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ORIGINAL PAPER

Cross talk between 26S proteasome and mitochondria in human mesenchymal stem cells' ability to survive under hypoxia stress

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Abstract

Mesenchymal stem cells (MSCs) are regarded as unique cells which play an imperative role in the field of regenerative medicine. They are characterized by the self-renewal capacity, multi-lineage differentiation abilities and immunomodulation properties which render them perfectly ideal cell type for treating a wide range of chronic diseases. Despite these enchanted features, there are many hurdles that need to be circumvented to ensure their long-term survival and viability after transplantation. Recently, hypoxia has been indicated as one of the most baffling stress conditions that can affect the survival rate of MSCs either positively or negatively depending on the level of hypoxia. MSCs can survive well under moderate hypoxia, but die shortly if they were exposed to severe hypoxia without clearly convincing explanation for this enigma. The current study reveals a novel mechanism of 26S proteasome in controlling the ability of BM-MSCs to withstand hypoxic stress by maintaining proper mitochondrial function. The results indicated that 26S proteasome remains functioning once BM- MSCs are exposed to moderate hypoxia ($2.5\%O_2$) and preserves their survival and proliferation mediated by intact mitochondrial performance, whereas 26S proteasome becomes inactive when BM-MSCs faces severe hypoxia that lead to poor mitochondrial function and less chance to survive longer. The outcomes of this study demonstrated the importance of 26S proteasome machinery in enhancing the resistance of BM-MSCs to hypoxic stress condition which may help in better planning future studies that target this system.

Graphic abstract

Schematic representation summarizing the findings of the current study. 26S proteasome function preservation in normoxia and moderate hypoxia leads to maintain appropriate proliferation and mitochondrial activity in human BM-MSCs and promote their survival. On the opposite side, severe hypoxia disrupts the 26S proteasome function leading to significant reduction in the proliferation, survival and mitochondrial dynamics in human BM-MSCs causing their death.

Ramada R. Khasawneh and Ejlal Abu-El-Rub equally contributed.

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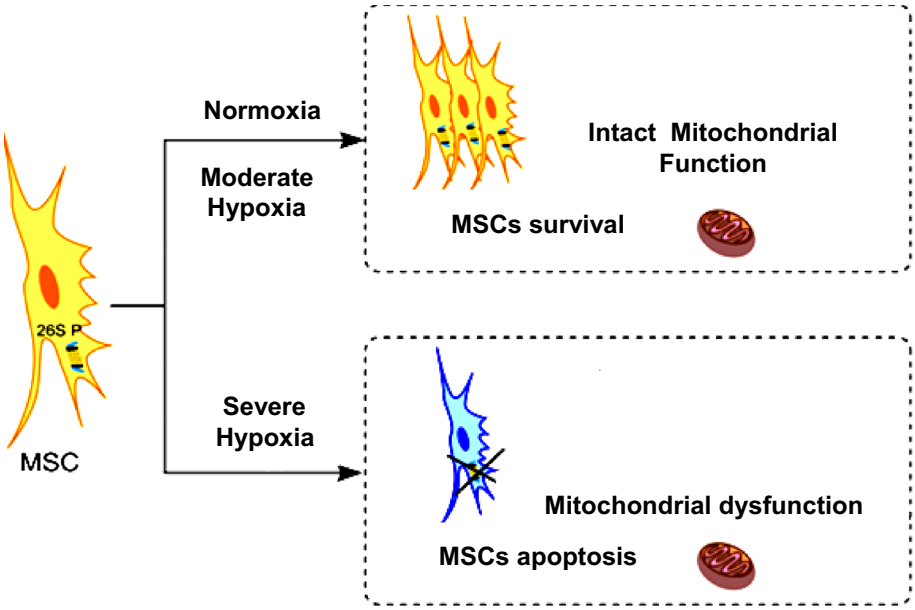

Keywords Mesenchymal stem cells · Hypoxia · Survival · Proteasome · Mitochondria

Introduction

Mesenchymal stem cells (MSCs) are considered to be the ideal candidate in regenerative medicine [31, 38]. They hold unique characteristics that place them at the top of cell therapy pyramid, including their ability to differentiate into various cell lineages; they release many cellular regulatory cytokines [6, 17, 39, 52], and they are able to migrate to the site of injury and use their self-renewal capacity [39]. These especially distinct traits intrigue great interest in MSCs for clinical purposes which have been translated currently by having huge number of clinical studies [25, 27, 37, 41, 45]. The findings of these studies have supported the idea that MSCs could repair and regenerate damaged tissues and restore their function. Furthermore, the outcome of many clinical trials reported that transplanted cells were safe to the patients as no significant side effects were observed [7, 27, 41]. However, the beneficial effects of transplanted MSCs were short-lived which have caused decline in the overall enthusiasm about MSC therapy [12, 16, 35]. These revelations have sent researchers back to the bench looking forward to develop strategies to overcome the limitations of cell therapy and to develop more efficient approaches. Accordingly, in this light, each step from bench to bedside needs to be punctiliously evaluated and increasingly optimized to enhance the benefits of stem cell therapy. There are many ongoing efforts which are trying to dodge the obstacles that hinder long-term transplantation success of MSCs therapy [7, 16, 41]. Regardless of that fact, the results mostly turned out to be indecisive in long-term follow-ups. One of the most important obstacles that faces MSCs therapy success is the shift in the poor MSCs survival when they are placed in stress environment [18, 23]. Hypoxia, which is a harsh hallmark that presents in many chronic illnesses, is found to be hugely impacts the success rate of MSCs transplantation [2, 13]. Recently, researchers have focused on the effect of oxygen tension on MSCs behavior in regenerative medicine. Many studies on bone marrow-derived MSCs have shown that moderate hypoxia enhances the proliferation of MSCs and elucidates a protection against apoptosis [13, 14, 28]. Moreover, many animal models have showed that MSCs could migrate efficiently from the bone marrow to remote organs and tissues under moderate hypoxic condition. On the other side, more severe hypoxia that dominates in many diseases such as myocardial infarction, renal diseases, reduces the proliferation of MSCs and induces their death [8, 48]. However, the exact mechanism of why MSCs can tolerate moderate hypoxia not severe hypoxia needs to be investigated. The 26S proteasome is the master degradation machinery that is involved in the degradation of ubiquitinated and misfolded proteins in the cell [3, 9]. Moreover, it has been found that 26S proteasome orchestrates vital processes in the cell such as transcription initiation and activation, cell cycle progression, apoptosis, DNA repair, autophagy and many more processes [3, 5]. In addition, these diverse functions of 26S proteasome are governed by the unique nature of each cell type. Therefore, inactivation of 26S proteasome in different cell types may influence varied

