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TUNNELING NANOTUBE (TNT) MEDIATED INTERCELLULAR CROSSTALK AND AUTOPHAGY IN HYPOXIA-INDUCED MESENCHYMAL STEM CELLS

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Abstract: Intercellular communication is a critical process and multicellular organisms rely on *communication* networks to coordinate and maintain physiological functions. Tunneling nanotubes (TNTs) defined as a novel cell to cell communication mechanism and characterized by F-actin. TNTs allow the rapid exchange of cellular cargos including organelles, vesicles, molecules etc. Hypoxia plays an essential role in stem cell functions and also a known stimulus of autophagy. Autophagy and Wnt/β-catenin signalling pathways have important roles during essential cellular processes like tissue homeostasis. This study was aimed to investigate the effect of hypoxia on autophagy, Wnt/β-catenin signalling and the formation of TNTs in bone marrow mesenchymal stem cells (BM-MSCs). Western blotting was applied for HIF-1α protein expression. Immunolabeling was applied to investigate LC3B, p62 and β-catenin protein expressions. Immunofluorescence staining was assessed to evaluate TNT formations and HIF-1α protein. HIF-1α protein expression was significantly increased in $CoCl_2$ induced hypoxic BM-MSCs compared to the normoxia. As a result of the immunofluorescence staining, HIF-1α was positively stained in the cell nuclei of hypoxic BM-MSCs. Number and lengths of TNT formations was increased in hypoxic BM-MSCs compared to the normoxia. Also, we showed that hypoxia upregulates LC3B and downregulates p62 expression. In conclusion, our study indicates that TNT-mediated intercellular communication increases under the hypoxia in BM-MSCs and hypoxic microenvironment may be a significant factor for stem cell functions. Our findings may also draw attention to a possible TNT-mediated crosstalk for autophagy and Wnt/β-catenin signalling mechanism between distant cells.

Keywords: Autophagy, Hypoxia, Intercellular communication, Mesenchymal stem cell, Tunneling nanotubes, Wnt signalling.

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1. Introduction

Intercellular communication is essential for the maintenance of multicellular organisms. These communications can occur via different mechanisms such as secretion of cytokines, exosomes etc. (Mittelbrunn and Sánchez-Madrid, 2012). In 2004, a novel cell to cell communication mechanism based on the formation of thin membranous channels was reported in the neuron-like PC12 cell line. These unique structures were defined as tunneling nanotubes (TNT) and characterised by F-actin (Rustom et al., 2004). They provide a direct communication way to cells over long distances. TNTs vary in diameter and length in different cell types (Gerdes and Carvalho, 2008; Gurke et al., 2008; Sowinski et al., 2008). These TNT formations allow the rapid transport of cellular cargos including organelles, vesicles, ions and various molecules. They have important communication roles in early development, stem cell functions, regeneration and pathological mechanisms of some diseases (Austefjord et al., 2014; Gerdes et al., 2013; Roehlecke and Schmidt, 2020; Zurzolo, 2021).

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regeneration and pathological mechanisms of some diseases (Austefjord et al., 2014; Gerdes et al., 2013; Roehlecke and Schmidt, 2020; Zurzolo, 2021).

Hypoxia is a known stimulus of autophagy (Wu et al., 2014). Autophagy is a cellular homeostatic process and involves degradation of nonfunctional cellular components. Cellular adaptation to environmental conditions are mainly regulated by autophagy and its crosstal with various pathways and one of them is Wnt/β-catenin signaling pathway (Xu et al., 2017). Autophagy and Wnt/β-catenin signaling are the essential pathways which contribute to tumor growth and anticancer therapies. LC3B and p62 are well-known molecules in autophagy mechanism. β-catenin, an essential Wnt signaling protein, are targeted by LC3 for autophagic degradation. β-catenin also acts as a corepressor of the p62, when Wnt signaling is activated. There is a regulatory feedback mechanism coordinating with autophagy, and this mechanism include β-catenin at a key integration point with implications for targeting these pathways for development cancer therapies (Lorzadeh et al., 2021; Petherick et al., 2013; Wu et al., 2014).

