Food and Chemical Toxicology 134 (2019) 110844

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

The biological response of mesenchymal stromal cells to thymol and carvacrol in comparison to their essential oil: An innovative new study

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ARTICLE INFO

Keywords:
MSCs
Biology
Combination
Medicinal plants
Thymol and carvacrol
Essential oil

ABSTRACT

Mesenchymal stromal cells (MSCs) represent a progenitor cell population with several biological properties. MSCs are thus of therapeutic interest for cell-based therapy but great efforts are needed to enhance their efficiency and safety. Herbal remedies and in particular their bioactive molecules, are potential candidates for improving human health. The novelty and originality of this study is to develop an efficient cell-therapeutic product by combining MSCs with medicinal plant derived bioactive molecules. Thus, the impact of Essential Oil, Thymol and Carvacrol from Ptychotis verticillata on several BM-MSC biological features were studied. These compounds have shown positive effects on MSCs by preserving their morphology, sustaining their viability, promoting their proliferation, protecting them from cytotoxicity and oxidative stress. Accordingly, the combined administration of P. verticillata extract and MSCs may represent a new approach to enhance the therapeutic issue. Further investigations should greatly improve the manufacturing of these compounds as well as our understanding of the therapeutic effects of these bioactive molecules on the biology and functions of MSCs.

1. Introduction

Improvement of our knowledge of cellular therapy based on the use of stem/progenitor cells imply their well-identification and most importantly strengthening their potential. Mesenchymal stromal cells (MSCs) are non-hematopoietic cells with a fibroblast-like shape that can be virtually exist in almost all tissues. Due to their simple and easier isolation procedure as well as their great expansion potential, MSCs are increasingly considered as ideal candidates for different therapeutic applications (Najar et al., 2019a). They present a specific immunological profile and are considered hypo-immunogenic. Accordingly, their use as immunotherapeutic strategies will present new hopes for treating patients with immunological and inflammatory diseases. The therapeutic effect of MSCs is mainly a result of their potent immunomodulatory functions. MSCs are not true immune cells but tissue precursor cells harboring a spectrum of therapeutically active molecules (Najar et al., 2019b). Depending on the signals present in the microenvironment, MSC may differentially influence almost all the cells of the innate and adaptive immune system. Indeed, MSCs are environmentally responsive as they can actively sense their surroundings and modulate accordingly their fate and behavior. By nature, MSCs demonstrate plasticity in their immunomodulatory effects as a way of responding to these challenges (Wang et al., 2014). We have thus demonstrated that BM-MSCs are highly sensitive to inflammation and respond to such signal by properly adjusting their gene and protein expression of regulatory factors (Najar et al., 2019b). Recent strategies

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https://doi.org/10.1016/j.fct.2019.110844
Received 5 July 2019; Received in revised form 4 September 2019; Accepted 24 September 2019
Available online 25 September 2019
0278-6915/

to enhance the function of MSCs have evaluated the influence of different signals to strengthen their immuno-biology and therefore improve their therapeutic efficiency (Kim and Cho, 2016). In line, different medicinal plants with several therapeutic properties are reported as potential candidates for improving human health. Ptychotis verticillata is an aromatic and medicinal plant that has been used in folk medicine, phytopharmaceutical preparations, food preservatives, and as an aromatic ingredient (El Ouariachi el et al., 2011). In this context, the major active component isolated from these plants have been reported to harbor the main bioactive properties. The oil was dominated by phenolic compounds (48.0%) with Carvacrol (44.6%) and Thymol (3.4%) as the main compounds. Classified as phytochemicals, thymol and Carvacrol have shown several effects including antimicrobial and antioxidant activities (Salehi et al., 2018). However, as all the phytochemicals were extracted under different conditions or by using different parts of the plant, it is difficult to evaluate and compare accurately their effect. In the present study, the effects of Ptychotis verticillata derived Essential Oil and its isolated components Thymol and Carvacrol were combined to bone marrow (BM) derived MSCs. We have thus evaluated several biological features of MSCs including morphology, viability, apoptosis, proliferation and cytotoxicity, metabolic activity and oxidative stress response. The novelty and originality of this study is to develop an efficient cell-therapeutic product by combining MSCs with medicinal plant derived bioactive molecules. Further investigations should greatly improve our understanding of the effects of bioactive molecules on the biology and functions of MSCs.