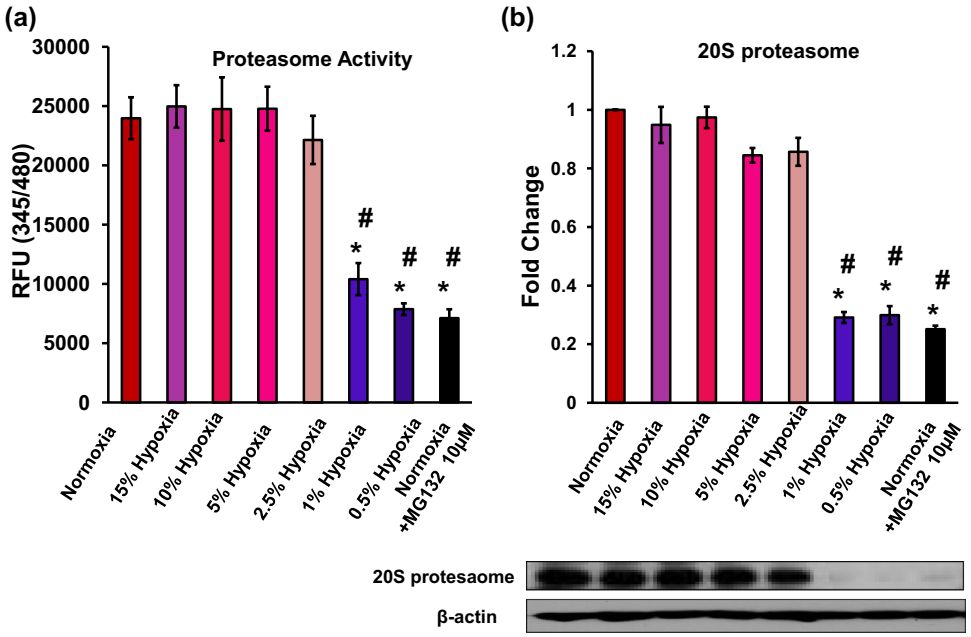

Fig. 1 Hypoxia effect on 26S proteasome function in human BM-MSCs: Human BM-MSCs were incubated in hypoxia chamber under different hypoxia levels (0.5–15% O_2). **a** 26S proteasome activity was assessed by measuring fluorescence intensity (RFU) of breaking down 26S specific substrate (Succ-LLVY-AMC), which showed a significant decrease in 26S proteasome activity in severe hypoxia (0.5–1% O_2) compared to normoxia and moderate hypoxia (2.5–15% O_2), whereas 26S proteasome function remained stable in human BM-MSCs under moderate hypoxia (2.5–15% O_2); $n=5$. **b** Western blot detection of 20S proteasome protein levels showed a significant reduction in 20S proteasome protein levels with the gradual raise in hypoxia severity; $n=3$.*$p<0.05$ compared to normoxia group; #$p<0.05$ compared to moderate hypoxia groups. Each experiment was repeated 3 times

pathways and determine the ability of these cells to tolerate various stress conditions [30]. In this light, it is of paramount importance to study the role of 26S proteasome in the ability of MSCs to withstand hypoxic stress. Thus, in this study we aim to explore the dynamic state and intactness of 26S proteasome under moderate and severe hypoxia, and its correlation with proper mitochondrial function in MSCs that ultimately influence their ability to survive hypoxia stress, which may help in developing various approaches and planning future studies that can enhance the tolerance abilities of MSCs to different hypoxia levels by targeting this pathway.

Results

Exposure to severe hypoxia, not to moderate one, triggers the inactivation of 26S proteasome in human MSCs

26S proteasome regulates many functions within the cell, and its dynamics fluctuates under different stress conditions. We want to determine whether there was any change in the activity of the 26S proteasome under different hypoxic levels in human BM-MSCs. Therefore, human BM-MSCs were incubated in the hypoxia chamber under different hypoxic oxygen percentages: 15, 10, 5 and 2.5% O_2 which are considered as moderate hypoxia, and 0.5 and 1% O_2 which are considered as severe hypoxia for 24 h. The 26S proteasome activity was assessed by measuring the fluorescence intensity (RFU) of breaking down 26S specific substrate (Succ-LLVY-AMC). It was noticed that there was a considerable decrease in the 26S proteasome activity in the severe hypoxia (1 and 0.5% O_2) compared to normoxia (Fig. 1a). On the other hand, the 26S activity did not get affected under moderate hypoxia oxygen levels (2.5–15% O_2) compared to normoxia (Fig. 1a). Furthermore, the 26S proteasome activity level decreased remarkably in the severe hypoxia (0.5% O_2) compared to moderate hypoxia (2.5% O_2) as well (Fig. 1a). Therefore, these results indicated that the severe hypoxia induced the loss of 26S proteasome function in human BM-MSCs, whereas under moderate hypoxia, the 26S proteasome continued to function properly and in commensurate fashion to normoxia. In order to confirm the proteasome activity outcomes, we performed Western blot analysis for protein expression of 26S proteasome in different hypoxia levels, and the 20S proteasome proteolytic core was ubiquitously expressed in normoxia and moderate hypoxia oxygen levels (2.5–15% O_2) (Fig. 1b), while its protein levels dumped significantly in MG132 treated