Mesenchymal stem cells (MSCs) are sources for cell therapies, immune-modulation and regenerative medicine-based applications (Arthur and Gronthos, 2020; Baker et al., 2015). MSCs are self-renewal, multipotential and highly proliferative cells. They represent a promising cellular strategy in regenerative medicine because of their highly proliferative, self-renewal and multipotential properties (Bornes et al., 2014). The MSC microenvironment is characterized by a low oxygen tension. Since MSCs are quite resistant to oxygen limitations hypoxia is believed to play an essential role in controlling the behavior and function of mesenchymal stem cells (MSCs) (Chen et al., 2019). Despite several studies, the influence of hypoxia on the regulatory mechanisms of MSC functions and other related cellular pathways is still a matter of discussion. In our study, we aimed to mimic the hypoxic microenvironment in order to examine the effects of hypoxia on the formation of tunneling nanotubes in bone marrow mesenchymal stem cells (BM-MSCs) and to investigate the possible relation with autophagy and Wnt/β-catenin signalling.

2. Materials and Methods

2.1. Cell Culture

Bone Marrow-Derived Mesenchymal Stem Cells (BM-MSCs; Cat No: PCS-500-012, *ATCC*) were used for cell culture experimental studies. Cells were cultured in DMEM (Dulbecco's Modified Eagle Medium; Cat No: 11965092, Capricorn), supplemented with 10% fetal bovine serum (FBS; Cat No: 10270106, Gibco) and 1% antibiotics (penicillin/streptomycin; Gibco Penicillin-Streptomycin (10,000 U/mL)) at 37°C and 5% CO_2 in a standard incubator until they reached the required numbers. Inverted microscope (IX70 Olympus) was used for cell culture experiments.

2.2. Hypoxia Mimetic Treatment of BM-MSC

Cobalt chloride ($CoCl_2$) (Cas no: 7791-13-1, Sigma Aldrich) was used to create chemical hypoxia. Equal numbers of BM-MSCs were incubated with $CoCl_2$ (100 μM, final concentration) for 24 h. The optimum concentration for experiments was chosen based on the results from literature (Bhandi et al., 2021; Ciavarella et al., 2016; Liu et al., 2018; Nugraha et al., 2021; Teti et al., 2018; Yoo et al., 2016; Yu et al., 2013). Cell viability was measured using a trypan blue assay after 24 h of incubation with 100 μM concentration of $CoCl_2$ and evaluated with an hemocytometer. To compare hypoxia with normoxic conditions, MSCs were also incubated in $CoCl_2$ free medium for 24 h.

2.3. Western Blotting

The BM-MSCs were cultured with 100 μM $CoCl_2$ for 24 hour and cytosolic content was obtained by using RIPA lysis buffer (with protease inhibitor cocktail). After determining protein concentrations with BCA assay, adequate amount of samples were boiled in sample buffer and loaded to Bis Tris 4–12% gradient gel (Invitrogen) and transferred into a nitrocellulose membrane using a semi-dry transfer system. The membranes were blocked with 3% dry milk in PBS for 1 hour at room temperature and were then incubated with rabbit polyclonal HIF-1α (1:1000, St John's Laboratory), and beta actin (1:1000, Bioss, USA) at 4°C overnight. After washing with PBS Tween, each blot was incubated with secondary anti-rabbit antibody (1:1000, Bioss, USA) at room temperature for 1 hour. After washing, membranes were exposed to Chemiluminescent Substrate (Thermo Fisher Scientific, Monza, Italy) for 2-3 min. After the visualisation, the band densities was evaluated with Image J software programme (National Institutes of Health). Intensities of the protein bands were corrected with equal beta-actin loading.

2.4. Immunolabeling

To evaluate the expression levels of the β-catenin, LC3B and p62, we performed the immunolabeling analysis. Firstly, BM-MSCs were cultured in 6-well culture plates on coverglass. When cells reached ~60% confluency, $CoCl_2$ (100 μM) administered for 24h. At the end of culture period, culture medium was collected and immunolabeling protocol was applied. In summary, cultured cells were fixed with 4% paraformaldehyde (PFA) for 15 minutes at room temperature, washed two times with PBS and blocked with serum Ultra V block (TA-125-UB, Thermo) for 1 hour. After blocking reagent, cells were washed two times with PBS and incubated with primary antibodies; β-catenin (sc7963, 1:100, Santa Cruz Biotechnology), LC3B (ab48394, 1:50, Abcam) and p62/SQSTM1 (H00008878-M01, 1:500, Abnova) at 4°C overnight. After washing, cells were incubated with biotinylated HRP (Cat No: TP-125-BN, Thermo) for 1 hour at room temperature. A streptavidin-peroxidase (Cat No: TS-125-HR, Thermo) was used to identify the antigen–antibody complexes for 30 min at room temperature. Cells were shortly incubated with AEC chromogen (Cat No: TA-125-HA, Thermo) and counterstained with Mayer's

