RESEARCH ARTICLE

CYPRUS
JOURNAL OF MEDICAL SCIENCES

DOI: 10.4274/cjms.2025.2024-107
Cyprus J Med Sci 2025;10(Suppl 1):87-91

Does RAB31 Continue to Play a Role in Exosome Biogenesis of Adipose-Derived Mesenchymal Stem Cells in 2D and 3D Culture Conditions in a Hypoxic Environment?

Hafize Seda Vatansever[1,2], Hilal Kabadayı Ensarioğlu[1], Aslınur Aktaş[1], Burak Kutlu Aydın[1], Nadire Kıyak[3], Mehmet Vatansever[4]

[1]Department of Histology and Embryology, Manisa Celal Bayar University Faculty of Medicine, Manisa, Türkiye
[2]DESAM Research Institute, Near East University, Nicosia, North Cyprus
[3]Department of Histology and Embryology, Near East University Faculty of Medicine, Nicosia, North Cyprus
[4]Department of Occupation Therapy, Near East University Faculty of Health Sciences, Nicosia, North Cyprus

Abstract

BACKGROUND/AIMS: Mesenchymal stem cells (MSCs) are considered potential candidates in regenerative medicine due to their angiogenic, anti-apoptotic, and immunomodulatory properties. Among the secretory products of MSCs are biomolecules such as cytokines, chemokines, growth factors, and particularly exosomes. Exosomes are nanovesicles, ranging from 20 to 100 nm, that can be isolated from various body fluids. RAB31 plays a role in an endosomal sorting complex required for transport-independent pathway. Our study aims to determine the changes in exosome biogenesis and secretion of RAB31 from adipogenic mesenchymal stem cells (AMSCs), cultured in 2D and 3D culture conditions under normoxic and hypoxic environments.

MATERIALS AND METHODS: AMSCs were cultured in 2D and 3D conditions, and the study groups were formed as follows: normoxic 2D AMSC culture (group 1), normoxic 3D AMSC culture (group 2), hypoxic 2D AMSC culture (group 3), and hypoxic 3D AMSC culture (group 4). After culturing all groups of cells under normoxic and hypoxic conditions for 48 hours, exosomes were collected from culture medium, and the presence of RAB31, Rab7, CD9, and CD63 proteins was evaluated using indirect immunocytochemistry.

RESULTS: The exosome secretion was different after normoxic and hypoxic conditions. In addition, the distributions of RAB31 and RAB7 were different, but the intensity of CD9 and CD63 immunoreactivity was similar in 2D conditionions. CD9 immunoreactivity was not affected under hypoxic or normoxic conditions. However, increased immunoreactivity of CD63 was observed. Moderate and negative RAB7 immunoreactivity was observed in 3D hypoxic and normoxic conditions, respectively. RAB31 immunoreactivity was higher in hypoxic conditions than in normoxic conditions.

CONCLUSION: Exosome secretion from AMSCs was affected after culture conditions. Especially hypoxic conditions triggers the secretion of RAB31, and co-localization of CD63 and RAB31 under these conditions points out that exosome biogenesis controls RAB31 in AMSCs under hypoxic conditions.

Keywords: Exosome, biogenesis, RAB31, adipogenic mesenchymal stem cells

To cite this article: Vatansever HS, Kabadayı Ensarioğlu H, Aktaş A, Aydın BK, Kıyak N, Vatansever M. Does RAB31 continue to play a role in exosome biogenesis of adipose-derived mesenchymal stem cells in 2D and 3D culture conditions in a hypoxic environment? Cyprus J Med Sci. 2025;10(Suppl 1):87-91

ORCID IDs of the authors: H.S.V. 0000-0002-7415-9618; H.K.E. 0000-0001-7429-1478; A.A. 0009-0000-4168-5753; B.K.A. 0000-0002-5837-0026; N.K. 0000-0002-7126-2775; M.V. 0009-0005-6155-3076.