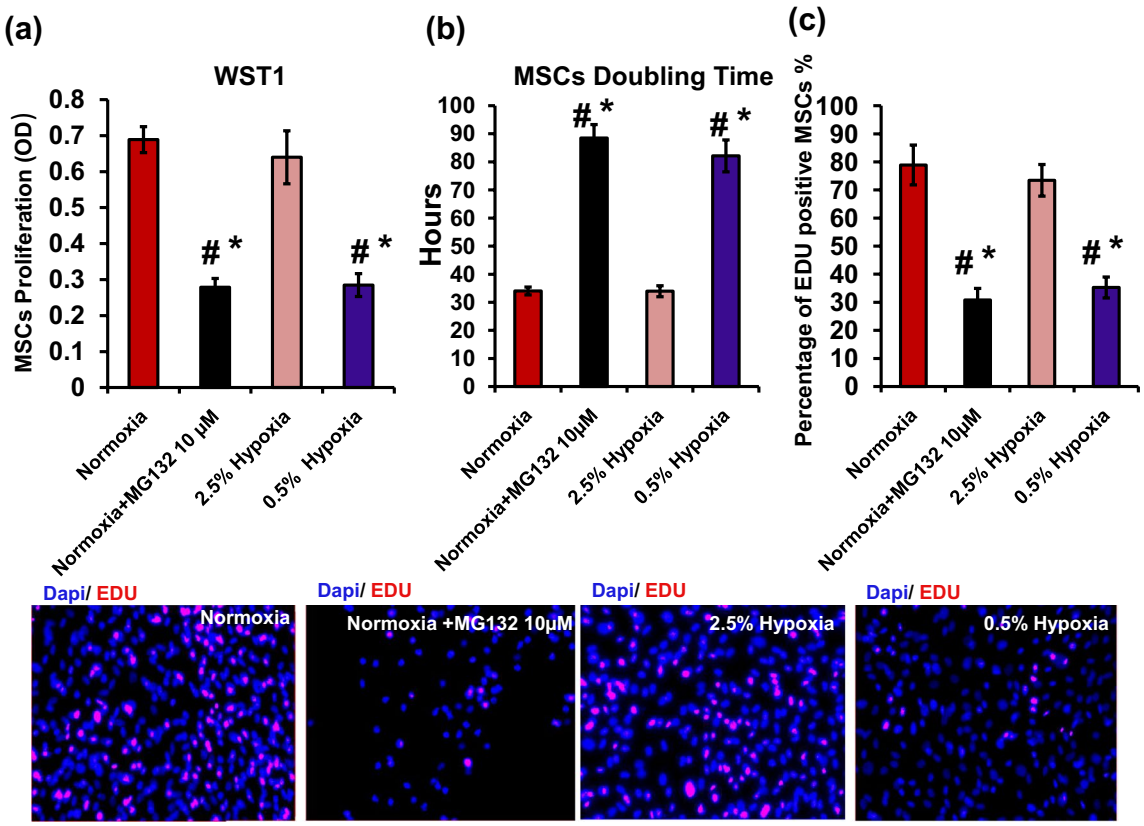

Fig. 2 26S proteasome influence human BM-MSCs proliferation under different hypoxia levels. **a** Human BM-MSCs proliferation was measured using WST1 Quick assay; the results showed a remarkable decline in the cells proliferation after treating the cells with MG132 inhibitor and severe hypoxia compared to normoxia and moderate hypoxia where it remained unaffected. $n=7$; **b** the doubling time was assessed using trypan blue viability assay. The results depicted a significant rise in the doubling time of human BM-MSCs incubated with MG132 and severe hypoxia weighed against normoxia and moderate hypoxia. However, in moderate hypoxia, the doubling time of human BM-MSCs fell within the normal range. $n=6$. **c** EDU staining for proliferation was performed. The percentage of positive Dapi/EDU purple cells conspicuously high normoxia and moderate hypoxia and less in human BM-MSCs incubated with MG132 and severe hypoxia; $n=3$. *$p<0.05$ compared to normoxia group; #$p<0.05$ compared to moderate hypoxia group. Each experiment was repeated 3 times

human BM-MSCs as well as in severe hypoxia (1 and 0.5% O_2) (Fig. 1b).

26S proteasome strongly affects the proliferation and survival of human MSCs under hypoxia stress

Next, we want to determine whether the fluctuation in 26S proteasome dynamics can affect the proliferation ability of human MSCs under different hypoxic levels. To investigate that, human BM-MSCs were treated with 26S proteasomic inhibitor (10 µm of MG132) and with either moderate hypoxia (2.5% O_2) or severe hypoxia (0.5% O_2) for 24 h. Then, the proliferation of human BM-MSCs was measured by using WST1 assay as well as assessing their doubling time. The addition of 26S proteasome inhibitor caused a significant slump in the proliferation and increased the doubling time of human BM-MSCs compared to normoxia (Fig. 2a, b). Moreover, exposing human BM-MSCs to severe hypoxia (0.5% O_2) caused a noticeable decline in the proliferation and increased their doubling time in comparison with normoxia, and also in a comparable fashion to the treatment with 26S proteasome inhibitor (Fig. 2a, b). On the other hand, human MSCs under moderate hypoxia continued to proliferate with a doubling time similar to normoxia (Fig. 2a, b). Therefore, appropriate 26S proteasome function maintained the proliferation of human BM-MSCs in moderate hypoxia (2.5% O_2), while under severe hypoxia 26S proteasome lost its function in human BM-MSCs which conspicuously led to decrease in their proliferation and increase their doubling time. Besides, the percentage of EDU positively stained human BM-MSCs which is an indicator of active proliferation, found to be high in human BM-MSCs treated with moderate hypoxia (Fig. 2c), while the percentage of EDU positively stained human BM-MSCs is considerably less with equivalent fashion in cells treated with MG132 and severe hypoxia (Fig. 2c). Furthermore, LDH cytotoxicity assay and live/dead assay demonstrated high

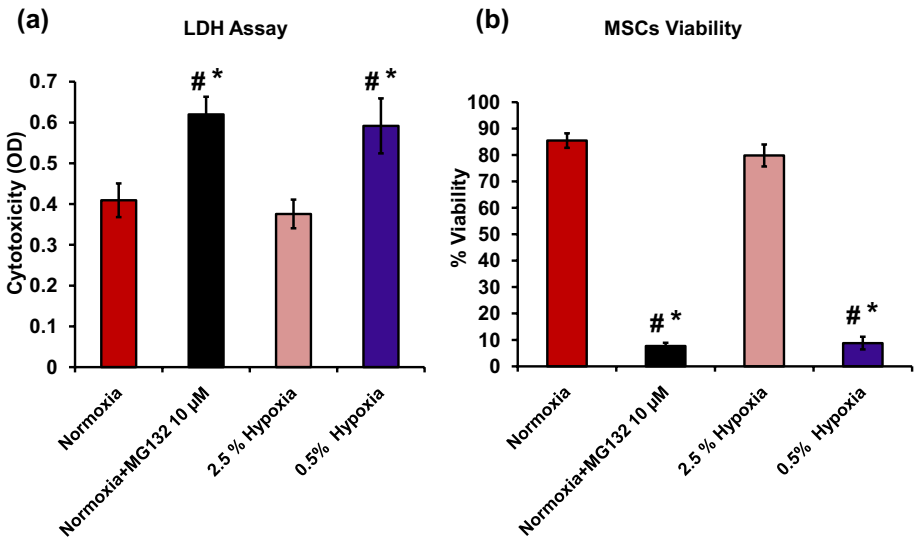

Fig. 3 26S proteasome functional status in human BM-MSCs under hypoxia controls their survival. Human BM-MSCs were treated with MG132 inhibitor (10 µm) and two different hypoxia percentages: 2.5% (moderate hypoxia) and 0.5% (sever hypoxia) for 24 h. **a** LDH cytotoxicity test was carried out after human BM-MSCs being exposed to the above corresponding treatments. The cytotoxicity of human BM-MSCs was hugely elevated after treating them with MG132 inhibitor as well as with severe hypoxia compared to normoxia and moderate hypoxia. Moderate hypoxia instigated no significant increase in the cytotoxicity level in human BM-MSCs. $n=8$. **b** Viability assay was performed using live detection dye calcein-AM and dead detection dye ethidium homodimer-1; the results showed a significant decline in human BM-MSCs viability after using MG132 inhibitor with similar results in severe hypoxia compared to normoxia and moderate hypoxia. In moderate hypoxia, the survival of human BM-MSCs was high in parallel to normoxia. $n=4$: $*p<0.05$ compared to normoxia group; $\#p<0.05$ compared to moderate hypoxia. Each experiment was repeated 3 times

