**Extracellular vesicles for lung regeneration in alpha-1-antitrypsin deficiency**

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In the last years, several studies suggested that mesenchymal stem/stromal cells (MSCs) act as Medicinal Products, via the secretion of soluble proteins and nano/microstructured extracellular vesicles (EVs), collectively known as secretome [1]. Recently, we defined a scalable GMP-compliant production process for MSC freeze-dried secretome (lyo-secretome) [2]: proteomic investigation revealed that this product contains alpha-1-antitrypsin (AAT). AAT is a 52 kDa single-chain glycoprotein abundantly produced in the liver by hepatocytes and, once released into the blood circulation, it enters the tissues, especially in the lungs, and protects them from being damaged by proteolytic enzymes, such as trypsin, elastase, and protease-3. In Europe, it is estimated that about 1 in 2000 people have AAT deficiency, an inherited genetic disorder, totalling about 370,000 people with low levels of AAT in plasma. The reduced level of AAT induces an excess of protease activity, that leads to the damage and destruction of the pulmonary tissue, predisposing it to a greater risk of bacterial infections and triggering the inflammatory response. Given these premises, here we investigate the potential employment of MSC-secretome in the treatment of AAT deficiency-associated lung diseases.

MSC-secretome release was obtained by platelet lysate starvation (ST) or by ST and dexamethasone (DEX) and/or IL-1β stimulation. Supernatants were purified by ultrafiltration, added with mannitol and freeze-dried. Expression of SERPINA1 transcripts in MSCs was evaluated by RT-PCR. MSC-secretome was fractioned to separate the EV-enriched fraction (EV, >300 kDa), the low molecular weight protein fraction (LMW, 5-100 kDa) and the high molecular weight protein fraction (HMW, 100-300 kDa). Protein content was determined by the BCA Protein Assay Kit, AAT was measured by a rate immune nephelometric method, and proteomic analysis was performed by LC-MS/MS. Elastase inhibition activity was assessed spectrophotometrically and calculated as Abscontrol-Abssample/Abscontrol x 100. Logarithmic lowering of the bacterial population was calculated against Gram-positive (*S.aureus, S. aureus* MRSA) and Gram-negative (*K. pneumoniae, Ps. aeruginosa*) bacteria. Immunomodulation potency was assessed as ability of lyo-secretome to modulate cytokines (IFN-γ, IL-10 and IL-6) produced by Peripheral Blood Mononuclear Cells (PBMCs) stimulated with phytohemagglutinin. The response was quantified after 3-days incubation, as cytokine amount (ELISA quantification), and as a function of MSCs (as a control) or the cell-equivalent mg of lyo-secretome. Results are reported as mean ± standard deviation, n=3.

Proteomic investigation revealed that MSC-secretome contains AAT, 72 other proteins involved in protease/antiprotease balance and 46 proteins involved in the response to bacteria [3]. Treatment with DEX and/or IL-1β increased MSC-secretome total protein content, but not AAT. The expression of AAT transcripts increased after ST and after IL-1β+DEX treatment. Secretome fraction separation revealed that the AAT content, expressed as µg/mg of freeze-dried sample, was 1.37 ± 0.25 for EV fraction, 1.38 ± 0.64 for LMW fraction and 0.16 ± 0.05 for HMW fraction. The EV fraction showed the highest anti-elastase activity (29.80 % ± 4.336, at 100 mg/ml) with respect to protein fractions. All the batches exhibited a good dose-dependent anti-elastase activity: at the highest dose (100 mg/ml), the inhibition rates were 46.17% ± 13.546, 39.97% ± 9.609, 41.77% ± 10.446 and 32.74% ± 15.196, for ST, DEX, IL-1β and IL-1β+DEX stimulated secretome, respectively. Lyo-secretome showed antimicrobial activity on Gram-negative bacteria, especially for *K. pneumoniae*, and modulated the cytokine production of phytohemagglutinin-activated PBMCs. In conclusion, we proved that MSC-secretome contains AAT with high anti-elastase *in vitro* activity. AAT is present both in the soluble fraction and associated with EVs, that can act as a natural carrier, promoting AAT *in vivo* stability and activity. Finally, lyo-secretome was active on Gram-negative bacteria and showed immunomodulatory properties. These results pave the way for the use of MSC-secretome in the treatment of AAT-deficiency lung diseases.

**References:**

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