Understanding the synergistic potential of phytocannabinoids and umbilical cord derived mesenchymal stem cells for regenerative medicine

Pargana GD.[1,2], Serras AS.[2], Camões SP.[2], Santos JM.[3], Miranda JP.[2]

[1] Instituto Superior Técnico, Universidade de Lisboa, Lisbon, Portugal
[2] Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal
[3] ITQB-UNL, Universidade NOVA de Lisboa, Lisbon, Portugal

Abstract

The properties of *Cannabis* and their derived phytocannabinoids have been researched for therapeutic applications thanks to their anti-inflammatory, anxiolytic or anti-epileptic effects. Mesenchymal stem cells (MSCs) and their derived secretome are already commonly used in regenerative applications, due to their anti-inflammatory and immunomodulatory properties. Therefore, combining phytocannabinoids and MSCs appears to be a great strategy applied for regenerative medicine. Hence, this work aims at assessing the synergistic potential between phytocannabinoids and umbilical cord derived MSCs (ucMSCs), which will be divided in two main parts, consisting in characterizing ucMSCs' cannabinoid receptors, and evaluating the potential of phytocannabinoids for modulating its secretome. The results obtained confirm the presence of cannabinoid receptors in ucMSCs and their induction upon exposure to an inflammatory stimulus. Furthermore, phytocannabinoids modulate ucMSCs' expression and secretory profile, showing their influence in these cells' therapeutic potential. Ultimately, this thesis demonstrates the potential of possible therapies formulated from the interaction between these factors.

Keywords: Phytocannabinoids, Cannabinoid receptors, Mesenchymal stem cells, Secretome, Tissue regeneration.

Introduction

Cannabinoids

The use of *Cannabis* plants as medicinal products has been documented since ancient times[1]. The endocannabinoid system (ECS) is involved in important physiological processes, such as homeostasis maintenance, regulation of anxiety and feeding behaviors and pain sensation. The ECS is composed of cannabinoid receptors, cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2), G-protein coupled receptors (GPCR) that bind to cannabinoids, their endogenous ligands, endocannabinoids, anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), and the enzymes responsible for their synthesis and degradation[2].

CB1 and CB2 modulate various intracellular signaling pathways important to control cell survival, differentiation and apoptosis, like inhibition of adenylyl cyclase, stimulation of mitogen-activated protein kinases (MAPK) and recruitment of beta-arrestins[2]. CB1 receptors are mainly present in the central nervous system (CNS) and regulate memory, cognition, and mood processes, inhibit nociception and activate reward pathways[1], while CB2 receptors are mainly expressed in the peripheral nervous system and in immune cells[3], influencing inflammation and immunomodulation. Other receptors can interact with cannabinoids, such as transient receptor potential (TRP) channels, from TRP vanilloid (TRPV), TRP ankyrin (TRPA) and TRP melastatin (TRPM) subfamilies, working as sensors for pain, itch and temperature, and influencing inflammation processes. Cannabinoids can desensitize these channels, causing analgesic effects[4].

Phytocannabinoids are plant-derived cannabinoids, mainly from *Cannabis* plants, and are interesting for their therapeutic effects. The major phytocannabinoids are Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD), however others also exert therapeutic effects, such as cannabidivarin (CBDV)[5]. THC is a partial agonist for CB1 and CB2, while CBD and CBDV are inverse agonist for these receptors, blocking their access to other ligands. For TRP channels, both compounds work as agonists for TRPV1 and TRPA1, and antagonists to TRPM8[6].

Both CBD, CBDV, and THC, have purported anxiolytic, analgesic, anti-epileptic and anti-inflammatory effects[7] and are being tested in treatment of inflammatory conditions[8], neuropsychiatric disorders[9], epilepsy[10] and other conditions, giving these compounds high interest in regenerative applications.

Mesenchymal stem cells

Stem cells are undifferentiated cells characterized by their capacity of self-renewal and differentiation into different tissues. Mesenchymal stem cells (MSCs) have the

capacity to differentiate in tissues originated from mesoderm like bone, cartilage and adipose tissue and can be extracted from bone marrow (bmMSCs), adipose tissue (atMSCs) or umbilical cord blood or matrix (ucMSCs). MSCs present high therapeutic potential due to their ability to differentiate and replace damaged tissues, low immunogenicity, and paracrine effects leading to release of cytokines and growth factors[11]. Thanks to these properties, MSCs have varied effects in tissue regeneration across the human body, being a helpful tool to aid the recovery of several tissues, such as bone, cartilage, nerve, liver, heart and skin, among others[12].

The set of molecules secreted by MSCs is called secretome or conditioned medium (CM), including growth factors, cytokines, exosomes and microvesicles that play important roles in cell communication, signal transduction and inflammatory responses[13]. MSCs have immune plasticity and possess two distinct secretory profiles depending on the microenvironment they are exposed to: a pro-inflammatory MSC1 profile, characterized by release pro-inflammatory factors and activation of T cells, or an anti-inflammatory MSC2 phenotype, promoting secretion of anti-inflammatory cytokines[14]. The secretome is advantageous since it has shown that it can improve regeneration of injured tissue, overcoming problems associated with live cells administration, such as risk of rejection, and can be easily modulated through changing the factors present in MSCs' microenvironment, a process called priming[15]. Therefore, priming of MSCs and the derived secretome have become useful tools to obtain the therapeutic effects desired for a specific application.

