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HYPOXIC PRECONDITIONING OF ADIPOSE-DERIVED MESENCHYMAL STEM CELLS UPREGULATES EXPRESSION OF EXTRACELLULAR MATRIX PROTEINS

Zahra Rashidbenam[1], Mohd Hafidzul Jasman[2], Reynu Rajan[3], Fatimah Mohd Nor[4], Guan Hee Tan[2], Eng Hong Goh[2], Xeng Inn Fam[2], Christopher Chee Kong Ho[5], Mohamad Aznan Shuhaili[3], Nik Ritza Kosai[3], Zulkifli Md Zainuddin[2], Min Hwei Ng*[1]

[1]Tissue Engineering Centre, 12th Floor, Clinical Block, University Kebaangsaan Malaysia Medical Centre, Jalan Yaa`cob Latiff, Bandar Tun Razak, 56000, Cheras, Kuala Lumpur, Malaysia.
[2]Urology Unit, Department of Surgery, 8th Floor, Clinical Block University Kebangsaan Malaysia Medical Centre, Jalan Yaa`cob Latif, Bandar Tun Razak, 56000, Cheras, Kuala Lumpur, Malaysia.
[3]Minimally Invasive, Upper Gastrointestinal and Bariatric Surgery Unit, Department of Surgery, 8th Floor, Clinical Block University Kebangsaan Malaysia Medical Centre, Jalan Yaa`cob Latif, Bandar Tun Razak, 56000, Cheras, Kuala Lumpur, Malaysia.
[4]Plastic and Reconstructive Surgery Unit, Department of Surgery, 8th Floor, Clinical Block University Kebangsaan Malaysia Medical Centre, Jalan Yaa`cob Latif, Bandar Tun Razak, 56000, Cheras, Kuala Lumpur, Malaysia.
[5]School of Medicine, Taylor`s University, No. 1 Jalan Taylor's 47500 Subang Jaya, Selangor Daru Ehsan Malaysia.

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*Corresponding author
Min Hwei Ng
email:
angelaster3@gmail.com

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SUMMARY

Stem cell derivatives are vastly studied for their therapeutic effects. In this study, adipose-derived stem cells conditioned medium (ASC-CM) were collected under normoxic (21%O_2) and hypoxic conditions (1%O_2) and analyzed. It was found that protein concentration and number of detected proteins in CM derived from hypoxic ASCs were higher compared to that in normoxic condition. Among detected proteins, 59 were unique to hypoxic and 17 proteins that were originally present in normoxic condition, were absent in hypoxic CM. Interesting a number of these unique proteins are associated with the extracellular matrix (ECM) components such as collagen, laminin and vitronectin, suggesting that low O_2 tension can be applied in improving ECM produced by ASCs.

1.0 Introduction

Stem cells are immature cells, which have the ability to self-renew and are able to differentiate towards multiple cell lineages. Owning to these characteristics, stem cells are vastly studied for their application in tissue regenerations and engraftment. Stem cells exert their therapeutic effects via three key mechanisms of action including homing to the site of acute injury, differentiation towards multiple cell types and secretion of bioactive factors. Studies focusing on bioactive compounds in stem cell secretome and their role in regulation of key biological processes came into focus since it was shown that implanted stem cells do not survive for so long, therefore their therapeutic effects mostly rely on bioactive factors produced by them [1]. It is believed that physiological preconditioning (culturing under low O_2 condition) of mesenchymal stem cells (MSCs) can increase their stemness [2] and can improve the therapeutic effects of their secretome [3]. Prolonged exposure of adipose-derived stem cells (ASCs) into low oxygen tension increases the angiogenic properties of ASC-derived conditioned medium (ASC-CM), which in turn can make it an ideal option to be used for cell free therapies [4]. Recently, it was showed that fibroblast growth factor-10 released by hypoxia activated ASCs (ASCs cultured in 1%O_2) has antiapoptotic effect on irradiation-induced salivary hypofunction, which is resulted from radiation therapy for treating head and neck cancer [5]. Pezhman et al. [6] showed that preconditioning of human cardiomyocyte in 1% O_2 level

for 3 hours results in positive expression of hypoxic-inducible factor-1α (HIF-1α) in 100% of exposed cells [6]. HIF-1 is a marker, which is expressed in hypoxic cells. Knowing the fact that exposing ASCs into low O_2 condition promotes their secretome therapeutic properties, this study applying the same method as pezhman et al. [6], aimed to screen and profile all components present in CM derived from ASCs growing under normoxic (21% O_2) and hypoxic (1%O_2) conditions.

2.0 Materials and Method

2.1 Sample Collection and Cell Isolation

This study was approved by Universiti Kebangsaan Malaysia Medical Research and Ethics Committee (FF-2017-227). Human adipose tissue was collected from an individual undergoing abdominoplasty in Hospital Universiti Kebangsaan Malaysia, Faculty of Medicine, Universiti Kebangsaan Malaysia, after obtaining patient`s written consent. Adipose tissue was washed with Dulbecco`s phosphate buffered saline and was digested with 0.06% collagenase type I. Isolated stem cells then were cultured in Dulbecco`s Modified Eagle`s Medium/Nutrient Mixture F-12 Ham (DMEM-F12) containing 10% (v/v) fetal bovine serum (FBS), 1% glutamax and 1% antibiotic-antimycotic mixture.