levels of cytotoxicity and poor viability in human BM-MSCs after being treated with 26S proteasome inhibitor and severe hypoxia in comparable trend (Fig. 3a, b), while we found low cytotoxicity and high viability in human BM-MSCs under moderate hypoxia similar to normoxia (Fig. 3a, b), which additionally confirmed the importance of 26S proteasome.

Inactivation of 26S proteasome triggers the loss of mitochondrial function and induces apoptosis in human BM-MSCs

Many studies revealed that severe hypoxia can stimulate many death pathways in MSCs, while under moderate hypoxia MSCs continued to function in a healthy way, but no study so far explained the reasons behind this observation. In this light, we wanted to explore the role of 26S proteasome system in cell death signaling stimulation. Multiple lines of evidence indicated that 26S proteasome orchestrates many stages of mitochondrial protein biogenesis. Mitochondria is the powerhouse organelle in the cells. It has a complex proteome of proteins that are encoded by nuclear and mitochondrial genomes which require their transport in an unfolded state. Any changes in the folding or localization of mitochondrial proteins are deleterious to the cell as long as the nature of the electron transport chain in mitochondria is a reservoir of reactive oxygen species that cause severe damage. In this context, mitochondrial dysfunction with the loss of cellular protein homeostasis (proteostasis) can strongly manipulate the behavior of the cells and induce critical changes in their biological features. Several studies reported that hypoxic stress can enkindle excessive mitophagy leading to mitochondrial loss and dysfunction [15, 42]. PTEN-induced putative kinase 1(PINK1) contributes extensively in mitophagy initiation and acts in normal conditions as a quality control check inspector to keep eradicating damaged or unfunctional mitochondria [20, 32]. The expression of PINK1 in normal conditions is maintained at the lower levels and tightly controlled to avoid excessive mitophagy and mitochondrial loss. PINK1 initiates mitophagy via the activation of Parkin, an E3 ligase that binds to mitochondrial outer membrane proteins (OMM) and triggers their ubiquitination and degradation by lysosomal proteases [50]. PINK1/Parkin pathway is controlled by 26S proteasome which cleaves and inactivates PINK1/Parkin mitophagy induction [46]. No study so far finds an answer to excessive mitophagy seen in hypoxia stress. We checked the protein levels of PINK1 and Parkin in human BM-MSCs under different hypoxia levels as well as after treating the cells with MG132. Gradual increase in the severity of hypoxia stress enhances the accumulation of PINK1 and Parkin (Fig. 4). Treating human BM-MSCs with MG132

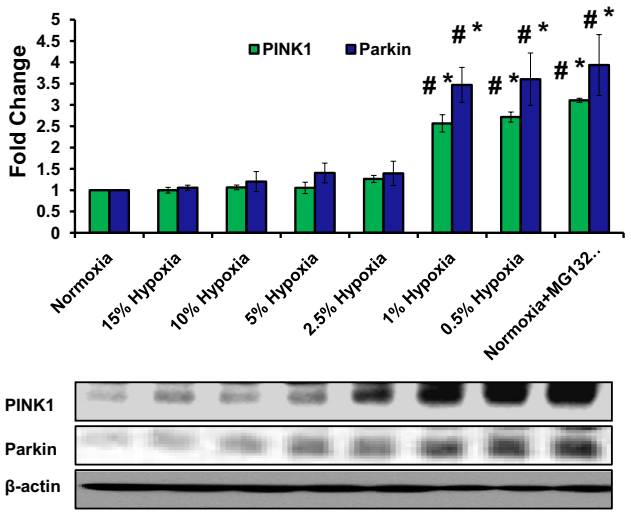

Fig. 4 PINK1 and Parkin proteins accumulation in severe hypoxia levels as a result of 26S proteasome inactivation. Human BM-MSCs were incubated in hypoxia chamber under different hypoxia levels (0.5–15% O_2). Cell lysate was prepared, and Western blot was carried out to check the levels of PINK1 and Parkin proteins. As the severity of hypoxia raises (low oxygen levels), PINK1 and Parkin proteins upregulated and accumulated gradually as a result of poor 26S proteasome degradation function; $n=3$. *$p<0.05$ compared to normoxia group; #$p<0.05$ compared to moderate hypoxia

causes tremendous increase in the levels of PINK1 and Parkin parallel to severe hypoxia affect which indicates that 26S proteasome inactivation observed in severe hypoxia contributes strongly to the massive upregulation in PINK1 and subsequently Parkin in human BM-MSCs (Fig. 4). These results prompted us to examine the mitochondrial function. Consequently, we carried out a screening assay to measure the mitochondrial membrane potential and performance in human BM-MSCs after treating them with 26S proteasome inhibitor, moderate hypoxia (2.5% O_2) and severe hypoxia (0.5% O_2) for 24 h. We used the TMRE Mitochondrial Membrane Potential Assay. TMRE (tetramethylrhodamine, ethyl ester) is red–orange positively charged dye that is so efficient in labeling and accumulating only in active mitochondria due to their negative charge, while depolarized or inactive mitochondria fails to sequester TMRE dye. Fluorescent intensity detection was used to interpret this assay. Our results discovered that TMRE fluorescent intensity in human BM-MSCs treated with 26S proteasome inhibitor is tremendously weak compared to normoxia and moderate hypoxia (2.5% O_2) (Fig. 5a, b). These outcomes are parallel with severe hypoxia (0.5% O_2) TMRE results. On the contrary, moderate hypoxia did not instigate any significant changes in the TMRE fluorescence intensity which is high and comparable to the results of normoxia group (Fig. 5a, b). Mitochondrial membrane potential is tightly regulated by electron transport chain complexes (I–V) which is incredibly important to maintain appropriate mitochondrial potential gradient with the generation of ATP that is crucial for the perseverance of vital cellular processes. So in order to have a better understanding of the negative effect of PINK1/Parkin accumulation on the mitochondrial membrane potential, mitochondrial electron transport chain complexes activity was evaluated which demonstrated the disturbance of many electron transport chain complexes after the treatment with MG132 and severe hypoxia (0.5% O_2) (Fig. 5c), while all complexes continue to function properly in moderate hypoxia (2.5% O_2) in a parallel fashion with normoxia results (Fig. 5c). These outcomes give convincible explanations of the intact mitochondrial membrane potential in moderate hypoxia and normoxia, while it is negatively affected when human BM-MSCs were exposed to 26S proteasome inhibitor and severe hypoxia. These findings delineated the importance of 26S proteasome in maintaining the mitochondria dynamics in human BM-MSCs under hypoxia, and the disturbance in 26S proteasome machinery that is presented under severe hypoxia (0.5% O_2) causes a huge dump in mitochondrial membrane potential and function which further explained the inability of human BM-MSCs in tolerating severe hypoxia levels, while they continued to function normally under moderate hypoxia.

