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Role of extracellular vesicles derived by human gingival mesenchymal stem cells in cardiomyocytes acute hypoxia

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Abstract. Hypoxia has an impact on pathological conditions of different tissues and especially on the heart where it can have different consequences depending on the duration of exposure to the hypoxic state. Acute hypoxic exposure can result in reversible acclimatization in heart tissue, maintaining a good systemic oxygen supply, while chronic hypoxic exposure leads to tissue damage exacerbating hypoxia-induced cardiac dysfunction. Extracellular vesicles (EVs) are small membrane vesicles, of the order of nanometers, secreted by different cell types. EVs are mediators of intercellular communication in both physiological and pathological conditions. EVs produced by oral-cavity-derived Mesenchymal Stem Cells (MSCs), including human gingival mesenchymal stem cells, have pro-angiogenic and anti-inflammatory effects. For this reason, the EVs can be identify as a new therapeutic potential for tissue regeneration. The aim of the present work was to evaluate the effect of treatment with EVs produced by human gingival mesenchymal stem cells (hGMSCs) on an in vitro model of HL-1 cardiomyocytes cultured under acute hypoxia state (0,2% hypoxia) followed by normoxia conditions. The HIF-1 α expression was downregulate with EVs treatment. EVs could represent an innovative platform to prevent the hypoxic damages.

Keywords: extracellular vesicles, human gingival mesenchymal stem cells, cardiomyocytes, acute hypoxia.

INTRODUCTION

The heart is one of the tissues most affected by variations in oxygen levels and therefore in the hypoxic state, mainly due to phenomena such as nitric oxide (NO) and reactive oxygen species (ROS) formation and increased superoxide formation through NADPH, which lead respectively to an alteration of cardiac contractility, mitochondrial damage, and formation of atherosclerosis [1-3]. Therefore hypoxia-inducible factor (HIF), key molecule of the

hypoxic pathway activation [4], has an impact on several cardiac phenotypes including heart failure [5].

A mild exposure to hypoxia can provoke a reversible physiological acclimatization in the heart tissue as happens following an acute and systemic hypoxic exposure in which there is an immediate increase in heart rate and lung function maintaining a good supply of systemic oxygen, while the prolonged exposure to hypoxia (chronic hypoxia) leads to tissue damage [6,7]. Furthermore, oxygenation after acute or prolonged exposure has been shown to cause oxidative damage exacerbating hypoxia-induced cardiac dysfunction [8].

Extracellular vesicles (EVs) are nanometer-sized vesicles containing lipid, proteins and different types of nucleic acids, enclosed by lipid membranes. These are secreted by different cell types and act as important mediators of intercellular communication in both physiological and pathological conditions [9,10]. EVs isolated from oral-cavity-derived Mesenchymal Stem Cells (MSCs) have been shown to carry pro-angiogenic and anti-inflammatory factors showing themselves as therapeutic potential for tissue regeneration [11,12].

The aim of the present work is to evaluate the effect of EVs produced by human gingival mesenchymal stem cells (hGMSCs) on an in vitro model of HL-1 cardiomyocytes cultured under 0.2% hypoxia (acute hypoxia) followed by normoxia evaluating the HIF-1 α expression.

MATERIALS AND METHOD

Hypoxic culture

The HL-1 cells (Sigma-Aldrich, Milan, Italy) were cultured in Claycomb medium completed with 10% fetal bovine serum (Euroclone, Milan, Italy), 2 mM l-glutamine, 0,1 mM norepinephrine, and 100 μ g/mL penicillin/streptomycin (Lonza, Basel, Switzerland) under hypoxia 0,2% for 24h and then in normoxia at 37 °C in a humidified atmosphere of 5% of CO₂ in air for 24h. The hypoxia state was performed using ProOx Model P110 (BioSpherix, 25 Union Street, Parish, NY 13131) hypoxia chamber, and following the referred Manual.