Corresponding author: Hafize Seda Vatansever
E-mail: seda.vatansever@cbu.edu.tr/seda.vatansever@neu.edu.tr
ORCID ID: orcid.org/0000-0002-7415-9618

Received: 01.01.2025
Accepted: 03.03.2025
Publication Date: 04.06.2025

INTRODUCTION

Exosomes are nanovesicles with a size of 20-100 nm, are secreted from many cells in the body, and have been isolated from body fluids such as blood, urine, breast milk, amniotic fluids, and bronchial alveolar lavage.[1] Exosomes belong to the group of extracellular vesicles (EVs) along with ectosomes, microvesicles, membrane vesicles, and apoptotic bodies. Although the content of exosomes varies depending on the cells of origin, they can generally be considered vesicles containing proteins, lipids, and nucleic acids.[2] Its structure includes cytoskeleton proteins (actin, tubulin, cofilin, moesin); adhesion molecules tetraspanins (CD9, CD37, CD53, CD63, CD81, CD82); integrins; endosomal sorting complex required for transport (ESCRT) proteins; transport/binding proteins (annexins, galectin, rab family, GTPases); as well as ALIX and TSG101 proteins and heat shock proteins (HSP70, HSP90) that act as markers and participate in exosome biogenesis.[3] Due to the similarity of the exosome membrane with the cell membrane structure, due to its fusion with other cells, and due to its ability to easily pass through many barriers due to its small diameter, research on exosome biogenesis continues.[4-6]

Although the content of exosomes varies depending on the cells of origin, they can generally be considered as vesicles containing proteins, lipids, and nucleic acids.[2,7] Various proteins are involved in exosome biogenesis. Two main pathways have been explained: ESCRT-dependent and independent pathways.[8] There are three Rab-GTPases described in EVs biogenesis and secretion: RAB11, RAB35, and RAB27A.[9,10] Due to the secretion of intraluminal vesicles (ILVs) from ESCRT-depleted cells, when the level of the Rab-related protein in brain (RAB) 31, which is a member of the RAB5 subfamily of Rab GTPases, is higher than RAB7, RAB31 inactivates RAB7, rescuing lysosomal degradation, and therefore ILVs are secreted as exosomes.[11]

Stem cells can be obtained from fetal and adult tissues. They have the potential to proliferate on their own and differentiate in appropriate culture environments. Mesenchymal stem cells (MSCs), which are favored in the field of medicine due to their angiogenic, anti-apoptotic, and immunomodulatory properties, are often used because they can be taken from the person himself/herself. In addition, MSCs do not cause ethical problems in their use even when sourced from different people. MSC-derived EVs retain the biological activity of parental MSCs and demonstrate a similar therapeutic potential.[11-14] Several studies have explored the potential of stem cell-derived exosomes collected under normoxic and hypoxic conditions for advancing regenerative medicine.[14] Hypoxia modulates the secretion, composition, and function of exosomes, especially various cancers.[8] While hypoxia increased exosome release via upregulation of RAB27a and reducing RAB7[9,10] the secretion of RAB31 via hypoxia remains unclear.

We aim to determine the changes in exosome biogenesis and the secretion of RAB31 in the context of the culture of adipogenic mesenchymal stem cells (AMSCs) in 2D and 3D culture conditions under normoxic and hypoxic environments.

MATERIALS AND METHODS

2D and 3D Culture of AMSCs in Hypoxic and Normoxic Conditions

AMSCs were thawed rapidly in a water bath at 37 °C, then transferred to Dulbecco's Modified Eagle Medium culture medium containing 10% exosome-free fetal bovine serum (Capricorn Scientific, FBS-ED-12B), 1% penicillin/streptomycin, and 1% L-glutamine, and centrifuged at 1000 rpm for 5 minutes. After the supernatant is discarded, 5 mL of culture medium is added to the pellet, and it is cultured in a 25 cm^2 flask, with the culture medium changed every 2 days until it reached 80% confluency. AMSCs were then subdivided into four groups: normoxic 2D culture, normoxic 3D culture, hypoxic 2D culture, and hypoxic 3D culture. For 2D conditions, they were cultured in 24-well plates according to standard cell culture protocols. For 3D conditions, matrigel was prepared at a concentration of 5 mg/mL, and 100 µL was added to each well of a 24-well culture plate. After incubating for 30 minutes at 37 °C and 5% CO_2, 30 µL of AMSCs suspension (at a concentration of $5x10^6$ cells/mL) was mixed into 270 µL of Matrigel solution, resulting in a final cell density of $5x10^5$ cells/mL. This mixture was incubated for 30-45 minutes at 37 °C and 5% CO_2. Afterward, 2 mL of culture medium was added to each well, and the cells were cultured. AMSCs were incubated for 48 hours in normoxic (5% CO_2, 95% O_2) and hypoxic (5% CO_2, 5% O_2, 90% N2) conditions. All cell culture studies were performed in three replicates.