hematoxylin. The light microscope was used to view the samples (Olympus BX43F JAPAN, Olympus DP26 camera JAPAN) and photographed (Photography program: Olympus DP21, JAPAN). The results were evaluated with Image J software for 10 randomly chosen fields. Cytoplasmic expression levels were evaluated as; 0: no expression, 1: low expression, 2: high expression and 3: very high expression. Data were represented as Mean ± Standard Deviation (SD).

2.5. Immunofluorescence Staining

In order to visualize the HIF-1α expression and TNT formations, immunoflorescence staining protocol was applied. After $CoCl_2$ administration, cultured cells were fixed with 4% PFA for 15 minutes, washed two times with PBS, permeabilized with 0.5% Triton X-100 for 15 minutes and washed two times with PBS, blocked with 5% fetal bovine serum for 30 minutes, then incubated with HIF-1α polyclonal antibody at 4°C overnight. After washing, secondary antibody (Texas Red, Abcam) was applied at room temperature for 1 h. Cells were then washed again and incubated with diluted (1:100) Flash Phalloidin™ Green 488 (Cat No: 424201, Biolegend) at the dark for 1 hour. Lastly, the slides were mounted with DAPI mounting medium (ab104139, Abcam) and imaged with Olympus BX53 Fluorescence microscope. TNT formations were assessed at x200 magnification in 10 randomly chosen fields.

2.6. Statistical Analysis

Western blotting and immunolabeling data were evaluated with Image J software (National Institutes of Health). Statistical analysis was done with SPSS Statistics (IBM, version 21.0) and Graphpad Prism (version 7.0). Data was analysed using one-way ANOVA, Mann Whitney-U and Kruskal Wallis tests. All data were represented as mean ± standard deviation (SD) of three independent experiments. The significance levels were defined as P≤0.05 (*), P≤0.01 (**) and P≤0.001 (***).

3. Results

3.1. Cobalt Chloride As a Hypoxia-Mimicking Agent Enhances Hif-1 Alpha Expression in BM-MSCs

In order to examine cytotoxic effect of $CoCl_2$ on BM-MSCs, we first applied Trypan blue staining protocol. We observed that $CoCl_2$ (100 μM) administration for 24h did not affect the cell viability of BM-MSCs, both normoxic and hypoxic cells were 90-92% alive. After the analysis of cell viability, we have continued with same concentration of $CoCl_2$ for hypoxia induction. Expression of HIF-1α is a part of the systemic response to low oxygen levels. Therefore, HIF-1α can act as an effective molecular marker of hypoxia. We performed the western blotting to analyse the expression levels of HIF-1α by BM-MSCs in normoxia and hypoxia. Beta-actin was used for the normalization of HIF-1α protein levels. According to the results of western blotting, HIF-1α protein level was significantly increased in BM-MSCs in hypoxia compared to normoxia (P=0.034, P≤0.05) (Figure 1). In addition to western blotting, our immunofluorescence imaging results also demonstrate that HIF-1α antibody was positively stained in the cell nuclei of hypoxic BM-MSCs (Figure 2).