2. Materials and methods

2.1. Cell isolation, culture, expansion and characterization of MSCs

The samples were collected after approval by the local ethics committee and according to the recognized guidelines of the Helsinki Declaration. Informed written consent was obtained for all donors. BM was harvested from the sternum or iliac crest of five healthy volunteers. The mean age of the donors was 33 ± 2 years (range 18–41 years). Mononuclear cells (MNCs) were isolated from bone marrow aspirates by density-gradient centrifugation (Linfosep; Biomedics) and washed in the Hank's buffered salt solution (HBSS; Lonza). MNCs were seeded at a cell density of 2×10^4 cells/cm^2 in a low-glucose DMEM (DMEM-LG; Lonza) supplemented with 15% (v/v) heat inactivated FBS, 2mML-glutamine, and 0.5% (v/v) antibiotic/antimycotic solution (all from Life Technologies). Cells were incubated at 37 °C in a 5% CO_2 humidified atmosphere. After 48 h, non-adherent cells were removed by washing and the medium (DMEM-LG) was changed twice a week. When sub-confluency (80–90%) was achieved, adherent cells were recovered by adding TryplSelect solution (Lonza) and expanded by replating at a lower density (200 cells/cm^2) using DMEM-LG as culture medium.

MSCs were defined according to ISCT criteria (Dominici et al., 2006). First, MSCs were immunophenotypically characterized by flow cytometry using the following monoclonal antibodies (MAb): anti-CD166-fluorescein isothiocyante (FITC; DakoCytomation, Glostrup, Denmark), anti-CD45-FITC and anti-HLA DR-phycoerythrin (PE; Exalpha Biologicals, Maynard, MA, USA), anti-CD34_PE and anti-CD73_PE (BD Biosciences Pharmingen, San Diego, CA, USA), anti-CD14-PE, anti-CD105-FITC and anti CD90-PE (R&D Systems, Minneapolis, MN, USA). Secondly, MSCs were evaluated for their multilineage potential by culturing them in appropriate induction medium to assess adipogenic, osteogenic and chondrogenic differentiation.

3. Essential oil, thymol and carvacrol combined to MSCs

3.1. Essential oil isolation

Fresh vegetal material was water distillated (3 h) using a clevenger-type apparatus according to the method recommended in the European Pharmacopeia. The essential oil yields were 2% (w/w). They were dried over anhydrous sodium sulfate and then stored in sealed glass vials at 4–5 °C prior to use.

3.2. Preparation of essential oil, carvacrol and thymol solutions

1. **Essential Oil**: 50 µl of pure essential oil was dissolved in 50 µl dimethylsulfoxide (DMSO, Merck, Germany) and diluted (0.01%, 0.025%) with culture medium before experiments.
2. **Carvacrol**: 100 µl of pure Carvacrol was dissolved in 900 µl DMSO and diluted (6 µM and 25 µM) with culture medium before experiments.
3. **Thymol:** 100 mg of Thymol powder was dissolved in 1 ml DMSO and diluted (3 µg/ml and 6 µg/ml) with culture medium before experiments.

4. MSCs and drug combination

The different solutions of Essential Oil, Carvacrol and Thymol were added to BM-MSC culture for 24 h and 72 h of incubation.