Therapeutical applications of MSCs and their combination with cannabinoids

MSCs from the bone marrow[16,17] and adipose tissue[18] express both cannabinoid receptors and interact with phytocannabinoids[19,20]. Cannabinoid receptors are essential for survival, differentiation and regenerative potential of MSCs[21,22] and stimulation of this system can augment MSCs' abilities. Thus, the combination of phytocannabinoids and MSCs has high potential in regeneration therapies. However, more research is needed to solidify these findings.

Objectives

The objective of this work is to observe a potential synergistic activity between ucMSCs and phytocannabinoids. To achieve it, the work will be divided in two parts. Due to the lack of characterization of the endocannabinoid system of ucMSCs, first, the presence and activity of its cannabinoid receptors is determined. The second part evaluates the therapeutic potential of the ucMSCs-derived secretome, after priming by phytocannabinoids. This work aims to setup the grounds for future studies regarding combination of phytocannabinoids and MSCs, for regenerative therapies.

Results and Discussion

Characterization of the cannabinoid receptors in ucMSCs

Basal gene expression of *CB1, CB2, TRPV1* and *TRPA1* in ucMSCs was analyzed. Results are displayed in Figure 1a.

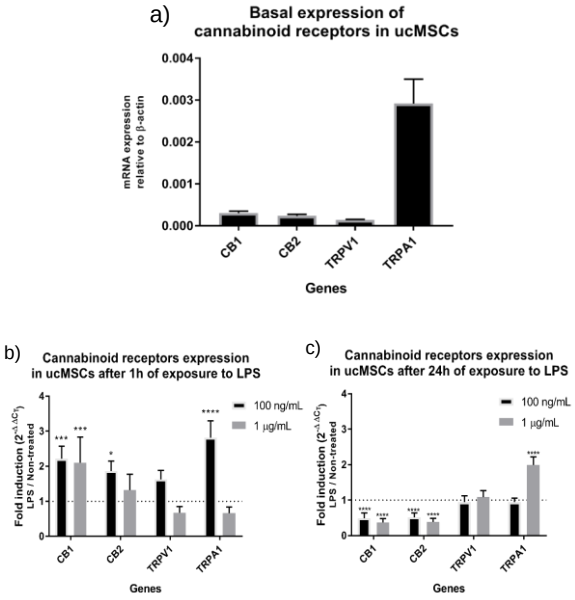

Figure 1- *Gene expression levels of the cannabinoid receptors CB1, CB2, TRPV1 and TRPA1 in ucMSCs. a) Basal levels, b) upon 1h induction with LPS, c) upon 24h induction with LPS. Results are presented as the level of mRNA expression of the receptors in non-treated ucMSCs relative to the housekeeping gene β-actin (a) and fold induction relative to the non-treated control group (represented by the dotted line; b, c). Data are represented as Average ± SEM and statistically significant results are presented as *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 (n=3-6). CB, cannabinoid receptor; CBD, cannabidiol; CBDV, cannabidivarin; LPS, lipopolysaccharides. TRPA1, transient receptor potential ankyrin 1; TRPV1, transient receptor potential vanilloid 1.*

All receptors show expression in ucMSCs, with *TRPA1* showing higher basal expression levels. Therefore, there is confirmation that ucMSCs constitutively express cannabinoid receptors

and can interact with phyto- and endocannabinoids.

Cannabinoid receptors are present in many cells in the human body, influencing vital functions in different tissues. These receptors are present in MSCs, with their expression being detected at bmMSCs[16,17] and atMSCs[18]. The results obtained in this work suggest that ucMSCs also express cannabinoid receptors, indicating that these cells possess and can regulate functions mediated by them.

For TRP channels, their expression has been confirmed in various types of stem cells[23]. The results acquired support their existence in MSCs, specifically *TRPV1* and *TRPA1* expression in ucMSCs, allowing for interaction with agonists CBD or CBDV.

Next, ucMSCs were exposed to two different LPS concentrations, 0.1 and 1 µg/mL, for 1h and 24h, and their cannabinoid receptor expression was measured. The exposure of cells to LPS have shown to modulate the expression of these cannabinoid receptors[20] in bmMSCs and atMSCs. Results of gene expression analysis are shown in Figure 1b, c.

For 1h of exposure to LPS, there is increase of expression of all genes exposed to low concentration, while for high concentration, both TRP channels analyzed present expression similar to basal level, which could mean these receptors only react to low amounts of LPS. After 24h of exposure, both *CB1* and *CB2* have reduced expression in all concentrations tested, which could signify their response may be time-dependent, increasing reaction to inflammatory stimulus immediately after contact and reducing after. For TRP channels, inflammatory stimulus seems to minimally affect *TRPV1*, while for *TRPA1*, low concentrations of LPS seem to exert the same effect as observed in cannabinoid receptors, while high concentrations of LPS seem to have the opposite effect, increasing receptor's expression level overtime, since its action seems to affect ucMSCs response to inflammation.