Statistical Analysis

Immunocytochemical Analysis

All groups of cells were fixed with 4% paraformaldehyde (Merck, Cat. No: TP70404 415) and distributions of RAB31 (Affinity Biosciences, DF4401), RAB7 (Affinity Biosciences, DF6288), CD9 (Santa Cruz, sc-13118), and CD63 (Santa Cruz, sc-5275) were analyzed using indirect immunoperoxidase staining. For the permeabilization process, the samples were kept in 0.1% Triton-X 100 solution on ice for 10 min. Cells were washed with phosphate buffered saline and then treated with 3% H_2O_2 for 5 min to inhibit tissue endogenous peroxidase. The blocking solution was incubated for 60 min. After removal of the blocking solution, primary antibodies were added overnight. After washing steps, biotin-streptavidin horseradish peroxidase secondary (Thermo Scientific, Cat. No: TP-125-HL) antibodies were applied. Diaminobenzidine (Thermo Scientific, Cat. No: TA-125-HD) was used for chromogen and Mayer's hematoxylin (Atom Scientific Ltd, Cat. No: TTSP60) for background staining. Immunocytochemical staining was performed in three replicates.

RESULTS

Fusiform structures of AMSCs were observed after 2D, and culture under normoxic and hypoxic conditions (Figure 1). The shape of AMSCs after 3D culture was spheroid in both normoxic and hypoxic conditions (Figure 1). Intensities of CD9 and CD63 were similar in both 2D hypoxic and normoxic conditions, while CD9 immunoreactivity was negative, but moderate staining of CD63 was detected (Figure 2). CD9 immunoreactivity was similar in both conditions in 3D culture, and it was very weak (Figure 2). The intensity of CD63 was strong and similar in both 3D hypoxic and normoxic conditions (Figure 2). RAB7 and RAB31 immunoreactivities were higher in the 2D hypoxic conditions than in the 2D normoxic conditions (Figure 2). Moderate and absent RAB7 immunoreactivity was observed in 3D hypoxic and normoxic conditions, respectively, (Figure 2). However, RAB31 immunoreactivity was higher in 3D hypoxic conditions than in normoxic conditions (Figure 2).