The disturbance in mitochondrial membrane potential and function lead to initiation of the release of many apoptotic markers; therefore, we performed Western blot to detect the protein levels of apoptotic and survival marker: BAX, BCL-XL, P-AKT, and cleaved caspase 3, besides measuring caspases 3/7 activity. P-AKT/AKT ratio is a survival marker which showed a discernable downregulation after the inhibition of 26S proteasome with equivalent trend found in severe hypoxia in comparison with normoxia and moderate hypoxia (Fig. 6a). On the other hand, P-AKT/AKT remained high in moderate hypoxia similar to normoxia (Fig. 6a). The BAX/BCL-XL ratio is a good indicator for the occurrence of apoptosis, which increased significantly after treating human BM-MSCs with 26S proteasome inhibitor and severe hypoxia compared to normoxia and moderate hypoxia (Fig. 6b). The BAX/BCL-XL ratio showed a negligible change under moderate hypoxia, in parallel to normoxia (Fig. 6b). Next, we conducted caspases 3/7 activity assay through measuring the fluorescence intensity of the tetrapeptide DEVD, a specific substrate known to be broken by caspases that caused the emission of bright green color. Live fluorescent images of the cells were also obtained to confirm the assay. The results revealed a momentous rise in caspase 3/7 activity after using the 26S proteasome inhibitor as well as in severe hypoxia compared to normoxia and moderate

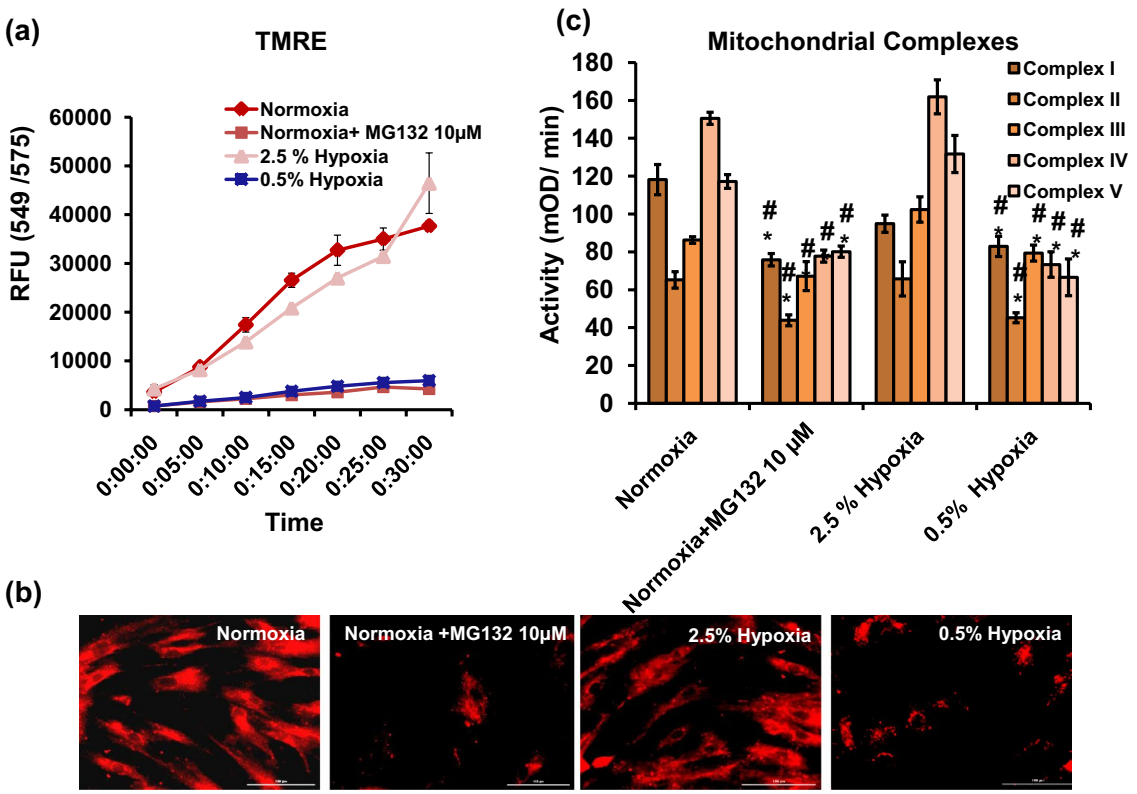

Fig. 5 26S proteasome functional status under hypoxia determines the integrity of mitochondria dynamics in human BM-MSCs. Human BM-MSCs were incubated with MG132 inhibitor(10 μm), and two different hypoxia percentages: 2.5% (moderate hypoxia) and 0.5% (sever hypoxia) for 24 h. TMRE Mitochondrial Membrane Potential Assay was performed. **a**, **b** The TMRE fluorescence intensity readings and imaging in human BM-MSCs treated with MG132 inhibitor is enormously low with similar results found in severe hypoxia compared to normoxia and moderate hypoxia. On the contrary, moderate hypoxia-treated human BM-MSCs showed sufficient TMRE intensity readings close to those found in normoxia. $n=6$; **b** live fluorescent images showed a weak signal of TMRE stain in human BM-MSCs after using the 26S MG132 and severe hypoxia weighed against normoxia and moderate hypoxia where the signal was strong and bright. **b** Mitochondrial complexes activity assays of human BM-MSCs after being subjected to experimental treatments. The activity of most complexes was negatively affected in human BM-MSCs treated with MG132 or severe hypoxia with the most discernable decrease in complexes IV and V, while the activity of mitochondrial complexes continued in normal mode in human BM-MSCs treated with moderate hypoxia similar to normoxia state; $n=3$. $*p<0.05$ compared to normoxia group; $\#p<0.05$ compared to moderate hypoxia. This experiment was repeated 3 times