5. Viability assay

The viability was determined using Trypan blue exclusion assay (Lonza) and 7 amino-actinomycin (7-AAD) staining assay (BD Biosciences). After drug incubation, the supernatants of the culture were removed and the cell layer washed with PBS (Lonza). The adherent cells was then recovered by adding TryplSelect solution (Lonza) and washed by centrifugation. The cell pellet was re-suspended and stained with 0.4% trypan blue solution (Lonza). We calculated the % of cell viability as follow = [1.00-(Number of blue cells ÷ Number of total cells)] × 100. We also evaluated the viability by using the BD Via-Probe™ viability staining solution (7-AAD). Absolute volumetric cell counting was performed with the MACSQUANT® flow cytometer.

6. Apoptosis assay

Apoptosis was determined by staining of the cells with Annexin V-FITC (Biosource International, Camarillo, CA, USA) and propidium iodide (PI), according to the instructions of the manufacturer, and analyzed using by flow cytometer (Miltenyi Biotec) as previously reported (Fayyad-Kazan et al., 2016).

7. MTS assay

The CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega Benelux) is a colorimetric method for determining the number of viable cells in proliferation, cytotoxicity or chemosensitivity assays. The solution contains a yellow tetrazolium compound that is metabolized by viable cells to purple formazan crystals. Briefly, after 24 h of adherence (removing non-adherent cells), MSCs were recovered from the petri dishes and seeded in flat-bottom 96-well plates and treated with the different solutions of Essential Oil, Carvacrol and Thymol for 24 h and 72 h of incubation at 37 °C in a 5% CO_2 atmosphere. After the indicated incubation period, 10 µl of MTS solution was added to each well and the incubation was continued for an additional 4 h. Then, the absorbance of each well at 485 nm was determined on a microplate enzyme-linked immunosorbent assay (ELISA) reader. The quantity of formazan product as measured by the amount of absorbance is directly proportional to the number of living cells in culture. The experiments were performed in triplicate wells and repeated at least three times. Viability percentage was calculated by the following formula: [(absorbance of treated cells/absorbance of corresponding control) × 100.

8. ROS assay

Reactive oxygen species (ROS) are measured by using 2′-7′-Dichlorodihydrofluorescein diacetate (DCF-DA) probe (Sigma) which is a cell-permeable non-fluorescent probe that upon oxidation is de-esterified intracellularly and converted to highly fluorescent 2′,7′-dichlorofluorescein (DCF). Briefly, after the related culture and drug combination steps, MSCs are recovered and stained with 15 μM DCF-DA probe for 15 min at 37 °C according to manufacturer's guidelines. The cells were then washed with PBS (Lonza) and subjected to a flow cytometric analysis of DCF fluorescence intensity as a measure of ROS production.

9. $DiOC_6(3)$ assay

$DiOC_6(3)$ (3,3′-Dihexyloxacarbocyanine Iodide) (Thermo Fisher Scientific) is a cell-permeant, green-fluorescent, lipophilic dye used to discriminate functional cellular states by detect mitochondrial membrane potential. Briefly, after the related culture and drug combination steps, MSCs are recovered and washed twice with PBS. Then, the cells are re-suspended in 100 μl of buffer, stained with 5 μl of $DiOC_6(3)$ and incubated at 37 °C for 15 min. Before flow cytometric analysis, the cells are washed and re-suspended in 100 μl of buffer.

10. Flow cytometry

Data were acquired and analyzed on a MacsQuant analyzer (Miltenyi Biotec).

11. Statistical analysis

Data are presented as the mean ± SEM of at least seven independent experiments and were analyzed using the paired Wilcoxon test.