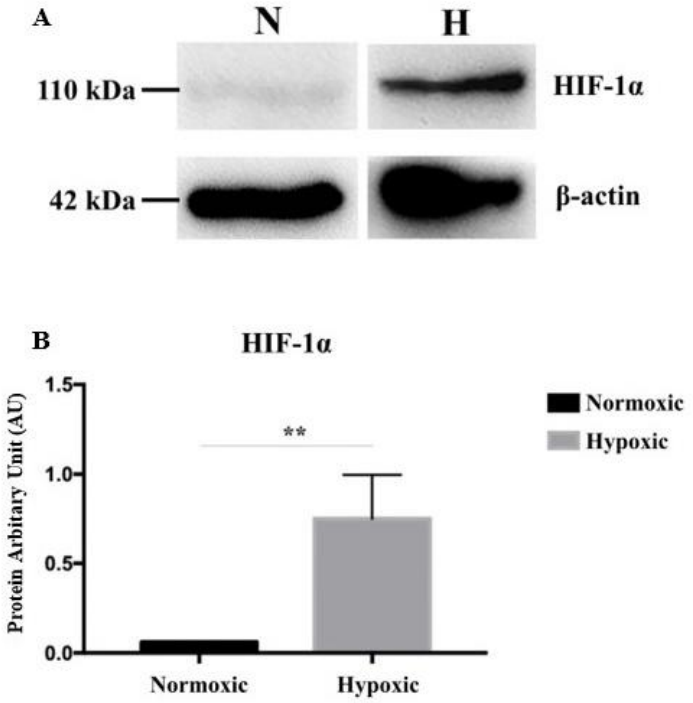

Figure 1. Western blotting analysis of HIF-1α protein for hypoxic and normoxic BM-MSCs (A). The significance level was defined as P≤0.01 (**) (B).

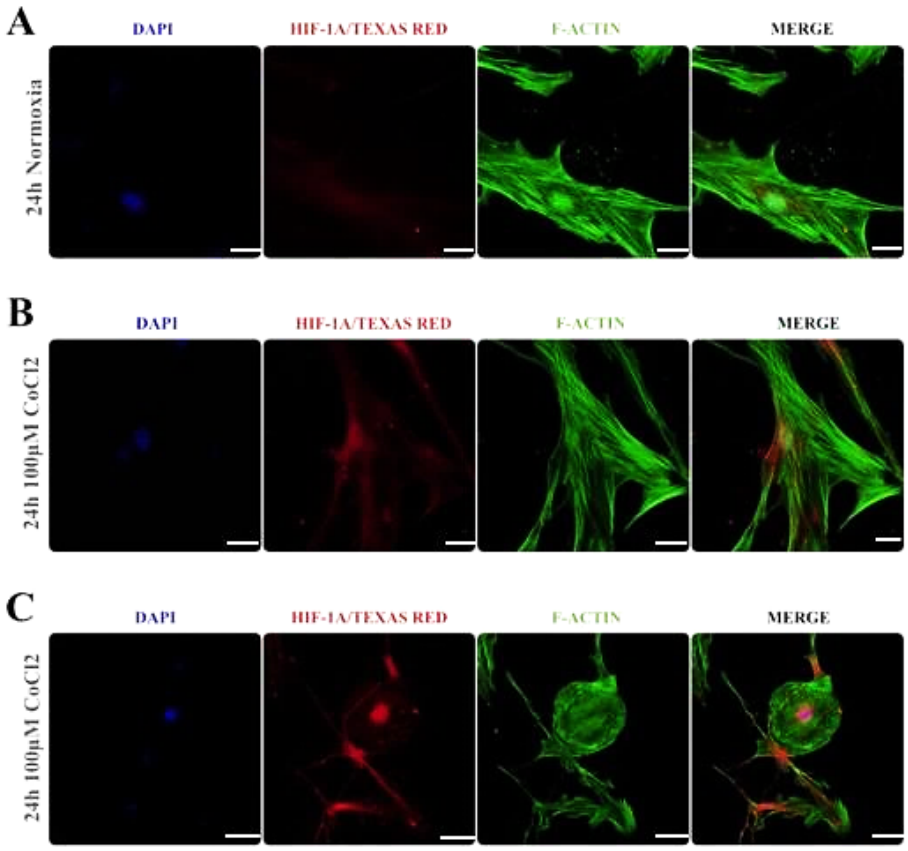

Figure 2. Immunofluorescence imaging of HIF-1α protein (red), F-actin (Phalloidin) (green) and DAPI (blue). (A) 24h normoxic group of BM-MSCs. (B-C) 24h 100μM $CoCl_2$-induced hypoxic group of BM-MSCs. Scale bars: 40 μm.

3.2. Hypoxic Microenvironment Increases the Number and Lengths of TNTs in BM-MSCs

We performed immunoflorescence staining to visualize and analyse TNT formations in BM-MSCs. F-actin containing nanotube structures between BM-MSCs and vesicle-like cargos throughout the TNTs were observed (Figure 3). When TNT formation was assessed we observed that the lengths of TNTs were significantly increased in hypoxic BM-MSCs compared to normoxia (P=0.004, P≤0.05) (shown in the Table 1 and Figure 4A). Also, there were an increased number of TNT formation in hypoxic BM-MSCs compared to the cells in normoxia. However, this finding was not statistically significant (P=0.167, P>0.05) (Figure 4B).