Cannabinoid receptors activate anti-inflammatory functions, and exposure of cells to LPS could induce the expression of these receptors. In fact, *CB1* and *CB2* have been reported to be activated upon exposure to inflammatory stimulus such as ultraviolet radiation[24]. Even for LPS, *CB1* and *CB2* expression increases in keratinocytes and fibroblasts after uptake of this agent[25]. A different study showed ucMSCs have lower response to inflammatory stimulus caused by LPS when compared to bmMSCs or atMSCs[26]. In mice bmMSCs, exposure to LPS for 12h resulted in significative decrease in expression of *CB1* and *CB2* receptors[20], going according with results obtained here for 24h of exposure to LPS.

TRP channels affect processes in inflammatory responses, and exposure of ucMSCs to LPS could induce these receptors. Although their role in neurogenic inflammation and as response to LPS in sensory neurons is being studied[27,28], the response to this compound in MSCs remains to be elucidated. *TRPA1* has showed to be induced by exposure to LPS for 24h and 48h in dental pulp cells[29], proving *TRPA1* can react to this inflammatory promoter. Effects of LPS and other inflammatory agents in TRP channels of MSCs need clarification, as their mechanisms of action are investigated further.

Once LPS induces an inflammatory reaction in cells[14,20], depending on concentration or time of exposure, cell viability can decrease. It is imperative to verify if this agent causes viability reduction in ucMSCs, to use the most desirable concentrations or exposure times to LPS.

ucMSCs were subjected to LPS and an MTS viability assay was performed to see if cell viability is affected, with concentrations tested of 0.1, 0.5, 1 and 5 µg/mL, and exposure times of 1h or 24h. Results are presented in Figure 2. The conclusion is LPS does not alter cell viability in all conditions analyzed, denoting that LPS can be useful in ucMSCs, to induce an inflammatory state without affecting viability.

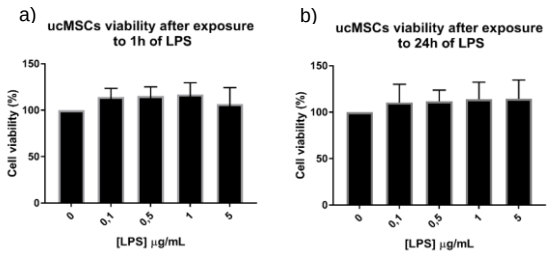

***Figure 2**- Viability of ucMSCs is not affected after exposure to different concentrations of LPS (0.1, 0.5, 1 and 5 µg/mL). Viability after a) 1h or b) 24h of exposure to LPS. Results are presented as a relative percentage to the non-treated control, with viability for this group being considered as 100% (n=3). LPS, lipopolysaccharides.*

This is concordant with previous studies, where 10 µg/mL of LPS showed to increase atMSCs proliferation after long-term exposure for 7 days[14], whereas in an experiment using bmMSCs, LPS increased proliferation at concentrations of 0.1 µg/mL, but decreased it for concentrations of 10 µg/mL[30], and an experiment using Wharton's Jelly-derived MSCs (WJ-MSCs), exposed to 1.0 µg/mL of

LPS showed no alteration on cell proliferation up to 120h[26].

Since exposure to low concentrations of LPS (100 ng/mL) for 1h was the only to increase all analyzed receptors' expression, this condition was applied in future experiments inducing ucMSCs' cannabinoid receptors, prior to phytocannabinoid application.

Evaluating the therapeutic potential of ucMSC's secretome primed with phytocannabinoids

Given the values obtained in previous studies for the plasmatic concentrations of phytocannabinoids[31], the concentrations of 100, 500 and 750 nM of CBD and CBDV were chosen.

Phytocannabinoids effect on gene expression profile of ucMSCs was assessed. The genes chosen were previously analyzed cannabinoid receptors, as well as cytokines involved in different stages of tissue repairs, with *IL-6, TNF-α* with pro-inflammatory, *G-CSF* with recruiting and *IL-10* with anti-inflammatory actions. *EGF, FGF2* and *VEGF-α* have proliferation and angiogenic roles, and *TGF-β1* with remodeling and immunomodulatory functions.

Conditions analyzed consisted in cells' exposure to CBD or CBDV for 24h, with or without previous induction of cannabinoid receptors by 1h of LPS. Results for cytokines and cannabinoid receptors are presented in Figures 3 and 4, respectively.