hypoxia (Fig. 7a), whereas under moderate hypoxia, there was no noticeable induction in caspases activity (Fig. 7a). To ascertain the results of the caspases activity assay, cleaved caspase 3 protein levels were detected using Western blot which were found to be significantly high after the inhibition of 26S proteasome and under severe hypoxia compared to normoxia and moderate hypoxia (Fig. 7b), while in moderate hypoxia, the cleaved caspase 3 protein level was as low as in normoxia (Fig. 7b). Collectively, these results give a proof that maintaining the 26S proteasome function under moderate hypoxia protects human MSCs from apoptosis and preserves its survival. However, the poor function of 26S proteasome under severe hypoxia acts as an impetus that enkindles apoptosis in human BM-MSCs which gives evidence on the importance of 26S proteasome's ability of human MSCs to endure hypoxic stress.

Discussion

The outcome of several MSCs-based preclinical and clinical trials corroborated that bone marrow-derived MSCs have the potential to treat many degenerative and chronic diseases and disorders [7, 31, 37, 41], and they are believed to be the future gold standard cure for repairing and regenerating the damaged tissues which cannot be completely healed by the current conventional medications; therefore, they will put an end to the excruciating anguish of many patients who are suffering from debilitating illnesses. Unfortunately, the miraculous beneficial effects of transplanted MSCs were short-lived [40], and such a fact has caused a decline in the overall eagerness about MSC therapy. In fact, recent analyses of MSCs-based studies demonstrated that after transplantation cells do not stay for long time and subsequently die after a short time. Also, it has been reported

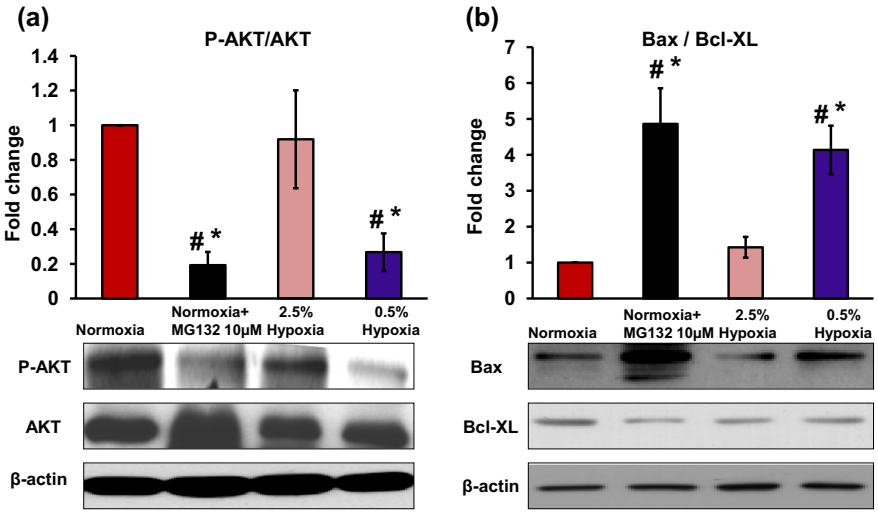

Fig. 6 26S proteasome poor function and mitochondrial damage under severe hypoxia triggers apoptosis in human BM-MSCs. Human BM-MSCs were treated with 26S MG132 inhibitor, (10 μm) and two different hypoxia percentages: 2.5% (moderate hypoxia) and 0.5% (severe hypoxia) for 24 h. **a** The survival P-AKT protein levels were analyzed by Western blot analysis. The results indicated a significant drop in P-AKT/AKT ratio after the inhibition of 26S proteasome and under severe hypoxia in comparison with normoxia and moderate hypoxia, whereas in moderate hypoxia, P-AKT/AKT protein ratio remained sufficient similarly to normoxia. $n=3$; **b** BAX/BCL-XL ratio, which is an indicator of apoptosis stimulation, showed a noticeable in human BM-MSCs treated with MG132 inhibitor with parallel trend in severe hypoxia compared to normoxia and moderate hypoxia. On the other hand, BAX/BCL-XL ratio inappreciably changed. $n=3$. $^{*}p<0.05$ compared to normoxia group; $\#p<0.05$ compared to moderate hypoxia. Each experiment was repeated 3 times

by many studies that MSCs, after being transplanted to the sites where the stress environment is high, start to lose their abilities to accommodate the stress, and they are not capable of surviving and proliferating for a long time [40]. However, the mechanisms of this change in the characteristics of MSCs which are originally known to be highly adaptive and tolerant cells with great aptitude to continue functioning, proliferating and maintaining their self-renewal proficiency despite the degree of stress they face [11] have not yet been studied conclusively. Hypoxia is considered as one of the major stress microenviroments that underlines in the pathogenesis of many diseases such as cardiovascular disorders, retinal degenerative diseases, renal diseases and many various types of illnesses. Additionally, several studies have illustrated the effects of hypoxia on proliferation and differentiation potential of MSCs [19, 26, 28, 29, 36, 51]; they stated that preconditioning MSCs by exposing them to moderate degree of hypoxia (2–7% of oxygen) inhibits apoptosis and senescence pathways and increases proliferation and differentiation potential of MSCs [13, 14, 28]. However, several preclinical and clinical trials reported that after transplanting MSCs into sites where severe hypoxia (1% or less than 1%) exists, they could not survive for long time, and ultimately they died and washed off [8, 26, 48]. Since the diseases such as myocardial infarction, where severe hypoxia dominates, were considered the target where MSCs transplantation is absolutely needed, it is of the top priorities to understand the mechanisms that affect the fickle tolerance abilities of MSCs under different hypoxia levels. No study so far has revealed the mechanistic medium that can solve the puzzle regarding the ability of MSCs to handle moderate hypoxia, but not the severe hypoxia. Currently, it has been discovered that 26S proteasome turned to be dysfunction machinery causing the rejection of MSCs in ischemic environment [1]. In this study, our results demonstrated that 26S proteasome machinery is the key to resolving this perplexing enigma of why MSCs tolerate and survive moderate hypoxia not severe one. We discovered that 26S proteasome was functioning effectively under moderate hypoxia; therefore, it helps in preserving MSCs proliferation aptitude and promotes their survival through preserving the mitochondrial dynamics by maintaining reasonable expression of PINK1 and Parkin proteins required for beneficial mitophagy that is essential for mitochondrial homeostasis and preventing the activation of apoptotic signaling pathways [33, 34]. Moreover, our data also evinced that under severe levels of hypoxia, 26S proteasome turned to be a paralyzed machine in MSCs which negatively impacted their survival and proliferation by causing the accumulation of PINK1 and Parkin proteins and disturbing the mitochondrial function with the activation of apoptosis markers [47]. Several studies have reported that the survival marker P-AKT protein levels were downregulated substantially under severe levels of hypoxia, but it is maintained unaffected in moderate hypoxia [4, 43, 44, 49]. Furthermore, it has been recently explored by one of the studies that 26S proteasome intactness is integrally pivotal