Table 1. Number and lengths of TNT formations for normoxic and hypoxic BM-MSCs

	Groups	Mean ± SD
TNT number	Hypoxic	4.50 ± 2.014
	Normoxic	3.30 ± 1.703
TNT length	Hypoxic	88.923 ± 26.873
	Normoxic	55.942 ± 16.497

SD= standard deviation

3.3. Expression of LC3B Upregulated and p62/SQSTM1 Downregulated in Hypoxia-Induced BM-MSCs

In addition to the evaluating TNT formations, we have also evaluated the expression of autophagy markers in BM-MSCs in normoxia and hypoxia conditions. According to the immunolabeling results, the cytoplasms of hypoxic BM-MSCs were stained positive, intensely. There were also positively stained small granules in their cytoplasms in normoxic BM-MSCs. Our results showed that LC3B protein expression is upregulated in hypoxic BM-MSCs compared to normoxia (P=0.001, P≤0.05) (Figure 5A). Also we have examined the p62 protein expression and our results showed that p62 is downregulated in hypoxic BM-MSCs. There was a few positive cytoplasmic staining in hypoxic BM-MSCs for p62 but it was decreased significantly (P=0.027, P≤0.05). (Figure 5B). Comparison of the expression levels can be seen in Figure 6.

3.4. Hypoxia Can Trigger Wnt/β-catenin Signaling through Increased Cytoplasmic Expression of β-catenin

In order to investigate the effect of hypoxia on the possible relation with Wnt signalling and autophagy, we have also examined the expression of β-catenin with immunolabeling. Our results showed that β-catenin protein expression was increased in hypoxic BM-MSCs. Although there was increased positive cytoplasmic staining in hypoxic BM-MSCs it was not statistically significant (Figure 5C and Figure 6).

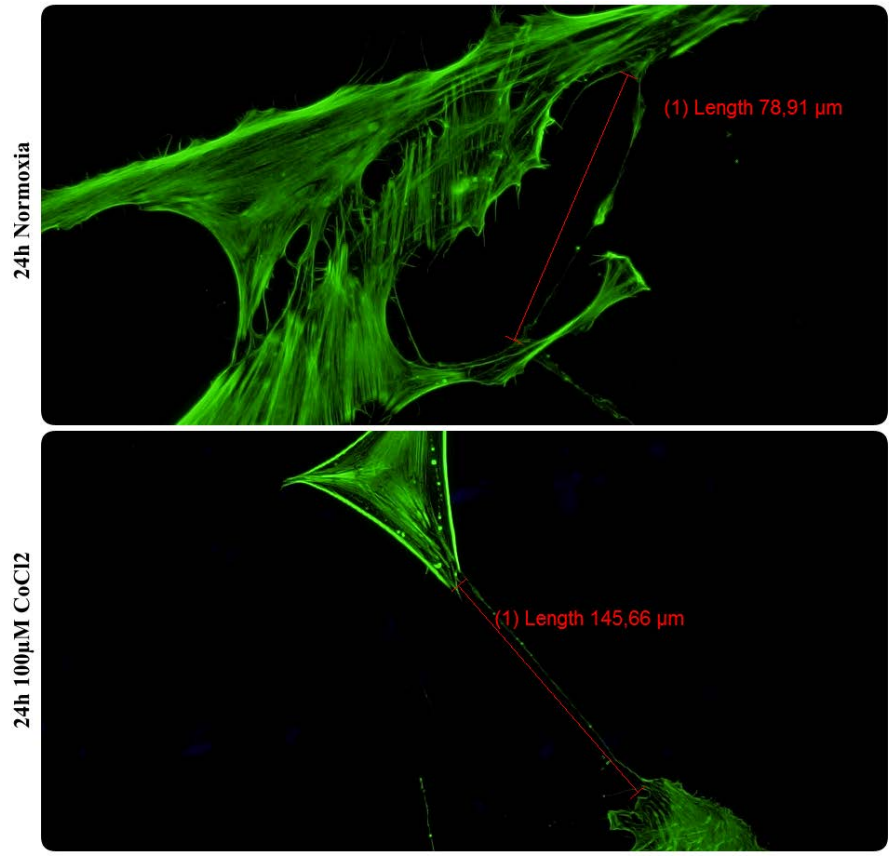

Figure 3. The measurement of TNT lengths in normoxic and hypoxic BM-MSCs. It can be seen there are tubular F-actin containing structures between BM-MSCs and the arrow (→) indicates vesicle-like cargos throughout the TNTs. Scale bars: 100 µm.