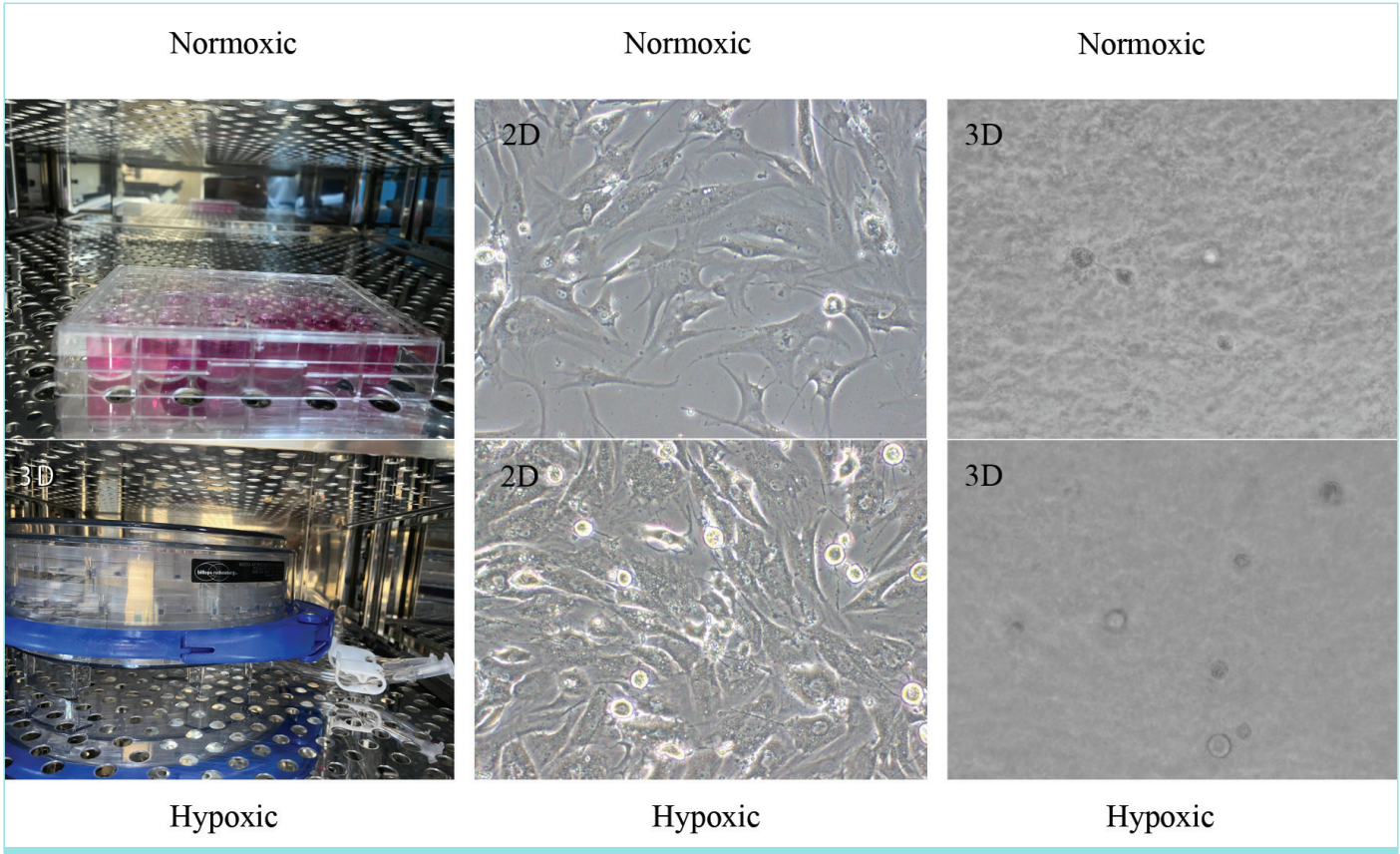

Figure 1. AMSCs were cultured with 10% exosome-free FBS, 1% penicillin/strepromycin, and 1% L-glutamine included DMEM for 48 h in 2D and 3D and normoxic and hypoxic conditions. Magnifications, x400.

AMSCs: Adipogenic mesenchymal stem cells, FBS: Fetal bovine serum, DMEM: Dulbecco's Modified Eagle Medium.

DISCUSSION

The exosome lipid bilayer is highly asymmetrical, which could be particularly advantageous for its interaction with the plasma membrane and especially with their target cells.[3,4] During the biogenesis of exosomes, endocytosis and plasma membrane invagination allow proteins, lipids, metabolites, small molecules, ions, and cell surface proteins to enter cells.[7] Improved understanding of the physical properties of exosomes and the mechanism of their biogenesis is leading to new approaches to increase yield and uniformity of their production. Both stem cells and exosomes have their place in a treatment protocol. Therefore, the EVs of MSCs are potential therapeutic tools, which have advantages over cell therapy in terms of safety, ease of storage/transportation, and clinical use.[11-14] Instead of examining the biogenesis of exosomes, it should be examined how exosome content is affected by environmental changes. In our results, we demonstrated that exosome secretion from AMSCs was affected by culture conditions. In both 2D and 3D conditions, RAB31 secretion was affected in hypoxic conditions. Higher intensity and intracellular co-localization of CD63 and RAB31 were observed. This indicating that hypoxia triggers exosome biogenesis via RAB31.

Study Limitations

During the studies, some proteins were not evaluated by immunocytochemical analysis, therefore, in future investigations, the protein levels will be evaluated by immunoblotting.

CONCLUSION

Understanding the mechanisms of exosome biogenesis and the influence of microenvironmental conditions on their content and secretion is critical to harnessing their full potential. This study highlights the impact of hypoxic conditions on exosome biogenesis in AMSCs, with RAB31 playing a significant role in this process. Modification of stem cell-derived exosomes and/or artificial synthesis of exosomes will be the new therapeutic approaches for treating diseases. It is very important to understand and control exosome secretion or biogenesis under different microenvironments.

