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Combination of urea-crosslinked hyaluronic acid and sodium ascorbyl phosphate for the treatment of inflammatory lung diseases: An *in vitro* study

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ABSTRACT

This *in vitro* study evaluated, for the first time, the safety and the biological activity of a novel urea-crosslinked hyaluronic acid component and sodium ascorbyl phosphate (HA-CL – SAP), singularly and/or in combination, intended for the treatment of inflammatory lung diseases. The aim was to understand if the combination HA-CL – SAP had an enhanced activity with respect to the combination native hyaluronic acid (HA) – SAP and the single SAP, HA and HA-CL components. Sample solutions displayed pH, osmolality and viscosity values suitable for lung delivery and showed to be not toxic on epithelial Calu-3 cells at the concentrations used in this study. The HA-CL – SAP displayed the most significant reduction in interleukin-6 (IL-6) and reactive oxygen species (ROS) levels, due to the combined action of HA-CL and SAP. Moreover, this combination showed improved cellular healing (wound closure) with respect to HA – SAP, SAP and HA, although at a lower rate than HA-CL alone. These preliminary results showed that the combination HA-CL - SAP could be suitable to reduce inflammation and oxidative stress in lung disorders like acute respiratory distress syndrome, asthma, emphysema and chronic obstructive pulmonary disease, where inflammation is prominent.

1. Introduction

The airway epithelium is directly exposed to the external environment and, consequently, it is strongly responsive to exogenous toxic substances like cigarette smoke, biophysical and biological stresses. All these factors, in combination with genetic predisposition and age, are involved in the pathogenesis of bronchopulmonary diseases. Respiratory pathologies - like asthma, chronic obstructive pulmonary disease (COPD), emphysema, acute respiratory distress syndrome (ARDS) and cystic fibrosis - affect millions of people every year and are amongst the main causes of mortality (Burney et al., 2015; Crotty Alexander et al., 2015; Ferkol and Schraufnagel, 2014; World Health Organization, 2017; Yang et al., 2017). Furthermore, patients require more effective treatments, as numerous of them will become non-responsive to the conventional corticosteroid therapy (Barnes, 2013; Jiang and Zhu, 2016). Considering that inflammation and oxidative stress represent the principal mechanisms underlying the development and progression of many lung diseases (Kleniewska and Pawliczak, 2017; MacNee, 2001; Moldoveanu et al., 2009; Nichols and Chmiel, 2015; Oudijk et al., 2003), the use of anti-inflammatory and antioxidant compounds as adjunctive therapy could be a promising approach to restore and maintain normal lung functions, possibly by reducing drug resistant refractory phenomena. To this end, several recent researches have investigated the therapeutic value of naturally occurring biomolecules –such as vitamins, hyaluronan, polyphenols, and herbal active compounds - and of their derivatives (Bharara et al., 2016; Garantziotis et al., 2016; Li and Li, 2016; Park et al., 2016; Pincikova et al., 2017; Yeo et al., 2017; Zemmouri et al., 2017).

Vitamin C (*i.e.* ascorbic acid) is a physiological low-molecular weight antioxidant. In the lung, it is able to regulate the innate immune

Abbreviations: ALI, air-liquid interface; ARDS, acute respiratory distress syndrome; COPD, chronic obstructive pulmonary disease; DCF, dichlorofluorescin; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; ELISA, enzyme-linked immuno assay; FBS, foetal bovine serum; H_2O_2, hydrogen peroxide; HA, hyaluronic acid; HA-CL, urea-crosslinked hyaluronic acid; IC50, half maximal inhibitory concentration; IL-6, interleukin-6; IL-8, interleukin-8; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MTS, methyl tetrazolium salt; PBS, phosphate buffer saline; ROS, reactive oxygen species; SAP, sodium ascorbyl phosphate; TEER, transepithelial electrical resistance; η_0, zero-shear rate viscosity