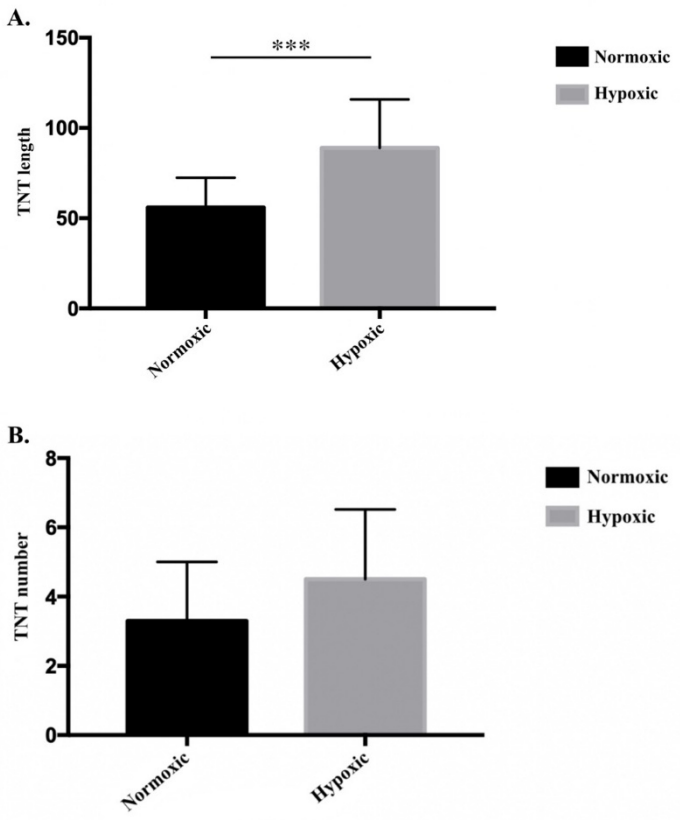

Figure 4. TNT lengths (A) and numbers (B) of normoxic and hypoxic BM-MSCs. (10 random field with x200 magnification and evaluated per cell). The significance level was defined as P≤0.001 (***).

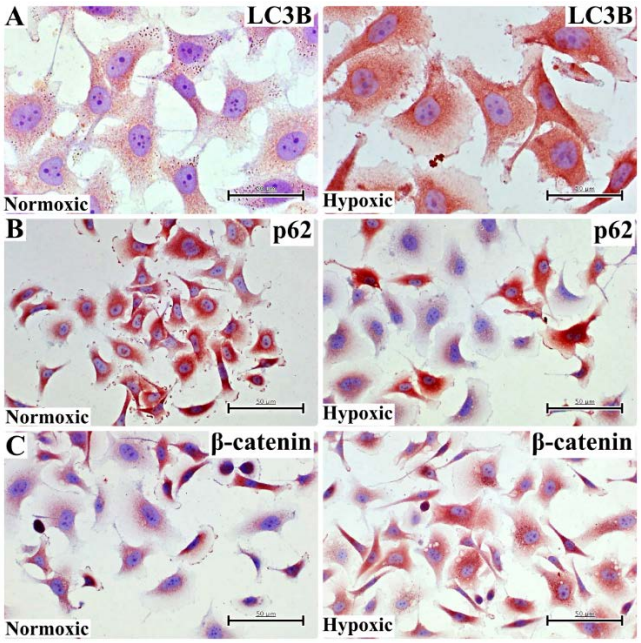

Figure 5. Immunolabeling images for the expressions of LC3B (A), p62 (B) and β-catenin (C) in normoxic and hypoxic BM-MSCs. Scale bars: 50 µm.