EVs isolation

EVs were isolated starting from supernatants (10mL) of hGMSCs culture after 48 hours of culture using ExoQuick-TC (System Biosciences, Euroclone SpA, Milan, Italy) following the manufacturer's protocol. The mixture was placed at 4 °C overnight and the following day was centrifuged 1500 \times g for 30 minutes

to settle the EVs and the pellets were resuspended in 200 μ L of PBS [13].

Experimental study design

The study design is reported as follows:

- (i) HL-1, used as a negative control (CTRL), were kept in hypoxia at 0,2% for 24 h and successively in normoxia for 24 h;
- (ii) HL-1 were kept in hypoxia at 0,2% for 24 h and treated with hGMSCs EVs in normoxia for successively 24 h (EVs Post-hypoxia);
- (iii) HL-1 treated with with hGMSCs EVs in hypoxia at 0,2% for 24 h and then in normoxia for successively 24 h (EVs Pre-hypoxia).

Confocal microscopy analysis

The HL-1 cells were seeded at 8500/well on 8-well culture glass slides (Corning, Glendale, Arizona, USA), under hypoxia state and treated with hGMSCs EVs as described in ii) and iii). The Confocal Microscopy analysis was performed as previously described [14] using HIF-1 α as primary mouse monoclonal antibody (1:200; Santa Cruz Biotechnology) and Alexa Fluor 568 red fluorescence conjugated goat anti-mouse antibody (1:200; Molecular Probes, Invitrogen, Eugene, OR, United States) as secondary antibody. The microscope used is Zeiss LSM800 confocal system (Zeiss, Jena, Germany).

Western Blotting Analysis

The proteins derived from cell cultures of the three experimental points were used at the concentration of 50 μ g for the electrophoresis and subsequent transfer on the membrane of polyvinyl-denifluoride (PVDF) as previously described [15]. The primary monoclonal antibody used (mouse, 1:500, Santa Cruz Biotechnology) is HIF-1 α . β -actin has been used as housekeeping protein. The ECL method was used to visualize the bands with an image documenter Alliance 2.7 (Uvitec, Cambridge, UK). The data were normalized with β -actin.

RESULTS

Under the light microscope, the cells show the typical morphology of the HL-1 mouse heart muscle cell line (Figure 1).

The immunofluorescence showed that the levels of HIF-1 α was significantly downregulated in HL-1 EVs

HL-1

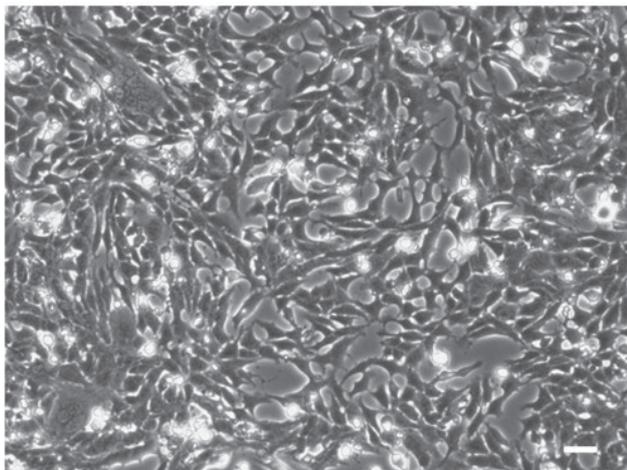


Figure 1. HL-1 Morphological features. Representative image of HL-1 maintained under normoxia conditions observed under inverted light microscopy. Scale bar: 20 μ m.

Post-hypoxia and EVs Pre-hypoxia groups.

On the other hand, HL-1 CTRL group HIF-1 α showed an high expression levels when compared to the other sample groups (Figure 2). The Western blot analysis confirmed the obtained results (Figure 3).