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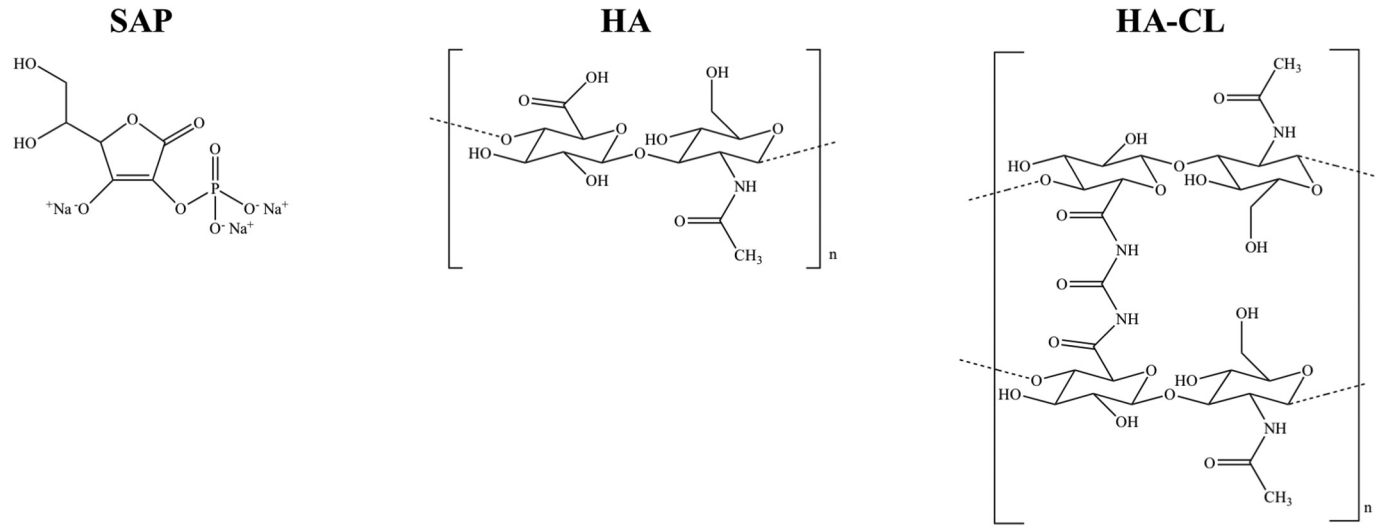

Fig. 1. Chemical structures of SAP, HA and HA-CL.

system, maintain host defence and function of the airway epithelial barrier (Li and Li, 2016). Indeed, vitamin C reduces the oxidative stress provoked by inhaled pollutants or irritants, thus limiting cellular damage induced by inflammatory-derived oxidants at the air-lung interface (Larsson et al., 2015). Moreover, vitamin C supplementation reduces acute lung inflammatory response, thus exhibiting a potential preventive and therapeutic role (Silva Bezerra et al., 2006). High doses of intravenous vitamin C with antioxidant and anti-inflammatory properties have been shown to be efficient as adjunctive therapy for recurrent ARDS (Bharara et al., 2016). Although the use of vitamin C to treat pulmonary impairments is still investigational, many studies have suggested its benefits against lung infections (Hemilä and Louhiala, 2007), COPD (Pirabbasi et al., 2016), asthma attacks, bronchial hypersensitivity (Hemilä, 2013) and smoke-induced pulmonary emphysema (Koike et al., 2014).

Sodium ascorbyl phosphate (SAP, Fig. 1) is a salt form of ascorbic acid 2-phosphate, and it is a hydrophilic derivative of vitamin C characterized by improved physico-chemical stability. SAP has already shown important antioxidant properties for dermal application (Klock et al., 2005; Spiclin et al., 2003). Additionally, even if to a lesser extend in comparison to magnesium ascorbyl phosphate (another salt of ascorbic acid 2-phosphate), SAP has been shown to stimulate collagen synthesis in cultures of human dermal fibroblasts (Geesin et al., 1993). Hence, SAP may be involved in wound healing processes like magnesium ascorbyl phosphate, which was found to be able not only to promote collagen synthesis (Amirlak et al., 2016; Geesin et al., 1993), but also to increase cell motility and fibroblast proliferation during skin reparation (Duarte et al., 2009; Stumpf et al., 2011).