Gene expression of cytokines involved in tissue regeneration

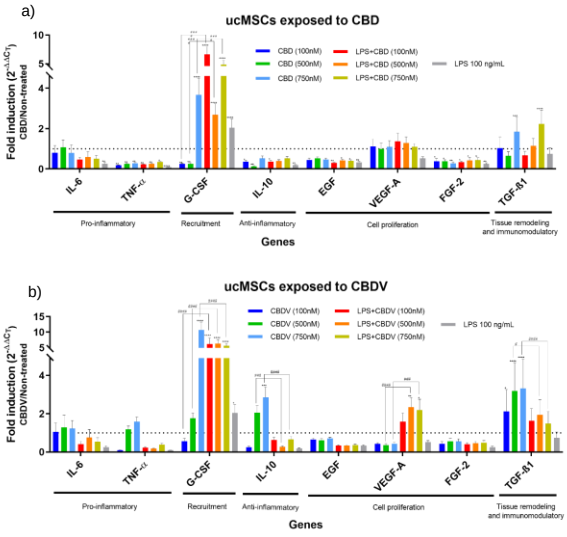

***Figure 3**- CBD (a) and CBDV (b) modulate gene expression of several cytokines involved in tissue regeneration, such as IL-6, TNF-α, G-CSF, IL-10, EGF, VEGF-α, FGF2 and TGF-β1, in ucMSCs. Exposure of cells to different concentrations of phytocannabinoids for 24h: 100 nM (blue); 500 nM (green); 750 nM (light blue); 100 ng/mL of LPS for 1h followed by: 100 nM (red); 500 nM (orange); 750 nM (yellow); 24h of culture with α-MEM without FBS (gray). Results are presented as fold induction relative to the non-treated control group (represented by the dotted line). Statistically significant results are presented as *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 in relation with the control, and of #p<0.05; ###p<0.001; ####p<0.0001 between the groups with the same concentrations, with or without previous exposure to LPS (n=2-4). CBD, cannabidiol; CBDV, cannabidivarin; EGF, epithelial growth factor; FGF-2, fibroblast growth factor 2; G-CSF, granulocyte-colony stimulating factor; IL, interleukin; LPS, lipopolysaccharides; TGF-β1, transforming growth factor-β1; TNF-α, tumor necrosis factor-α; VEGF-A, vascular endothelial growth factor α.*

Gene expression of cannabinoid receptors

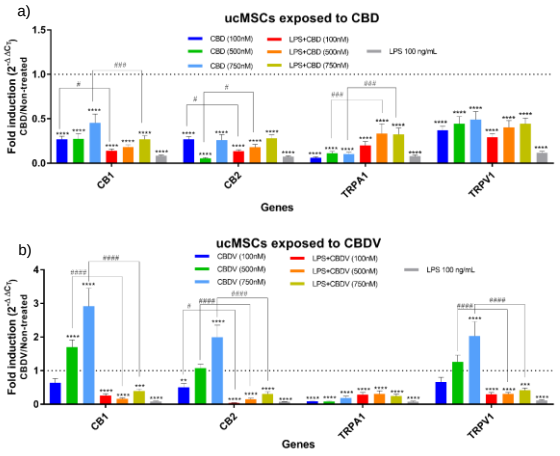

***Figure 4**- CBD (a) and CBDV (b) modulate gene expression of cannabinoid receptors, namely CB1, CB2, TRPA1 and TRPV1, in ucMSCs. Exposure of cells to different concentrations of phytocannabinoids for 24h: 100 nM (blue); 500 nM (green); 750 nM (light blue); 100 ng/mL of LPS for 1h followed by: 100 nM (red); 500 nM (orange); 750 nM (yellow); 24h of culture with α-MEM without FBS (gray). Results are presented as fold induction relative to the non-treated control group (represented by the dotted line). Statistically significant results are presented as *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 in relation with the control, and of #p<0.05; ###p<0.001; ####p<0.0001 between the groups with the same concentrations, with or without previous exposure to LPS (n=2-4). CB, cannabinoid receptor; CBD, cannabidiol; CBDV, cannabidivarin; LPS, lipopolysaccharides; TRPA1, transient receptor potential ankyrin 1; TRPV1, transient receptor potential vanilloid 1.*

Phytocannabinoids exposure to ucMSCs increase expression of key genes in regenerative processes. Exposure to CBD increases expression of both *G-CSF* and *TGF-β1*. A significant decrease in the expression levels of the pro-inflammatory gene *TNF-α* is also observed in all conditions. This may signify that CBD could present beneficial effects, such as anti-inflammatory, immunomodulatory, recruitment of other cells and remodeling effects, and previous exposure to an inflammatory agent with activation of cannabinoid receptors can potentially lead to higher sensitivity to this phytocannabinoid. With exposure to CBDV, *G-CSF* showed a similar expression profile to CBD, but with higher fold induction in the highest concentration tested, with sole exposure to CBDV. This condition seems to be the one that mostly increased the expression levels of *G-CSF, IL-10* and *TGF-β1*.

Stimulation of receptors by LPS seems to not affect expression of most genes analyzed, excluding *G-CSF* and *VEGF-α*, which were upregulated, suggesting this phytocannabinoid could be important not only on cellular recruitment but also on angiogenesis during inflammatory responses.