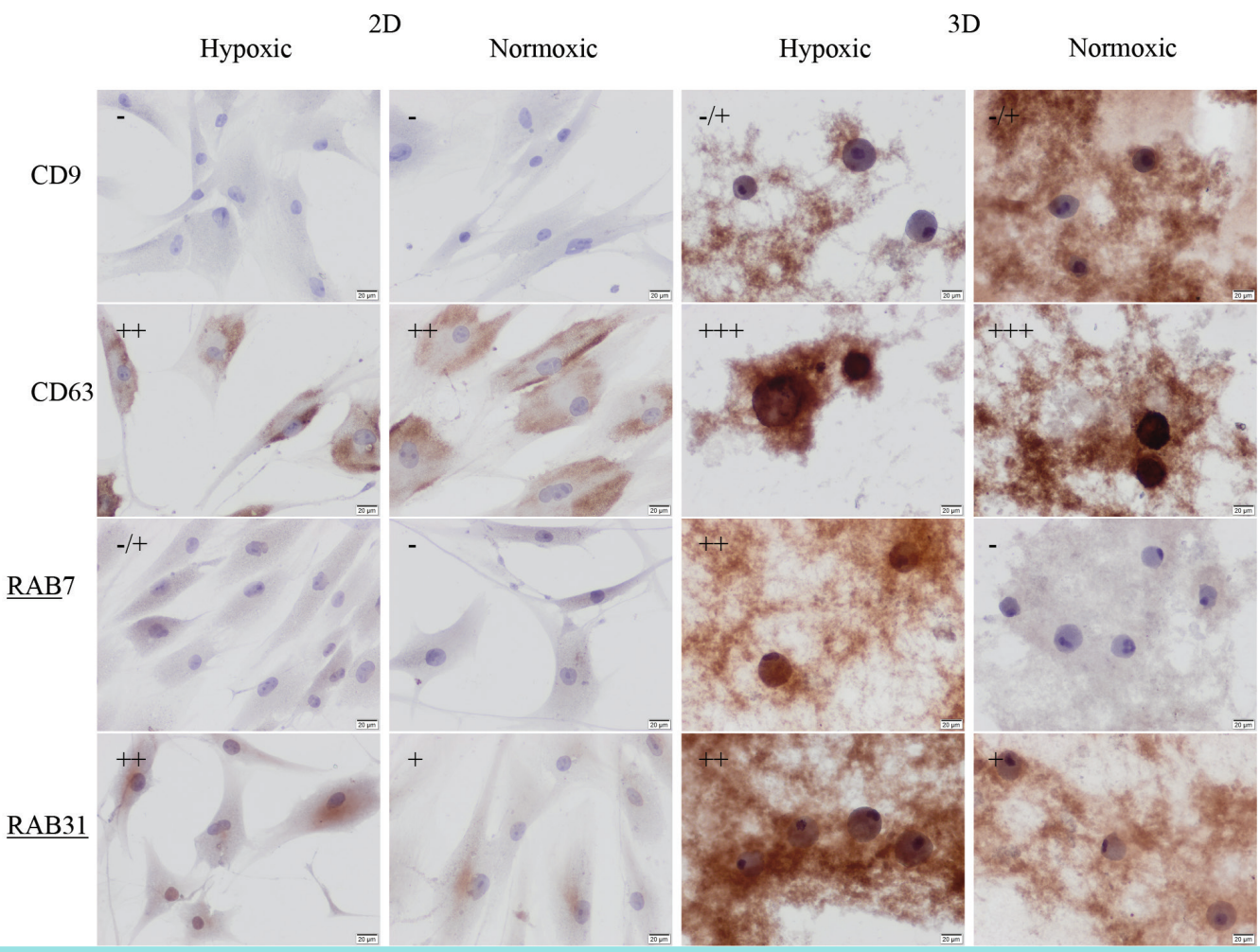

Figure 2. Immunoreactivity of CD9, CD63, RAB7, and RAB31 of AMSCs in 2D and 3D hypoxic and normoxic conditions. Scale bars: 20 µm.

