairs their activation (Bertolotti et al., 2000). CHOP is a leucine zipper transcription factor (McCullough et al., 2001) that is effectuated by ER stress (Oyadomari and Mori, 2004).The CHOP gene promoter accommodates binding sites for all of the major inducers of the UPR, including ATF4, ATF6 and XBP1, and as evidenced by various studies these transcription factors have causative roles in inducing CHOP gene transcription (Harding et al., 2003; Kim et al., 2008)which can induce apoptosis under the ER stress conditions via inhibition of the expression of anti-apoptotic B-cell lymphoma (*Bcl-2*) (McCullough et al., 2001; Puthalakath et al., 2007). Upon DOR activation via the agonist DADLE, hUCB-BFs death was significantly curtailed and there was a consequent down-regulation of the aforementioned UPR genes along with repression in the accumulation of aggregation of misfolded proteins, as shown by the Thioflavin-T staining images (Fig.3a–d). DOR activation distinctly inhibited intracellular ROS levels as shown by the CM-H_2DCFDA studies and the associated AP sites in cells treated with H_2O_2 alone (Fig.2d and e). Further evidence of the cytoprotective property of DOR in hUCB-BFs under oxidative stress was elucidated by upregulation of the apoptotic gene *Bcl-2* while a significant reduction in the expression of pro-apoptotic genes-*Bax* and *Bad* (Fig. 4a-d). Further studies necessary to dissect the intracellular mechanism(s) of such DOR mediated cytoprotection under oxidative stress are under way. The illustration in Fig.5 recounts the DOR-mediated enhanced hUCB-BFs viability. This was achieved via attenuation of the ROS-induced damage, which would necessitate further research, so as to discretely orchestrate the mechanisms through which

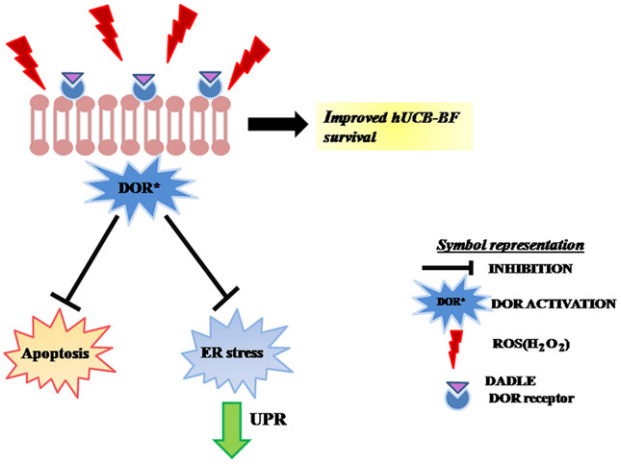

Fig. 5. Schematic representation of the proposed mechanism: DOR activation assuages the ER-stress levels induced by ROS and thereby improves the hUCB-BFs viability.

the ER-stress triggered UPR was restrained. Henceforth, the defined impediment of apoptosis observed on UPR repression would accord with the pronounced cellular viability. Nevertheless, the results represented in this study are in sync with the potential cytoprotective function of DADLE-mediated DOR activation (Borlongan et al., 2009; Borlongan et al., 2004) on hUCB-BFs and might light up new avenues in stem cell-based therapies.

5. Conclusion

The current study avers the cytoprotective role of DADLE-induced DOR activation on hUCB-BFs under ROS triggered oxidative stress that perturbs the UPR balance leading to ER-stress. DOR activation retrieves the hUCB-BFs by ameliorating the levels of the UPR genes alongside a remarkable regulation of the apoptotic genes. Thus, oxidative stress and UPR, which has been shown to be profoundly connected, thereby resulting in apoptosis of hUCB-BFs, were potentially subsided on DOR activation using DADLE. Hence, DOR activation under the conditions of oxidative stress-induced cell-death on gaining *in vivo* relevance might prove helpful in increasing the viability of the hUCB-BFs and thereby, direct the way in the pursuit of successful tissue-specific hUCB-BFs regeneration and translational therapy (Fig. 5).

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Conflict of interest

None.

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