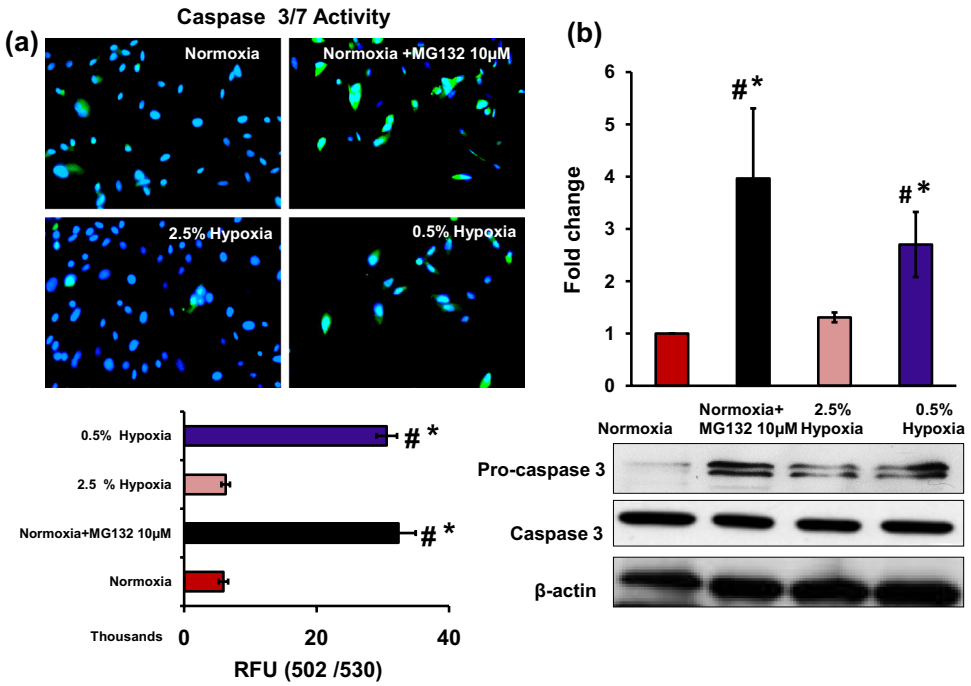

Fig. 7 Caspases levels detection in human MSCs under hypoxia. Human BM-MSCs were treated with MG132 inhibitor (10 μm), and two different hypoxia levels: 2.5% (moderate hypoxia) and 0.5% (severe hypoxia) for 24 h, followed by subsequent analysis of Caspases activation. **a** Caspases 3/7 activity assay was performed by measuring the fluorescent intensity of breaking down DEVD substrate. The findings demonstrated huge increase in the activation of Caspases 3 and 7 in MG132 and severe hypoxia-treated human BM-MSCs weighed against normoxia and moderate hypoxia, while in moderate hypoxia, Caspases 3 and 7 activity in human BM-MSCs was as low as in normoxia. $n=6$; Caspases 3/7 live fluorescent images were captured at FITC filter as further confirmation for the above assay and results. **b** Western blot detection of cleaved caspase 3 protein levels evinced high levels in MG132 and severe hypoxia-treated human BM-MSCs considerably to normoxia and moderate hypoxia where its levels were stayed low. $n=3$. $^{*}p<0.05$ compared to normoxia group; $\#p<0.05$ compared to moderate hypoxia. Each experiment was repeated 3 times

in preventing the senescence of MSCs and conserving their proliferation and expansion abilities with increasing passage number [21], which goes along with our findings regarding the importance of 26S proteasome in MSCs biology and performance [24]. In addition, it is reported that starvation is considered as a potent stress that leads to the inactivation of 26S proteasome [10], which is regarded another clue that espouses our results pertaining to the importance of 26S proteasome as a powerful system in forbearing severe hypoxia stress. Collectively, the findings provided by the current study give strong insights into the urgent need to develop future studies to improve the quality of MSCs so that they can survive in better fashion under severe hypoxia by developing strategies and approaches that can maintain proper functioning of 26S proteasome in MSCs, and eventually, they may assist in increasing the success rate of MSCs-based clinical trials and prolonging the survival rate of MSCs once they get exposed to severe ischemic/hypoxic environment and extending their long-term beneficial effects.

Materials and methods

Human mesenchymal stem cell line

Bone marrow-derived human MSCs (hMSCs) were purchased from ATCC Cat # PCS-500-012. The cells were cryopreserved in the second passage to ensure the highest viability and plating efficiency. According to the company safety analysis, the cells are not known to harbor any agent or infection recognized to cause disease in humans. The cells maintained and expanded in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% fetal bovine serum (FBS)[22], 100 units/ml penicillin G and 0.1 mg/ml streptomycin and incubated in 37 °C, 5% CO_2 incubator. The medium was replaced every 3 days, and the cells were sub-cultured when confluence exceeded 90%. These cells are characterized by the company. All the human MSCs-related in vitro studies were approved by Yarmouk University Ethics Board.

Experimental treatments

Different levels of hypoxia treatment were used: Moderate (2.5–15% O_2) and severe (0.5–1% O_2) were employed for 24 h. To block 26S proteasome activity in normoxic MSCs, the cells were treated with MG132 (10 μM) for 24 h.

26S proteasome activity assay

To determine the 26S activity of human MSCs, cells were subjected to the experimental treatment for 24 h, followed by measuring the fluorescent intensity of proteolysis of 26S proteasome substrate SUC-LLVY-AMC using a kit from Cayman (Cat# 10008041). The fluorescent intensity was read for each well at $\lambda_{ex}=350$ nm, $\lambda_{em}=480$ nm.

Doubling time

The population doubling time of MSCs under different treatments was analyzed using a trypan blue cell viability assay. The cells were plated at 5×10^4 cells/well in six-well dishes. Next, the cells were exposed to the treatment for 24 h and allow growing for 96 h, and then MSCs were detached using trypsin EDTA followed by staining with trypan blue and counting of the live cell number using an automated cell counter. The doubling time was calculated as follows:

Doubling time: time of the culture (h) × log(2)/
[log(final cell number)] − [log(initial cell number].