AMSCs: Adipogenic mesenchymal stem cells.

MAIN POINTS

- Exosome secretion from adipogenic mesenchymal stem cells (AMSCs) was affected after culture conditions.
- Hypoxic condition, trigger the secretion of RAB31 from AMSC.
- The biogenesis of exosomes should be evaluated under different culture conditions.

ETHICS

Ethics Committee Approval: Not applicable.

Informed Consent: Not applicable.

Footnotes

Authorship Contributions

Concept: H.S.V., H.K.E., Design: H.S.V., H.K.E., Data Collection and/or Processing: H.S.V., H.K.E., A.A., B.K.A., N.K., M.V., Analysis and/or Interpretation: H.S.V., H.K.E., A.A., B.K.A., N.K., M.V., Literature Search: H.S.V., H.K.E., A.A., B.K.A., N.K., M.V., Writing: H.S.V., H.K.E.

DISCLOSURES

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: This work was supported by Research Fund of Near East University (project number: SAG-2023-1-026).

REFERENCES

1. Choi DS, Kim DK, KimYK, Gho YS. Proteomics, transcriptomics and lipidomics of exosomes and ectosomes. Proteomics. 2013; 13(10-11): 1554-71.
2. Mathieu M, Martin-Jaular L, Lavieu G, Théry C. Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. Nat Cell Biol. 2019; 21(1): 9-17.
3. Kimiz-Gebologlu I, Oncel SS. Exosomes: large-scale production, isolation, drug loading efficiency, and biodistribution and uptake. J Control Release. 2022; 347; 533-43.
4. Du S, Guan Y, Xie A, Yan Z, Gao S, Li W, et al. Extracellular vesicles: a rising star for therapeutics and drug delivery. J Nanobiotechnology. 2023; 21(1): 231.
5. Wang X, Huang J, Chen W, Li G, Li Z, Lei J. The updated role of exosomal proteins in the diagnosis, prognosis, and treatment of cancer. Exp Mol Med. 2022; 54: 1390-400.

6. Gurunathan S, Kang MH, Jeyaraj M, Qasim M, Kim JH. Review of the isolation, characterization, biological function, and multifarious therapeutic approaches of exosomes. Cells. 2019; 8(4): 307.

7. Skotland T, Hessvik NP, Sandvig K, Llorente A. Exosomal lipid composition and the role of ether lipids and phosphoinositides in exosome biology. J Lipid Res. 2019; 60(1): 9-18.

8. Han QF, Li WJ, Hu KS, Gao J, Zhai WL, Yang JH, et al. Exosome biogenesis: machinery, regulation, and therapeutic implications in cancer. Mol Cancer. 2022; 21(1): 207.

9. Wei D, Zhan W, Gao Y, Huang L, Gong R, Wang W, et al. RAB31 marks and controls an ESCRT-independent exosome pathway. Cell Res. 2021; 31(2): 157-77.

10. Dorayappan KDP, Wanner R, Wallbillich JJ, Saini U, Zingarelli R, Suarez AA, et al. Hypoxia-induced exosomes contribute to a more aggressive and chemoresistant ovarian cancer phenotype: a novel mechanism linking STAT3/Rab proteins. Oncogene. 2018; 37(28): 3806-21.

11. Qiu G, Zheng G, Ge M, Wang J, Huang R, Shu Q, et al. Functional proteins of mesenchymal stem cell-derived extracellular vesicles. Stem Cell Res Ther. 2019; 10(1): 359.

12. Hade MD, Suire CN, Suo Z. Mesenchymal stem cell-derived exosomes: applications in regenerative medicine. Cells. 2021; 10(8): 1959.

13. Gomzikova MO, James V, Rizvanov AA. Therapeutic application of mesenchymal stem cells derived extracellular vesicles for immunomodulation. Front. Immunol. 2019; 10: 2663.

14. Braga CL, da Silva LR, Santos RT, de Carvalho LRP, Mandacaru SC, de Oliveira Trugilho MR, et al. Proteomics profile of mesenchymal stromal cells and extracellular vesicles in normoxic and hypoxic conditions. Cytotherapy. 2022; 24(12): 1211-24.