12. Results and discussion

Mesenchymal stromal cells (MSCs) represent a progenitor cell population with several biological properties including a high potential to self-renew. Although, they have been traditionally isolated from bone marrow, MSCs can be derived from a variety of tissues (Najar et al., 2019a). In addition to their tissue regenerative capacities, MSCs can display immunological features. MSCs are promising for different therapeutic applications but great efforts are requested to ensure the efficiency and safety of such cell-based therapy (Naji et al., 2019). Their popular appeal as cell-based repair therapy was initially based on their in vitro multilineage potential; however, there is lack of evidence of their differentiation to target cells in vivo. The failure of some MSC-based therapy in both animal models and human clinical trials may be explained by either lack of a proper activation signal for MSCs or a wrong timing and site in MSC administration (Krampera, 2011). Several strategies to strength the therapeutic potential of MSCs including drug-based combination approaches have been proposed (Kim and Cho, 2016). Thus, optimization of MSC use for therapeutic purposes is required to maximize their beneficial effects. A functional improvement of MSCs might be obtained after licensing (preconditioning or tailoring) these cells by different stimuli (Doorn et al., 2012). Inflammatory cytokines and TLR triggering described as important methods to license MSCs have shown contrasting results in the literature (Najar et al., 2017, 2018).

Nowadays, combination therapy based on the use of MSCs and either a cell-product (e.g. Tregs) or a medicinal drug is considered as an interesting tool to establish a potent therapeutic approach. Thus, combining adoptive Treg transfer with BM transplantation has shown great potency in preclinical animal models in terms of both safety and efficacy to induce immunological tolerance (Pilat et al., 2017). Indeed, it has been demonstrated that Treg facilitate MSC-based immunomodulation (Lee et al., 2015) as well as tissue healing (Li et al., 2018) by inhibiting local T-cell response. In the other hand, the interactions between MSCs and the combination of drugs in the recipient's body must be seriously considered (Nemeth, 2014). In this way, MSC therapy in combination with anti-cancer medicines/drugs may offer higher efficacy and lower toxicity to the patient compared to the existing therapeutic strategies (Hendijani and Javanmard, 2015). Due to their immunological plasticity, MSCs may potentiate the cytotoxic effects of these drugs through their immune-onco-regulatory protein-rich secretome. In parallel, because of their regenerative and healing capacities, MSCs protect and/or rescue normal as well as injured tissues from adverse cytotoxic drug reactions or systemic pathological events that occur during cancer treatment. Although such combinatory strategies have demonstrated some interesting results, its effectiveness has been hindered by several inherent shortcomings regarding safety (cytotoxicity) and efficacy (microenvironment influences). The extensive use of synthetic and semi-synthetic substances, i.e., recombinant cytokines and growth factors as proliferative and trophic (regenerative) mediators in cell therapy, may lead to side effects and toxicity while being exorbitantly expensive (Udalamaththa et al., 2016). Accordingly, alternative solutions that ensure safety and efficiency such as natural compounds should be investigated. Herbal remedies have been used in traditional medicine practices for centuries for a wide range of diseases and are a promising alternative, offering substantial improvement of patient conditions, although their mechanisms of action mostly remain undetermined. Indeed, medicinal plant are a reservoir of several bioactive molecules reported to be potential candidates for improving human health. As reviewed (Udalamaththa et al., 2016), several types of MSCs treated with individual or mixtures of herbal extracts, as well as with their purified compounds have resulted in increased proliferation, cell attachment and delayed senescence. These effects may vary due to cell type, inoculation number, culture conditions and product concentration (Xue et al., 2018).

Both synthetic and herbal effectors have their advantages and disadvantages for MSCs. Using different solvents and methods to prepare phytochemicals may produce different outcomes and even can lead to adverse effects. The lack of achieving predict clinical efficacy and quality control has been the major impediment to its in vivo application. Clinical applications are also challenging due to the variability and complexity of bioactive molecule formulations. With regards to this, we aimed to study the effects of Thymol and Carvacrol, two compounds derived from the medicinal plant Ptychotis verticillata (El Ouariachi el et al., 2011), known for their therapeutic properties (Salehi et al., 2018), on several biological features of MSCs. We found that the effects are depending on the type, concentration and time of compound incubation with MSCs. Thymol and Carvacrol preserved the morphology of MSCs, sustained their viability, were pro-proliferative, not cytotoxic and not inducer of oxidative stress.