In relation to cannabinoid receptor expression, in CBD's case, there is significant decrease in expression, meaning that long exposure of ucMSCs to CBD may deactivate the receptors, or they are only activated in initial exposure stage. For CBDV, in the highest concentration tested, it was observed high level of expression for most cannabinoid receptors, namely for *CB1*, *CB2* and *TRPV1*. These effects are concentration dependent and suggest that CBDV modulate the ECS of ucMSCs better when compared to CBD.

Phytocannabinoids are useful in MSCs affecting the immune system by activating cannabinoid receptors and TRP channels, making them great modulators of their secretome. *Ruhl et al.*, tested the change provoked by CBD in inflammatory profile of atMSCs, induced by LPS exposure. Presence of several cytokines in secreted medium was assessed, with conclusion that co-treatment of LPS and CBD increased the levels of *IL-6* and *VEGF*, however this effect was already seen after sole exposure to LPS[14]. However, concentrations of the compounds used were higher than the ones tested here, besides using MSCs from adipose tissue, explaining the differences observed between studies. In a study using mice bmMSCs, after LPS increased levels of inflammatory cytokines *TNF-α* and *IL-6*, CBD reduced their levels in cells. For cannabinoid receptors *CB1* and *CB2*, it was verified that administration of CBD after LPS increased *CB2*, but not *CB1* expression[20]. Another study using human gingival MSCs showed that treatment with CBD reduced pro-inflammatory and apoptosis-related genes expression, while increasing *CB1* receptor's[32]. A different study using gingival MSCs, demonstrated CBD increased *TGF-β1* expression in these cells[33]. These results could indicate that depending on the source of MSCs, CBD has different effects on their response to inflammatory environment.

For CBDV, there have not been studies focusing on its roles in MSCs, however several studies researched its function in inflammatory environments in other types of cells. A study researching the therapeutic effect of CBDV in ulcerative colitis found that after inflammation, CBDV reduced pro-inflammatory agents *like IL-6*, without increasing anti-inflammatory *IL-10*, in mice colon. This compound reduced *TRPA1* expression after its induction by inflammation in ulcerative colitis patients, signifying that its actions in this condition are mediated by this receptor[34]. Other study focused on the action of phytocannabinoids in muscular dystrophy. Inflammation markers in skeletal muscles of mice were analyzed. Administration of CBD or CBDV for 2 weeks reduced expression of markers, namely *IL-6*, *TNF-α* and *TGF-β1*, but only CBD was able to reduce all of them[35]. Another study focused on anti-inflammatory effects of phytocannabinoids in peripheral blood mononuclear cells (PBMC). Administration of CBDV was able to reduce levels of pro-inflammatory marker *IL-6* in monocytes[36]. Therefore, CBDV appears to present significant roles in amelioration of inflammatory processes.

Remarkedly, application of 750 nM of both phytocannabinoids suggests significant increase in cytokine expression. Therefore, this concentration was used for conditions tested in secretome production.

Next, the effect of phytocannabinoids in ucMSCs morphology was assessed during secretome production, finding that none of the compounds altered their morphology, showing that LPS, CBD or CBDV, do not affect these cells' conformation.

LPS had already been tested in WJ-MSCs and concluded that concentrations of 1 µg/mL did not affect cell conformation up to 72h of exposure[26]. This confirms the results, in which LPS exposure was only for 1h at concentrations of 100 ng/mL.

In atMSCs, CBD was tested to verify if it protects these cells from endoplasmic reticulum stress, maintaining their cytophysiological properties. 24h of cells' incubation with 5 µM of CBD succeeded in reverting atMSCs morphology[37]. Other study using gingival MSCs tested various concentrations of CBD for 24h to see if it altered cell morphology or viability. Cells treated with 5 µM of this phytocannabinoid did not show morphological changes or cause cell death[38]. These results go according to the ones obtained here, where exposure of ucMSCs to 750 nM of CBD for 24h did not alter cell morphology.

CBDV effects on MSCs morphology have not been researched, but the results presented here imply that this phytocannabinoid has similar effect on ucMSCs morphology to CBD, indicating that the concentrations used are safe.

Finally, the assessment of cytokine presence in the secretome obtained was performed by

western blotting. The proteins analyzed were G-CSF, TGF-β1, and IL-10, since their expression was augmented in the conditions tested for CM production. Results are displayed in Figure 5.