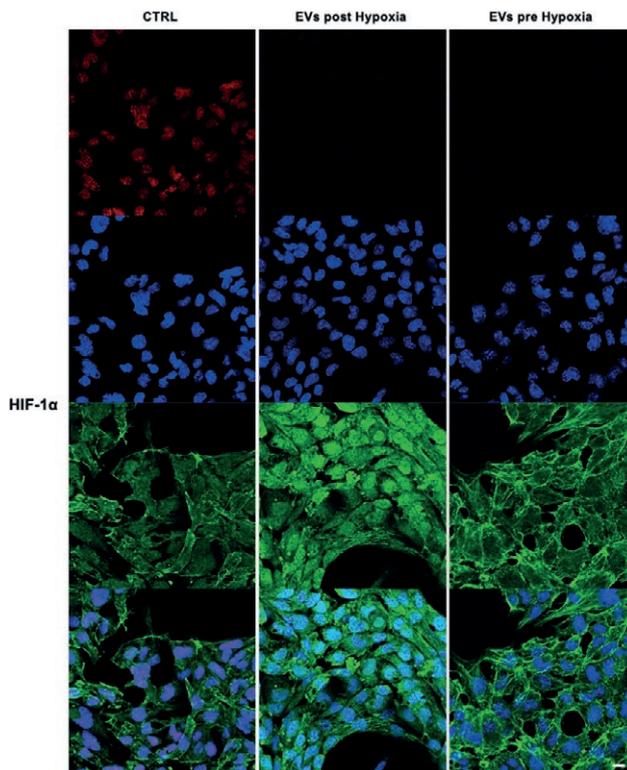


Figure 2. HIF-1 α expression in HL-1 at Confocal Microscopy in CTRL, post hypoxia and pre hypoxia. HIF-1 α is highly expressed in the CTRL while it decreases with EVs treatment both in post Hypoxia and pre hypoxia. Scale bar: 20 μ m.

DISCUSSION

The principal mechanism of response to hypoxia is that involves hypoxia inducible factors (HIFs)[16]. HIF-1 α proteins is continuously synthesized, but under normoxic

conditions they are rapidly degraded by the ubiquitin-proteasome system [17]. HIF-1 α is stabilized by low oxygen availability controlling the expression of a multitude of genes involved in different processes like angiogenesis,

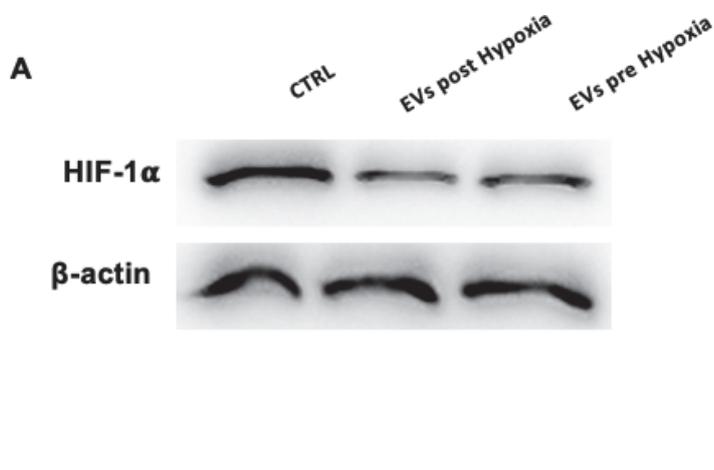


Figure 3. HIF-1 α protein expression in HL-1 in CTRL, post hypoxia and pre hypoxia. (A) HIF-1 α specific band protein expression. (B) Densitometric analysis of HIF-1 α normalized with β -actin. *** p <0.001; * p <0.05.

cell survival, metabolism, and metastasis [18]. Hypoxia, in which HIF-1 α is stabilized, can involve the heart following various pathological conditions leading to cardiomyopathies [19]. Even in vivo, an increase in HIF-1 α protein levels was detected in heart samples from patients with cardiomyopathy, indicating that the HIF pathway is activated during the progression of the disease [20]. The results obtained show that HIF-1 α protein levels decrease with EVs treatment both in post-hypoxia and pre-hypoxia conditions. These data indicate a protective effect against hypoxia from EVs at this molecular level.

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