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Research paper

Study of the stability of packaging and storage conditions of human mesenchymal stem cell for intra-arterial clinical application in patient with critical limb ischemia

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ABSTRACT

Critical limb ischemia (CLI) is associated with significant morbidity and mortality. In this study, we developed and characterized an intra-arterial cell suspension containing human mesenchymal stem cells (hMSCs) for the treatment of CLI. Equally, the stability of cells was studied in order to evaluate the optimal conditions of storage that guarantee the viability from cell processing to the administration phase. Effects of various factors, including excipients, storage temperature and time were evaluated to analyze the survival of hMSCs in the finished medicinal product. The viability of hMSCs in different packaging media was studied for 60 h at 4 °C. The best medium to maintain hMSCs viability was then selected to test storage conditions (4, 8, 25 and 37 °C; 60 h). The results showed that at 4 °C the viability was maintained above 80% for 48 h, at 8 °C decreased slightly, whereas at room temperature and 37 °C decreased drastically. Its biocompatibility was assessed by cell morphology and cell viability assays. During stability study, the stored cells did not show any change in their phenotypic or genotypic characteristics and physicochemical properties remained constant, the ability to differentiate into adipocytes and osteocytes and sterility requirements were also unaltered. Finally, our paper proposes a packing media composed of albumin 20%, glucose 5% and Ringer's lactate at a concentration of 1×10^6 cells/mL, which must be stored at 4 °C as the most suitable to maintain cell viability (>80%) and without altering their characteristics for more than 48 h.

1. Introduction

The recent advances of biomedical research and biotechnologies have opened new promising therapeutic strategies, and human mesenchymal stem cells (hMSCs) are attracting increasing interest for potential application in cell therapies for the treatment of several human diseases [1,2]. hMSCs offer considerable therapeutic potential through the development of different cell therapy medical products (CTMP) for clinical use [3], due to their regenerative and immunoregulatory capacities [4,5], which have made them one of the most promising candidates for cell therapy success including regenerative and immune therapies where other current conventional treatments are inadequate.

Critical limb ischemia (CLI) is one of the diseases most studied in the field of cell therapy, in particular in diabetic patients, CLI of the leg develops earlier and more intensely, avoiding revascularization [6,7]. CLI is a syndrome manifested by ischemic rest pain, non-healing ulcers, tissue loss and gangrene. The incidence of CLI is estimated to be approximately from 500 to 1000 patients per million and year [8]. Diabetic patients with CLI are at acute risk of amputation, which leads to a low quality of life, and severe morbidity and mortality, resulting in a significant social and economic impact [9].

Currently, the latest advances in this pathology have led to the development of new medicines with stem cells as an alternative to surgical and pharmacological treatment. hMSCs transplantation is one of the most studied therapeutic alternatives in preclinical and clinical stages, due to its paracrine, immunomodulatory, and differentiation effects [8,10,11].

hMSCs encompass a broad range of anchorage dependent fibroblast-like cells which can be obtained from bone marrow aspirates,

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skeletal muscle connective tissue, human trabecular bones, adipose tissue, periosteum, fetal blood and liver, and umbilical cord blood [12]. These cells are characterized by being able to adhere to plastic. They can proliferate *ex vivo* and exhibit multilineage differentiation capacity being capable to give rise to diverse cells like osteoblasts, chondrocytes, adipocytes, myocytes, tenocytes and vascular cells and to express several common cell surface antigens [13].

Adipose tissue-derived stem cells have emerged as a new and promising type of stem cells because adipose tissue is an abundant source of stem cells, it lacks donor limitation and it is possible to obtain by a minimally invasive method [14].

Conducting cellular therapeutics is a complex undertaking, and both safety and efficacy measures shall be considered in the establishment of the manufacturing process of a finished medicinal product for cell therapy [15] among all steps involved, research phase, translational phase for scaling-up the protocol for the clinical requirements, establishment of standard operation procedures (SOP), validation runs, regulatory registration, storage and transportation [16]. The use of hMSCs for clinical application requires a high number of cells, which entails the *ex vivo* expansion in a certified laboratory under good manufacturing practice (GMP) conditions [17]. After culturing and having obtained the necessary number of cells, hMSCs must be loaded in a suitable dosage form for their administration. hMSCs can be formulated into liquid and semisolid dosage forms. Current methods of cell delivery involve the use of injections and microencapsulation. The use of several biomaterials for microencapsulation results in an impenetrable membrane to cells, and requires strong mechanical disturbances such as pressurized nozzles, emulsification, or stirring during droplet generation leading to cell degradation [18]. A cell suspension can be parentally administered directly in the damaged organ/tissue whilst offering medical devices use possibility. Attempts of CLI treatment with intra-arterial hMSCs have been associated with significant therapeutics benefits [10].