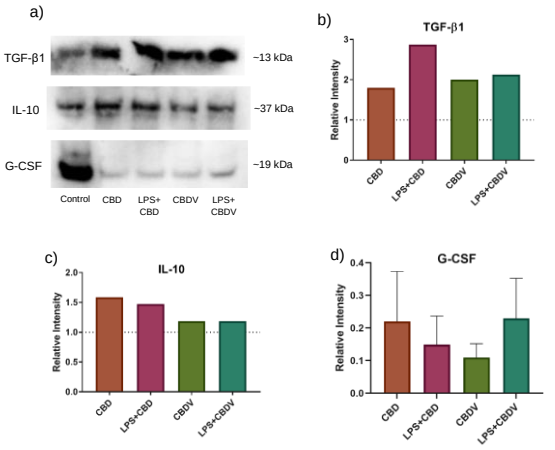

***Figure 5**- The secretome of ucMSCs modulated by phytocannabinoids contains several cytokines of interest. a) Western Blot (WB) bands of each of the proteins analyzed, namely TGF-β1, IL-10 and G-CSF in ucMSCs non-treated (control); exposed to 750 nM of CBD for 24h; to 100 ng/mL of LPS for 1h, followed by 750 nM of CBD during 24h; to 750 nM of CBDV for 24h; and to 100 ng/mL of LPS for 1h, followed by 750 nM of CBDV during 24h. Relative intensity of the WB bands of b) TGF-β1 (n=1), c) IL-10 (n=1) and d) G-CSF (n=3) in relation to the control (relative intensity equal to 1). CBD, cannabidiol; CBDV, cannabidivarin; G-CSF, granulocyte-colony stimulating factor; IL-10, interleukin-10; LPS, lipopolysaccharides; TGF-β1, transforming growth factor-β1.*

Results verify presence of the proteins analyzed in ucMSCs secretome, with bands obtained being in the molecular mass range of their respective proteins. Relative to band intensity, for TGF-β1 its protein is increased in all conditions, indicating the involvement of phytocannabinoids in inducing release of this cytokine. These results align with the expression profile observed previously, although the highest values were observed in the case of exposure to CBDV. For IL-10 an increase was observed in groups in all groups however with higher levels for groups exposed to CBD. These results are concordant with what was observed in gene expression analyses, although IL-10 was only increased when cells were solely exposed to CBDV. Interestingly, in G-CSF untreated control possesses greater amount of this protein compared to other conditions, contrary to gene expression analysis, where conditions exposed to phytocannabinoids had higher expression of G-CSF, suggesting the existence of post-transcriptional alterations after gene expression is verified.

The different results obtained may result from the experimental setup of each analysis. In protein analysis, cells were left in culture for 48h without exposure to any compound. Within this time ucMSCs could be responding to the withdrawal of phytocannabinoids, compared to what was observed in gene expression analysis, where expression observed was 24h after phytocannabinoids exposure, resulting in different secretory profiles.

Previous studies already derived results based on MSCs secretome. In a study researching ucMSCs-CM potential on wound healing, secretome reduced TGF-β1 levels in dermal fibroblasts, reducing fibroblast differentiation potential, and leading to anti-fibrotic properties[39]. Other study assessed expression of this cytokine in gingival MSCs pretreated with CBD, observing increase of TGF-β1 protein expression[33]. Regarding IL-10, a study using bmMSCs observed reduced secretion of this cytokine after exposure to LPS. However, the reduction was reverted after cell's treatment with a CB2 agonist, showing that this receptor mediates anti-inflammatory actions[17]. A different study using murine bmMSCs concluded that treatment with THC increases levels of IL-10 in the secretome, through a CB2 mediated pathway[16], showing again its important function in immune regulation. For G-CSF, a study researched secretory profile of bmMSCs after exposure to endotoxins, such as LPS. Exposure to different concentrations of LPS resulted in CM with induced G-CSF release, indicating this cytokine's secretion increases when cells are exposed to inflammation[40]. Nevertheless, in the results observed, there is a decrease in secretion of this cytokine, suggesting that the secretory profiles may vary depending on the time of CM collection. Further studies will be conducted to explore this hypothesis, to understand whether there is a potential post-transcriptional change that influences G-CSF secretion, or if it depends on the timepoint of analysis of protein secretion.

Conclusions

MSCs have become common in therapeutic applications thanks to their properties. Phytocannabinoids and their modulation of the ECS, although less explored, have been useful thanks to anti-inflammatory and analgesic roles. The combination of these therapies has recently started to be researched, proving advantageous in regenerative medicine.

This work assessed the synergistic activity between ucMSCs and phytocannabinoids, verifying their modulatory effects in ucMSCs secretome. The results obtained show that cannabinoid receptors *CB1* and *CB2*, and TRP channels *TRPV1* and *TRPA1* are expressed in ucMSCs and are inducible by inflammatory

stimulus. Phytocannabinoids, namely CBD and CBDV, influence expression of these receptors and cytokines characteristic of MSCs during tissue repair, modulating cytokine secretion in the secretome, indicating that these compounds affect ucMSCs functions and secretory profile. These results show the potential that phytocannabinoids have in ucMSCs and its secretome, and the usefulness of combining these two strategies for regenerative medicine, demonstrating the possible use of this secretome based therapy in future clinical applications.

However, more research is required to solidify the results obtained here, specifically by testing other cannabinoids, e.g., receptor agonists, that interact with the ECS in ucMSCs to understand the mechanism behind their function.