WST1 quick proliferation assay

We performed this assay using the kit from Biovision Company Catalog #K301. Briefly, and we seeded the MSCs in 96-well plates (5×10^4 per well in 100 μl/well culture medium) and incubate them overnight. Next day the cells were subjected to the study treatment for 24 h. After the treatment, 10 μl of WST1 solution was added to each well and it was incubated for 2 h. Once the incubation is finished, we shake it for 1 min and read the plate at the absorbance of $\lambda=450$ nm.

EDU proliferation assay

We investigated the proliferation of hMSCs treated with study experimental conditions using ethynyl-2′-deoxyuridine (EdU), Abcam iFluor 647 (Cat# ab222421). Briefly, hMSCs were seeded in 24-well plate and recovered overnight. Next, we added 100 μl of EDU solution (20 μM) and incubated for 4 h. After 4 h, the media containing EDU solution were aspirated and the cells were fixed using 4% formaldehyde for 20 min at room temperature. The fixation solution was aspirated and the cells washed twice using 3% BSA solution in PBS washing buffer. After aspirating the washing buffer, the cells were permeabilized for 30 min at room temperature followed by aspirating the permeabilization solution and washing the cells twice. Finally, 100 μl of iFluor 647 EDU reaction mix was added to each well and incubated in dark for 30 min. The cells were then washed and incubated for 10 min with 5 μg/ml Dapi working solution. Cells were visualized using fluorescent microscope. Purple cells represented Dapi/EdU positive cells.

Viability assay

The viability of MSCs was tested after the cells being exposed to the study treatments was measured by reading the florescence intensity of green-fluorescent calcein-AM (live detection dye) at $\lambda=530$ nm and red-fluorescent ethidium homodimer-1 (dead detection dye) $\lambda=650$ nm (Thermofisher Cat # L3224). The following formulas were used in final calculations: percentage of live cells $=(530_{sample}/530_{all\ live\ control})*100\%$. percentage of dead cells $=(650_{sample}/650_{all\ dead\ control})*100\%$. Percentage of viability = %live cells/(% live cells + % dead cells).

Cytotoxicity detection

The experimental treatment-mediated cytotoxicity on MSCs was detected by measuring the lactate dehydrogenase (LDH) released from the damaged MSCs using LDH Cytotoxicity kit (Sigma-Aldrich, Cat # 11644793001). Absorbance was measured at $\lambda=490$ nm.

Western blot

The protein levels for P-AKT (Cell Signaling Cat # 4060), BAX (Cell Signaling Cat # 5023), BCL-XL (Cell Signaling Cat #2764), cleaved caspase 3 (Cell Signaling Cat # 9661), 20S proteasome (R&D Systems Cat# MAB9554),PINK1 (Santa Cruz Biotechnology Cat # sc-518052), Parkin (Santa Cruz Biotechnology Cat # sc-133167) and β-actin (Santa Cruz Biotechnology Cat # sc-47778 HRP) were measured by Western blot. Briefly, total protein levels were measured by Bradford method, and 35 μg of protein was loaded onto SDS–PAGE. Following electrophoresis, proteins were transferred to PVDF membrane and incubated with appropriate primary and secondary antibodies. The membranes were developed using X-ray film, and bands were quantified using ImageJ for densitometry. Antibodies for P-AKT (Cell Signaling Cat# 4060), BAX (Cell Signaling Cat# 5023), BCL-XL (Cell Signaling Cat# 2764), cleaved caspase 3

(Cell Signaling Cat# 9661), Pro-Caspase 3 (Cell Signaling Cat# 9665S) and β-actin (Santa Cruz Biotechnology Cat# sc-47778 HRP) were used.

Caspase 3/7 fluorometric activity assay

Caspase 3/7 activity was detected using CellEvent™ Caspase-3/7 Green Detection Reagent (thermo scientific, Cat # C10423). In brief, human BM-MSCs were seeded in 96-well plates at 5×10^4 cells per well and allowed to adhere overnight. Cells were then subjected to the experimental treatment. After 24 h, media were aspired and cells were stained using 5 µM diluted Caspase-3/7 Green Detection Reagent in complete DMEM media without phenol red for 1 h in the incubator, plates were then read using microplate spectrophotometer at ($\lambda_{ex} = 502$ nm, $\lambda_{em} = 530$ nm), and immunofluorescence images were captured by fluorescent microscope using FITC filter ($\lambda = 488$).

Mitochondrial membrane potential

Mitochondrial membrane potential was assessed using TMRE kit (Abcam, Cat # ab113852). Briefly, human BM-MSCs were plated in 96-well plates at 1×10^5 cells per well and allowed to adhere overnight. After that, cells were treated for 24 h later, media were aspirated, and cells were stained using 400 nM TMRE in culture media for 20 min in the incubator, and then media were replaced with 100 µl PBS per well, plates were read over 30 min (5 min interval) using microplate spectrophotometer at ($\lambda_{ex} = 549$ nm, $\lambda_{em} = 575$ nm), and TMRE immunofluorescence images were captured by fluorescent microscope at Texas red filter.

Electron transport chain complexes assays

Human BM-MSCS were exposed to experimental treatments for 24 h followed by protein extraction using RIPA buffer. Thirty micrograms of protein extract was subjected to several complexes' activity kits including NADH Dehydrogenase (Complex I) Human SimpleStep ELISA Kit (Abcam, Cat# ab178011), Complex II Enzyme Activity Microplate Assay Kit (Abcam, Cat# ab109908), Cyt C Reductase (Complex III), Human Profiling ELISA Kit (Abcam, Cat# ab124537), Cytochrome c Oxidase (Complex IV), Human SimpleStep ELISA Kit (Abcam, Cat# ab179880) and ATP synthase (Complex V), Human Profiling ELISA Kit (Abcam, Cat# ab124539), and activity of mitochondrial complexes is expressed as mOD/min.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Comparison between experimental groups was achieved using one-way ANOVA followed by post hoc test (Dunnett test) using GraphPad Prism. Values of $p \leq 0.05$ are considered statistically significant.

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Author contributions RRK and EAR conceptualized the study; RRK and EAR designed the experiments; RRK, AOS, BOS and HAR carried out the experiments, acquired and analyzed the data; EAR and RRK interpreted the data and performed statistical analyses; EAR and RRK wrote the manuscript. All the authors have read and approved the final version of the manuscript.

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Compliance with ethical standards

Conflict of interest Authors declare no conflict of interest.

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