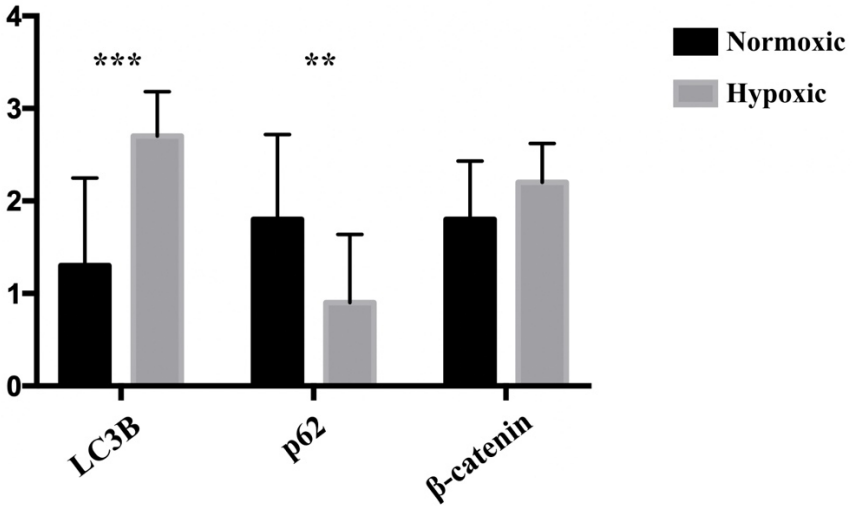

Figure 6. Comparison of expression levels for LC3B, p62 and β-catenin in normoxic and hypoxic BM-MSCs. The significance level was defined as P≤0.01 (**) and P≤0.001 (***).

4. Discussion

Hypoxia is an important microenvironment factor for stem cell behaviour and functions (Buravkova et al., 2012; Gleadle and Ratcliffe, 1998; Liu and Simon, 2004; Simon and Keith, 2008; Di et al., 2021). As previously mentioned, cobalt chloride ($CoCl_2$) is an easier and attractive method in order to induce hypoxia (Laksana et al., 2017). However, studies of the effect of $CoCl_2$ on BM-MSCs are limited and the influence of hypoxia on stem cell behaviour and intercellular communication is still a matter of discussion. This is a unique study to show the effect of $CoCl_2$ induced hypoxia on the formation of tunneling nanotubes (TNTs) in BM-MSCs.

Previous studies have showed that hypoxia induction by $CoCl_2$ increases the HIF-1α protein expression (Ciavarella et al., 2016; Nugraha et al., 2021; Teti et al., 2018; Yoo et al., 2016; Yu et al., 2013). Teti et al showed that the 100 µM concentration of $CoCl_2$ showed the highest cell viability at 24h for mesenchymal stem cells (Teti et al., 2018). Nugraha et al. have also reported that 100µM $CoCl_2$ can enhance significantly HIF-1 α expression of gingival derived mesenchymal stem cells for 24 hours in vitro (Nugraha et al., 2021). Consistent with previous studies, our results of western blotting analysis (Figure 1) and immunofluorescence staining (Figure 2) showed that hypoxia induction by $CoCl_2$ at 100 µM concentration for 24h increased the HIF-1α expression in BM-MSCs in hypoxia compared to normoxia.

In recent years, there has been considerable interest in the transfer of cytoplasmic material and organelles between

cells mediated by different intercellular communication mechanisms. In vitro studies have begun to elucidate the role of TNTs to make easier intercellular trafficking between cells. TNT formations in stem cell-based studies, cancer, as well as in other models of diseases, represent a novel and key topic for understanding how cellular communication takes place in different cell types (Gerdes and Carvalho, 2008; Gurke et al., 2008; Murray and Krasnodembskaya, 2019; Soundara et al., 2020; Roehlecke and Schmidt, 2020; Zurzolo, 2021).

Researchers, studied with SKOV3 and C200 cells, have reported that hypoxia stimulates and increases the number of TNT formations in ovarian cancer cells. They also indicated that cellular stress in the form of hypoxia could induce TNT-mediated intercellular communication (Desir et al., 2016). Another study also showed the TNT formations between K7M2 murine osteosarcoma cells, MC3T3 murine osteoblast cells and MG63 human osteosarcoma cells (Thayanithy et al., 2014). In 2022, Kato et al have studied with MCF-7 breast cancer cells and they showed that the chemotherapy agent 5-fluorouracil (5-FU) induced TNT formation in MCF-7 cells. They have also observed that mitochondrial exchange through TNTs following 5-FU treatment (Kato et al., 2022). Formicola et al identified structural differences of TNT formations by glioblastoma cells, in comparison with astrocytes. They have considered that thicker TNT structures are more effective in transportation of vesicles and organelles. So, their results suggest that tunnelling nanotube formations are potentially useful for drug delivery studies on cancer therapy (Formicola et al., 2019). In 2018, researchers observed and reported the TNT formations in mesenchymal stem cell spheroids as three-dimensional (3D) study (Zhang et al., 2018). Kolba et al reported that tunneling nanotubes (TNTs), which have been identified as a novel mechanism of intercellular crosstalk and mediate transfering cellular vesicles from stroma to leukemic cells (Kolba et al., 2019). Jackson et al have also demonstrated that mitochondrial transfer from MSC to innate immune cells via TNT-like structures (Jackson et al., 2016). In recent years, despite the increasing number of different studies about TNTs on different cell types, there are limited number of studies encountering TNT formations between mesenchymal stem cells (MSCs).