Materials and Methods

Reagents

Minimum essential medium alpha modification (α-MEM), Dulbecco's modified Eagle medium (DMEM), Lipopolysaccharides (LPS; LPS from Escherichia coli O55:B5) and trypan blue were obtained from Sigma-Aldrich® (St. Louis, MO, USA). Fetal bovine sera (FBS) and trypsin/ethylenediamine tetraacetic acid (Trypsin-EDTA) solution were obtained from Gibco® (Thermo Fisher ScientificTM, Waltham, MA, USA). 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from Promega (Madison, WI, USA). Cannabidiol and cannabidivarin were obtained from Cayman Chemical (Ann Arbor, MI, USA). $NaHCO_3$ was obtained from ITW Reagents© (AppliChem GmbH, Darmstadt, Germany). TRIzol® reagent was purchased from InvitrogenTM (Thermo Fisher ScientificTM, Waltham, MA, USA). Dimethyl sulfoxide (DMSO) was obtained from Fisher ChemicalTM (Fisher Scientific, Hampton, NH, USA). Bradford reagent was purchased from Bio-Rad® (Hercules, CA, USA).

Mesenchymal stem cells culture

This study was approved by the Ethics Committee of the Hospital Dr. José de Almeida (Cascais, Portugal), in the scope of a research protocol between ECBio (Research & Development in Biotechnology, S.A.) and HPP Saúde (Parcerias Cascais, S.A.). Umbilical cord donations, with written informed consents, as well as umbilical cord procurement, were made according to Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004 on setting standards of quality and safety for the donation, procurements, testing, processing, preservation, storage and distribution of human tissues and cells. Human umbilical cord matrix-derived mesenchymal stem cells were isolated as described by *Santos et al.*, according to a patented method (PCT/IB2008/054067; WO 2009044379), designed to produce a highly homogeneous population of cells that comply with the MSC standards defined by the ISCT[41]. ucMSCs were cultured and expanded in specific culture medium containing α-MEM, with 2 mM of L-Glutamine and 1 g/L of D-Glucose (11900-073, Gibco™), supplemented with 2.2 g/L of $NaHCO_3$ (131638, ITW Reagents©) and 10% (v/v) of heat inactivated fetal bovine serum (FBS) (10500-064, Gibco™), in a humidified atmosphere chamber at 37 ºC with 5% carbon dioxide (CO_2). ucMSCs were seeded in monolayer in t-flasks at 0.7 to $1.0x10^4$ cells/cm^2 and routine passages were performed every 2 to 3 days when cells reached up to 90% confluence. Cells were used from passage 12 up to 16. During each passage, cells were washed with phosphate-buffered saline (PBS) solution followed by trypsinization with 0.05% of Trypsin-EDTA solution (25300-062, Gibco™) for 5 minutes. Cell counting and viability assessment were performed using the trypan blue (T8154, Sigma-Aldrich®) exclusion method and an Olympus CK30 inverted phase contrast microscope. Cells were cryopreserved in FBS with 10% DMSO (Fisher Chemical™) and stored in liquid nitrogen until next use.

Cell morphology assessment

At different stages of the CM protocol, contrast-phase images of cells were captured with the Moticam 2500 5.0M Pixel USB 2.0 (Motic®) mounted on an Olympus CK30 inverted phase contrast microscope and viewed using Motic Images Plus 3.0 software (Motic®).

MTS cell viability assay

ucMSCs were seeded in 96-well plates at a density of $1.0x\ 10^4$ cells/cm^2 and kept in a humidified atmosphere at 37 ºC and 5% CO_2. Cells were cultured in α-MEM supplemented with 5% FBS until they reached confluence. LPS (L6529-1MG, Sigma-Aldrich®) was sequentially diluted to the final testing concentrations of 5, 1, 0.5 and 0.1 µg/mL. Cell medium was renewed and LPS was added to

each well and incubated for 1h or 24h. After this, cell culture medium was replaced by 100 µL of DMEM (D5523, Sigma-Aldrich®) and 20 µL MTS (CellTiter 96® AQ_{ueous} MTS Reagent Powder; G111A, Promega Corporation©) *per* well. After 2h of incubation at 37 ºC, absorbance was measured at 490 nm using a microplate spectrophotometer (SPECTROstar® Omega, BMG LABTECH). 10 % (v/v) DMSO was used as negative control and α-MEM supplemented with 5% FBS as positive control. Experiments were performed in triplicates, and results were expressed as percentage relative to positive control, which was considered 100% cell viability.

RNA extraction and gene expression analysis (qRT-PCR)