To begin, cellular therapy implies the use of a well-characterized cell-product in order to guarantee the safety and the success rate of the strategy. BM remains a major source of MSCs in most investigations with significant progress in their biology study as well as their widespread clinical application. Due to their simple and easier isolation procedure as well as their great expansion potential, MSCs are ideal candidates for different cellular therapies. Thus, we have cultured and characterized MSCs according to the ISCT criteria. ***Culture and Characterization of MSCs:*** MSCs derived from BM was expanded in culture and presented a fibroblastic-like morphology. MSCs displayed a high capacity to adhere to plastic. Immuno-phenotyping of the cells by flow cytometry revealed their positivity (> 95%) for CD73, CD90 and CD105 and negativity (< 5%) for CD14, CDthe19, CD34, CD45 and HLA-DR markers (data not shown). These MSCs are not immune cells or hematopoietic cells but stromal progenitors with a specific profile and role (Ankrum et al., 2014). The culture must be cell-type homogenous, purified and not immunogenic to help in minimizing host rejection

during cell therapy. Also, the understanding the product profile of the intended therapy is crucial to the development of the nonclinical safety study design (Sharpe et al., 2012). Historically (Pittenger et al., 1999), MSCs were defined as multipotent cells that can replicate as undifferentiated cells and that have the potential to differentiate to different lineages (multilineage potential). Based on the literature,.it has been demonstrated that the multipotency of MSCs is not a pivotal aspect of cell therapy and thus primarily referred to their paracrine function as a major activity in tissue repair. Indeed, MSCs are reported as promoters, enhancers, and playmakers of the translational regenerative medicine. Although cell replacement is an essential component of MSC-based therapy, their therapeutic effect is mainly due to their immunomodulatory functions. Stem cells can regulate the immune microenvironment during tissue repair and provide a good "soil" for tissue regeneration (Li et al., 2019). Such definition and characterization is important to be performed as they ensure a standardization process which would allow comparison across multiple studies and could facilitate potential clinical (Mendicino et al., 2014). Using phase contrast microscopy, we verified the impact of the drug combination on the morphology of MSCs. Morphological characteristics indicate the yield of early progenitors and represent a quality control for MSC culturing (Haasters et al., 2009). ***Morphology of MSCs after the drug combination:*** Despite the heterogeneous nature of MSC population that is apparent upon examination of the individual cell morphologies, the treatment has not affected the morphology of the cells. As shown in Fig. 1, none of the drug products has significantly altered the shape of the cells in culture, which maintained their typical fibroblastic appearance with an enlarged size and an agranular cytoplasm. Indeed, MSCs represent a population of cells with distinct self-renewing capacities. These observations suggest that the heterogeneity of MSC cultures is due to different mesenchymal progenitors. BM stroma is therefore a source of highly clonogenic MSCs suitable for our experiments (Smith et al., 2004). However, the safety of cell-therapy depends on many factors including the proliferation capacity of the cells and their long-term survival and/or engraftment. Indeed, MSCs after in vivo transplantation, may face an ischemic microenvironment characterized by nutrient deprivation and reduced oxygen tension that greatly reduces their viability, thus limiting their therapeutic potential (Ferro et al., 2019). Thus, we must provide MSCs with high viability rate to ensure that they are therapeutically active. ***Viability of MSCs after the drug combination:*** By trypan blue exclusion and 7-AAD staining, we have addressed the viability of MSCs and the influence of the drug. After 24H, only high-concentrated solution of Essential Oil (0.025%), Carvacrol (25 μM) and Thymol (6 μg/ml) compared to those of low-concentrated (0.01%), (6 μM) and (3 μg/ml) ones have increased the number of viable cells counted by trypan blue (Fig. 2 A). After 72H, the effect is inversed, as low-concentrated solution has slightly increased the number of viable cells. Furthermore, the Annexin/7-AAD flow cytometry analysis corroborated this observation by showing that the viability of MSCs following the drug combination is enhanced in a time- and dose-dependent manner (Fig. 2B). Therefore, increased survival of MSCs by these compounds may be associated with improving therapeutic efficacy. In agreement with our results, supplementing culture medium of adipose tissue-derived stem cells with Thymol-rich essential oil of Lippia origanoides increased the viability and cell proliferation (Brito et al., 2018). In line, Thymol/Carvacrol and the essential oil have been shown to improve wound healing by stimulating tissue development as well as promoting survival and growth of fibroblasts and keratinocytes (Costa et al., 2019). When reported, MSC engraftment is generally low and transient in nature. Priming MSCs by such bioactive molecules would be of benefit to increase MSC survival, integration and functionality when transplanted in vivo. Another issue related to MSC-