2.2 Collection of Conditioned Medium

ASCs at passage 2 were used for collecting CM. Once cells reached to 80% of confluence, the media were removed and cells were washed with DPBS. For normoxic condition, medium without FBS were added into the cells. ASCs were incubated in 5% CO_2 humidified incubator at 37°C and after 72 hours CM was collected. For hypoxic condition, medium without FBS, (medium was incubated in hypoxic condition 24 hours prior being added into ASCs) was added into the cells. ASCs were incubated in 1% O_2, 5% CO_2 humidified incubator at 37°C and after 3 hours, they were transferred into normoxic condition. At 72 hours, CM was collected.

2.3 Concentration of conditioned medium

To collect the proteins > 3KDa, CM was concentrated using Amicon ultra-15 centrifugal filter device and was centrifuged at 5000 × g at 25°C for 40 minutes in a fixed angel rotor.

2.4 Desalting Conditioned Medium

For desalting CM, 1kDa mini dialysis tube with a cap fitted with a dialysis membrane, was used. The capped tubes containing CM were inverted in a beaker containing DPBS and stored at 4°C overnight. The day after, mini dialysis tubes floating in DPBS were stirred at 2 rpm, at room temperature (RT) on a magnetic stirrer for 2 hours.

2.5 Protein Quantification

To measure concentration of protein in CM, Bicinchoninic acid assay (BCA assay; Singma-Aldrich, USA) was conducted according to manufacturer`s construction. Protein standard was prepared by serial dilution of bovine serum albumin (BSA; Sigma-Aldrich, USA). Protein concentration was measured by reading the absorbance at 562 nm. According to the plotted standard curve using BSA protein, the concentration of protein in the CM was calculated.

2.5 Protein Identification by Liquid Chromatography Mass Spectrometry (LCMS) Analysis

Protein sample was mixed with ammonium bicarbonate (NH_4HCO_3), followed by treating with 0.05% RapiGest ™SF. Protein was concentrated using 3K molecular weight cut-off Vivaspin column. Further protein processing and LCMS were performed by Malaysian Genome Institute, National Institutes of Biotechnology Malaysia. Data analysis was done using Thermo Scientific™ Proteom Discoverer™ software, version 2.1 with reference to *Homo sapiens* database. For classifying cellular components present in CM, PANTHER classification system was used.

3.0 Results

Culturing ASCs under hypoxic condition increased secretion of protein (640 µl/ml) in CM relative to that of normoxic condition (461 µl/ml) (**Figure 1**). Among total of 212 detected proteins present in CM (**Figure 2**), 136 proteins were common in between both normoxic and hypoxic conditions. 59 proteins were unique to hypoxic and 17 proteins that were originally present in normoxic condition, were absent in hypoxic CM. Using PANTHER Classification System, it was found that 3 groups of cellular components including extracellular matrix, extracellular region and membrane were uniquely found in CM derived from hypoxic ASCs. Interestingly, a number of these unique proteins are associated with the extracellular matrix components such as collagen, laminin and vitronectin.

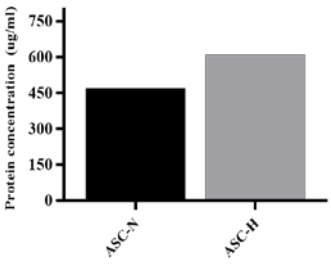

Figure 1. Concentration of protein in CM. Concentration of protein in secretome measured by BCA assay shows higher concentration of protein in ASC-hypoxic (ASC-H) compared to that of ASC-normoxic (ASC-N) derived CM.

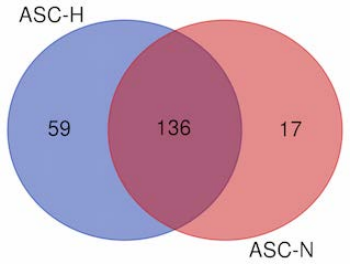

Figure 2. Number of detected common and unique proteins in ASC-derived CM under normoxic and hypoxic conditions. The Venn diagram displays detection of 153 and 195 number of proteins in ASC-derived CM under normoxic and hypoxic conditions, respectively.

4.0 Discussion & Conclusion

Preconditioning of MSCs in hypoxic condition improves the therapeutic properties of their secretomes.

Our study shows that culturing ASCs under stress condition alters gene expression and ultimately activates different biological pathways. This preliminary analysis suggests that preconditioning of ASCs with low O_2 level, increases extracellular matrix associated protein production by ASCs. Confirmation of the proteins and the determination of the relative levels of unique protein concentration will be performed via Western Blot. Further studies to investigate the role of these unique proteins during hypoxia and their associated pathways need to be investigated.

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