ucMSCs were seeded in 6-well plates at a density of 0.7 to 1.0 cells/cm^2, cultured in α-MEM supplemented with 5% FBS and kept in a humidified atmosphere at 37 ºC and 5% CO_2 until confluence was reached. Following this, culture medium was renewed and LPS 0.1 or 1 µg/mL was added to the wells, and incubated for 1h or 24h, after which total ribonucleic acid (RNA) of cell samples was isolated with TRIzol® reagent (15596-018, Invitrogen™) and extracted according to manufacturer's instructions. For the experiments using phytocannabinoids, after cells were seeded in 6-well plates and confluence was reached, medium was changed to α-MEM supplemented with 5% FBS with LPS 0.1 µg/mL for 1h, after which LPS was removed and wells were washed with PBS. Then, medium was replaced for α-MEM without FBS, and CBD or CBDV were added to the wells at concentrations of 100, 500 or 750 nM in 0.1% DMSO. Cells were exposed to these concentrations of CBD or CBDV during 24h, after which total RNA of cell samples was isolated with TRIzol® reagent and extracted according to manufacturer's instructions. For RNA quantification, absorbance was measured at 260 and 280 nm using LVis Plate mode on SPECTROstar® Omega. Purity measures were determined with standard 260/280 nm, for protein presence, considering ratios between 1.8 and 2.0. Complementary DNA (cDNA) was synthesized from samples of 0.7 to 2.5 µg RNA using the NZY First-strand cDNA Synthesis kit (MB12502, NZYTech®) following manufacturer instructions. qRT-PCR was performed using 7.5 µL PowerUp™ SYBR™ Green Master Mix (A25741, Applied Biosystems™) which was prepared for a final reaction volume of 15 µL, using 2 µL of template cDNA, 1 µL of forward and 1 µL of reverse primers. As to assure the inexistence of contamination, blank controls were also prepared without template cDNA. Reaction was performed on QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems™). The comparative Ct method ($2^{-\Delta\Delta Ct}$) was used to quantify gene expression, normalized to the reference gene *β-actin*. Results were expressed relative to the non-treated control condition.

Conditioned Media Production

ucMSCs up to 16 passages were seeded in 175 cm^2 t-flasks at a density of 0.7 to $1.0x10^4$ cells/cm^2, cultured in α-MEM supplemented with 5% FBS and kept in a humidified atmosphere at 37 ºC and 5% CO_2 until reaching 70% confluence. Upon this, medium was renewed and LPS 0.1 µg/mL was added to the t-flasks and incubated during 1h. After priming with LPS, t-flasks were washed with PBS to remove any vestigial remaining LPS. α-MEM without FBS and CBD or CBDV at concentrations of 750 nM were added to the t-flasks with a total volume of 18 mL, followed by their incubation at 37 ºC for 24h. After this, medium was removed and t-flasks were washed with PBS to remove residual CBD or CBDV. α-MEM without FBS was added to t-flasks with total volume of 25 mL and incubated for 48h. For the conditions where cells were only exposed to phytocannabinoids, CBD or CBDV were added to t-flasks with α-MEM without FBS right after cells reached 70% confluence. For the control group, a total volume of 25 mL of α-MEM without FBS was added when cells reached 90% confluence and were incubated for 48h. Post conditioning, the conditioned media (CM) produced was collected with each condition belonging to one of five different groups: i) Control; ii) CBD; iii) LPS+CBD; iv) CBDV; and v) LPS+CBDV. The corresponding CM was collected under sterile conditions, submitted to a cycle of centrifugation of 300 x g for 10 minutes at 25 °C and a following cycle of 2700 x g for 30 minutes at 4 °C to remove cell debris. CM was concentrated in 3 kDa cut-off centrifugal concentrators, Amicon® Ultra-15 Centrifugal Filter Unit (UFC900396, Millipore®), as *per* manufacturer's recommendations. All samples were stored aseptically at -80 ºC until further use. Cells were harvested for posterior qRT-PCR and total protein quantification.

Total Protein quantification

Total protein quantification was determined with a colorimetric assay based on the Bradford method, with protein dye reagent concentrate (500-0006, Bio-Rad®) according to manufacturer's instructions for microtiter plates. Absorbance at 595 nm was measured using microplate reader on SPECTROstar® Omega.

Western Blot

A total of 30 µg of each condition was resolved by SDS-PAGE in 12% polyacrylamide gels according to manufacturer's recommendations (Bio-Rad®). Gels were blotted onto PVDF transfer membranes, which were incubated with primary antibody diluted in 5% BSA blocking buffer overnight at 4ºC, namely anti-G-CSF (ab9691, Abcam plc©) diluted 1:2500, anti-TGF beta 1 (ab92486, Abcam plc©) diluted 1:100 and anti-IL10 (sc-8438, Santa Cruz Biotechnology©) diluted 1:200. Following this, membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (Jackson ImmunoResearch©) dilution 1:20000, for the first two, or anti-mouse antibody (R&D Systems, Bio-Techne®) dilution 1:1000, for the last, for 1h at room temperature. Protein loading control was performed with Ponceau S staining. Western blot bands were detected by enhanced chemiluminescence (Immobilon® Western Chemiluminescent HRP Substrate; WBKLS0100, Immobilon®) according to the manufacturer's instructions, and images were recorded using an iBright™ CL750 Imaging System (A44116, Invitrogen™).

Statistical Analysis

Statistical analyses of cell data were performed in GraphPad Prism 7.04 (GraphPad Software®) and Microsoft Excel (Microsoft Office®). Comparisons were analyzed by two-way ANOVA followed by Tukey's post hoc test. Results were expressed as average ± standard error of the mean (SEM), and *p*-values were presented for statistically significant results as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$.

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