On the other hand, the administration of the cells to the patient is not. Following the obtention of cells, formulation of the finished medicinal product is carried out, all quality checks should be performed before approving its release, hence cells must be stored and transported in the best conditions to maintain stability [19]. The finished medicinal product of a CTMP includes an active ingredient (hMSCs) and the selected excipients (packaging medium), which are different from the expansion media. The cell stability of the finished medicinal product is determinant for its therapeutic applications in clinical use since baseline characteristics of cells should be maintained [15].

The stability information should include biological (sterility including mycoplasma, endotoxin and adventitious viral agents, identity, purity and potency) and physicochemical tests including those related to the design of the dosage form such as cell sedimentation rate and resuspension [20]. Alternatively, these products are likely to have a short shelf-life, which often means that these products are administered to patients before current sterility test results are available [17]. Due to cells are highly fragile and sensitive to their surrounding environment, and in order to maintain their quality, their environment needs to be strictly controlled during the time gap between cell harvesting and administration. Therefore, for the formulation of a cell suspension, important key factors must be taken into account, such as selection of the excipients of the packing medium, which must be protein-free to avoid inflammatory responses affecting the efficacy and safety [21]. Temperature and time conditions of storage as well as transport should be also studied.

Even though characterization of hMSCs has been extensively studied for their *ex vivo* expansion, there are not data dealing with the cell characterization in the finished medicinal product. The present work studies the stability of MSCs from human adipose tissue elaborated in a cell suspension for intra-arterial application.

The choice of the packing medium and storage conditions for a hMSCs suspension have been studied through stability studies, with the purpose of formulating a finished medicinal product that assured the maintenance of the characteristics more similar as possible to those of native hMSCs. hMSCs have been characterized before and after formulation, studying cell viability, immunophenotypic and genotypic characterization, differentiation, microbiology and physicochemical properties.

2. Materials and methods

Clinical application of hMSCs requires a concentration approximately between 1 and 2×10^6 cells/kg [22], therefore hMSCs should be expanded in long-term culture that can affect their characteristics [23,24], specially their immunogenicity or a lack of safety of the medium components, resulting in chromosomal aberrations [25–28].

For intra-arterial administration of hMSCs the formulation of a suitable and safe finished medicinal product is a need absolutely vital. The storage condition study is a key factor to assure the viability of cells and hold their properties in the moment of administration [29–31]. For this reason, different excipients at different temperatures were studied in the finished medicinal product.

2.1. GMP environment

This study was performed in the context of a clinical trial, phase I/II under GMP conditions. All procedures were performed in a certified clean room at the Center for Molecular Biology and Regenerative Medicine (CABIMER), it was the first laboratory accredited by the Spanish Agency for Medicines and Medical Devices for production of stem cells as medicines in Andalucía (Spain).

All starting materials and reagents required for this study were according to GMP guidelines. All equipment was validated.

2.2. Human mesenchymal stem cell isolation and culture from adipose tissue

The donor source of the hMSCs was appropriately screened and tested for human pathogens. Procedures were performed at the San Lazaro Hospital (Spain). The therapeutic protocol was approved by the hospital ethics committee in accordance with Spanish law. All patients signed a detailed informed consent form before intervention and gave their consent for publication of the study results. This study was conducted in accordance with the ethical standards of the Helsinki Declaration (1975). In particular, the presence of Human Immunodeficiency Virus (HIV), hepatitis B and hepatitis C virus was analyzed. On the other hand, all starting materials and reagents required for the expansion were analyzed to certify that they were sterile and endotoxin-free.

Autologous hMSCs were isolated from adipose tissue by enzymatic digestion with collagenase (2 mg collagenase/1 g adipose tissue) (Roche Farma, Reinach, BL, Switzerland). Briefly, the sample was centrifuged at 400*g* for 10 min, filtered and washed with Phosphate Buffered Saline (PBS) (Sigma–Aldrich, St. Louis, MO, USA). The isolated cells were suspended and plated at medium density (passage 0) of $12–20 \times 10^4$ cells/cm^2 in culture flasks (Nunc, Roskilde, Denmark) with expansion medium composed by Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS), 1% of 10.000 IU/mL Penicillin, 10 mg/mL Streptomycin, 2% of 200 mM L-alanine solution and 1% of 200 mM L-glutamine, (all from Sigma–Aldrich, St. Louis, MO, USA). After 24 h non-adherent cells were removed by replacing

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