In this study, we have stimulated hypoxia in using $CoCl_2$. Our results showed that $CoCl_2$-induced hypoxia stimulates and enhances the formation of TNTs in BM-MSCs. In addition, the lengths of TNT formations were measured in our study and the result was significantly increased in hypoxic condition compared to normoxia. Overall, our data demonstrates that TNT-mediated intercellular communication increases under the hypoxia in BM-MSCs and hypoxia may be significant microenvironmental factor for stem cell studies. In addition, the increased length of TNTs in BM-MSCs under hypoxic microenvironment may be a target mechanism for the treatment of some hypoxia-related pathological situations such as cancer, metastasis, cardiomyopathy etc.

A key adaptive response to microenvironmental stress like hypoxia is autophagy. Oxidative stress induced by hypoxia may trigger autophagy to remove nonfunctional cellular structures to prevent further damage. In our study, we also investigated the effect of hypoxia on autophagy and Wnt/β-catenin signalling pathway and a possible relation with increased number and length of TNT formations. We showed that the cytoplasmic expression of LC3B, a key molecular component of autophagy mechanism, was upregulated, while p62/SQSTM1 (encoding the autophagy adaptor p62) was downregulated in hypoxic BM-MSCs. These findings were consistent with the previous studies (Jaakkola and Mursiheimo, 2009; Mitani et al., 2015; Monaci et al., 2020). Thus, our results indicate that hypoxia-activated autophagy with increased level of LC3B, accelerates degradation of SQSTM1/p62. In addition, our findings about increased expression of β-catenin in hypoxic group shows that hypoxia can activate the Wnt/β-catenin signalling pathway. This finding may support that β-catenin could also repress p62/SQSTM1 as previously studied (Petherick et al., 2013).

While accumulating data from recent studies reveal their importance for cellular functions, we still have a limited understanding of TNTs. The focus of the future studies must be to elucidate the mechanisms of formation and intercellular cargo transfer via TNTs and also their possible roles in diseases should be investigated. One question that remains to be answered regarding TNTs is whether longer TNTs are functional and allow for more material transfer between donor and recipient cells. Further studies may be conducted to find an answer to this question by lipid dye transfer. Our findings draw attention to a possible TNT-mediated crosstalk for autophagy and Wnt/β-catenin signalling mechanism between distant cells.

5. Conclusion

In conclusion, our study indicates that TNT-mediated intercellular communication increases under the hypoxia in bone marrow mesenchymal stem cells. Thus, hypoxic microenvironment may be a significant factor for stem cell-based regenerative medicine studies. In addition, our results showed that there is a relation between hypoxia-activated autophagy and Wnt/β-catenin signalling pathway. Targeting these pathways to develop stem cell-based therapies could be important for future studies. Also increased numbers and lengths of TNTs under hypoxic microenvironment draws attention to a possible TNT-mediated crosstalk for autophagy and Wnt/β-catenin signalling mechanism between distant cells and it may be a target mechanism for the treatment of some hypoxia-related pathological situations.

Author Contributions

The percentages of the author's contributions are presented below. All authors reviewed and approved the final version of the manuscript.

	G.İ.A	Ş.K	LD.K	H.M.Ö
C	25	25	25	25
D	30	30	20	20
S	30	30	20	20
DCP	30	30	20	20
DAI	30	30	20	20
LS	35	35	15	15
W	40	40	10	10
CR	25	25	25	25
SR	10	70	10	10

C= Concept, D= Design, S= Supervision, DCP = Data Collection Processing, DAI= Data Analysis Interpretation, LS= Literature Search, W= Writing, CR= Critical Review, SR= Submission and Revision.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because of there was no study on animals or humans.

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