Hyaluronic acid (HA, Fig. 1) is a biocompatible, biodegradable, visco-elastic and mucoadhesive glycosaminoglycan consisting of repeating dimeric units of D-glucuronic acid and *N*-acetyl-D-glucosamine (Fallacara et al., 2017a; Kakehi et al., 2003; Liao et al., 2005; Mayol et al., 2008; Toole, 2004). In humans, HA is ubiquitous, and in the lung its total content under physiological condition is approximately 160 mg (Turino and Cantor, 2003). Specifically, in the lung, the high-molecular-weight of HA exerts anti-inflammatory and anti-angiogenic actions, promotes cell survival, organizes extracellular matrix, stabilizes connective tissues, regulates hydration and water homeostasis (Garantziotis et al., 2016; Gerdin and Hällgren, 1997). Due to all these physico-chemical and biological properties, exogenous HA represents a promising multifunctional agent for the treatment of lung diseases, as it can be used as a drug carrier with intrinsic therapeutic potential (Garantziotis et al., 2016; Li et al., 2017; Martinelli et al., 2017; Surendrakumar et al., 2003). Emerging studies support the use of high-molecular-weight (> 1 MDa) HA to treat airway diseases whose pathogenesis involves inflammation, oxidative stress and epithelial remodelling (Allegra et al., 2008; Buonpensiero et al., 2010; Cantor et al., 2005, 2011; Furnari et al., 2012; Garantziotis et al., 2016; Gavina et al., 2013; Jiang et al., 2005, 2010; Lennon and Singleton, 2011; Maiz Carro et al., 2012; Petrigni and Allegra, 2006; Savani et al., 2001; Souza-Fernandes et al., 2006; Venge et al., 1996). Two inhalation products containing HA as active ingredient are currently marketed: Hyaneb® (Chiesi Farmaceutici, IT) and Yabro® (Ibsa Farmaceutici, IT). Hyaneb® is a hypertonic saline solution containing HA (molecular-weight 0.5 MDa, 0.1% w/v) to hydrate and consequently, reduce mucus viscosity in cystic fibrosis patients (Nenna et al., 2011). Yabro®, on the other hand, is a high viscosity nebuliser solution of hyaluronan (molecular-weight 0.8–1 MDa, 0.3% w/v) to treat bronchial hyper-reactivity due to irritants inhalation or physical stress (Gelardi et al., 2013).

To improve hyaluronan activity and potential for its use as therapeutic agent, several derivatives have been synthesized (Williams et al., 2017). In this study, a novel urea-crosslinked hyaluronic acid (HA-CL, Fig. 1) was investigated for pulmonary application. HA-CL is a patented (Citernesi et al., 2015, WO/2015/007773 A1) biocompatible and biodegradable polymer, with enhanced consistency in comparison to native HA (Citernesi et al., 2015, WO/2015/007773 A1; Fallacara et al., 2017a), due to its crosslinking with urea, a molecule naturally occurring and therapeutically employed as hydrating and re-epithelializing agent (Albèr et al., 2013; Charlton et al., 1996; Pan et al., 2013).

In this context, the focus of the present *in vitro* bio-investigation was to evaluate the cytotoxicity and the bioactivity - anti-inflammatory, antioxidant and wound healing properties - of these components, vitamin C and hyaluronic acid (HA) derivatives, used singularly and in combination, on Calu-3 lung carcinoma derived epithelia cells.

2. Materials and methods

2.1. Materials

Native hyaluronic acid (HA, *i.e.* sodium hyaluronate, molecular weight 1.2 MDa) and urea-crosslinked hyaluronic acid (HA-CL, molecular weight 2.0–4.0 MDa – raw material containing also pentylene glycol) were kindly donated by IRAlab (Usmate Velate, Monza-Brianza, Italy) and used as supplied. Sodium ascorbyl phosphate (SAP) was purchased from DSM Nutritional Products Ltd. (Segrate, Milano, Italy). Calu-3 cells were supplied by American Type Cell Culture Collection (ATTC, Rockville, USA). Transwell® polyester cell inserts (0.33 cm^2