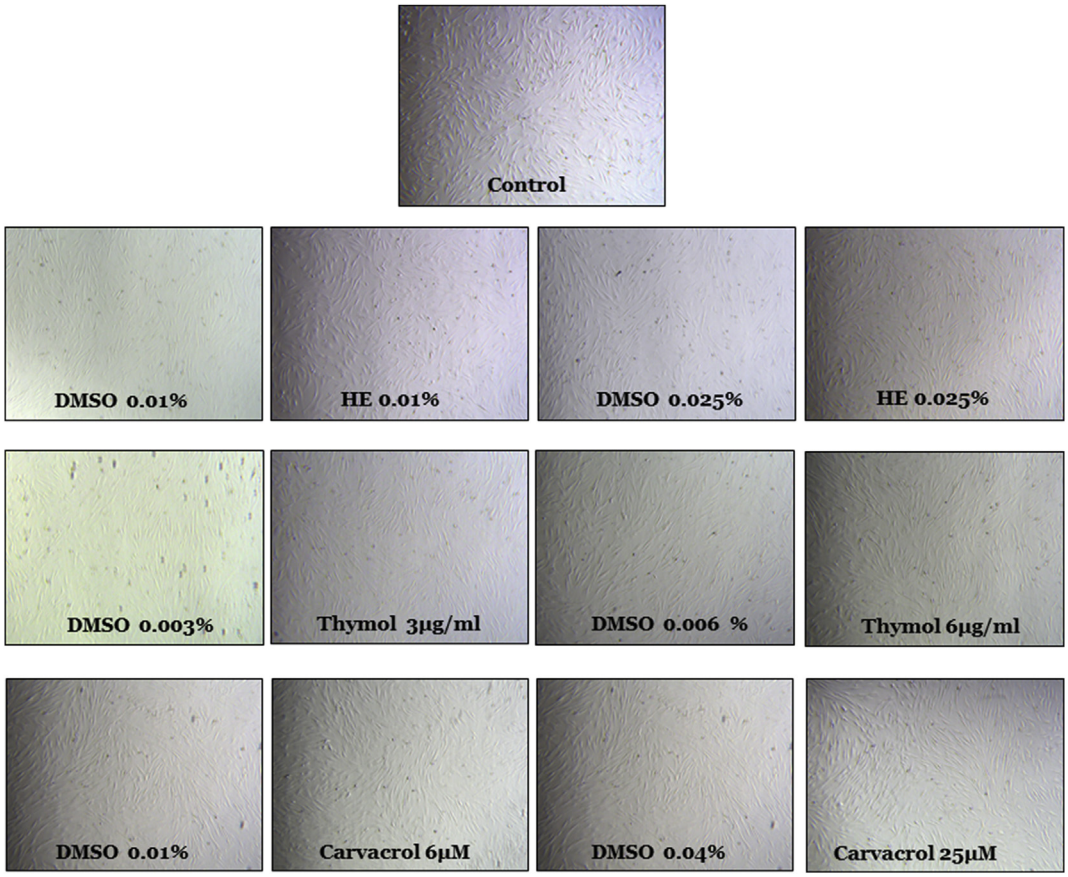

Fig. 1. Morphology of MSCs. MSCs were cultivated, under the indicated time, alone or in the presence of different concentrations of Essential Oil, Thymol and Carvacrol. The Morphology of MSCs was determined by using a microscope (100× objective).

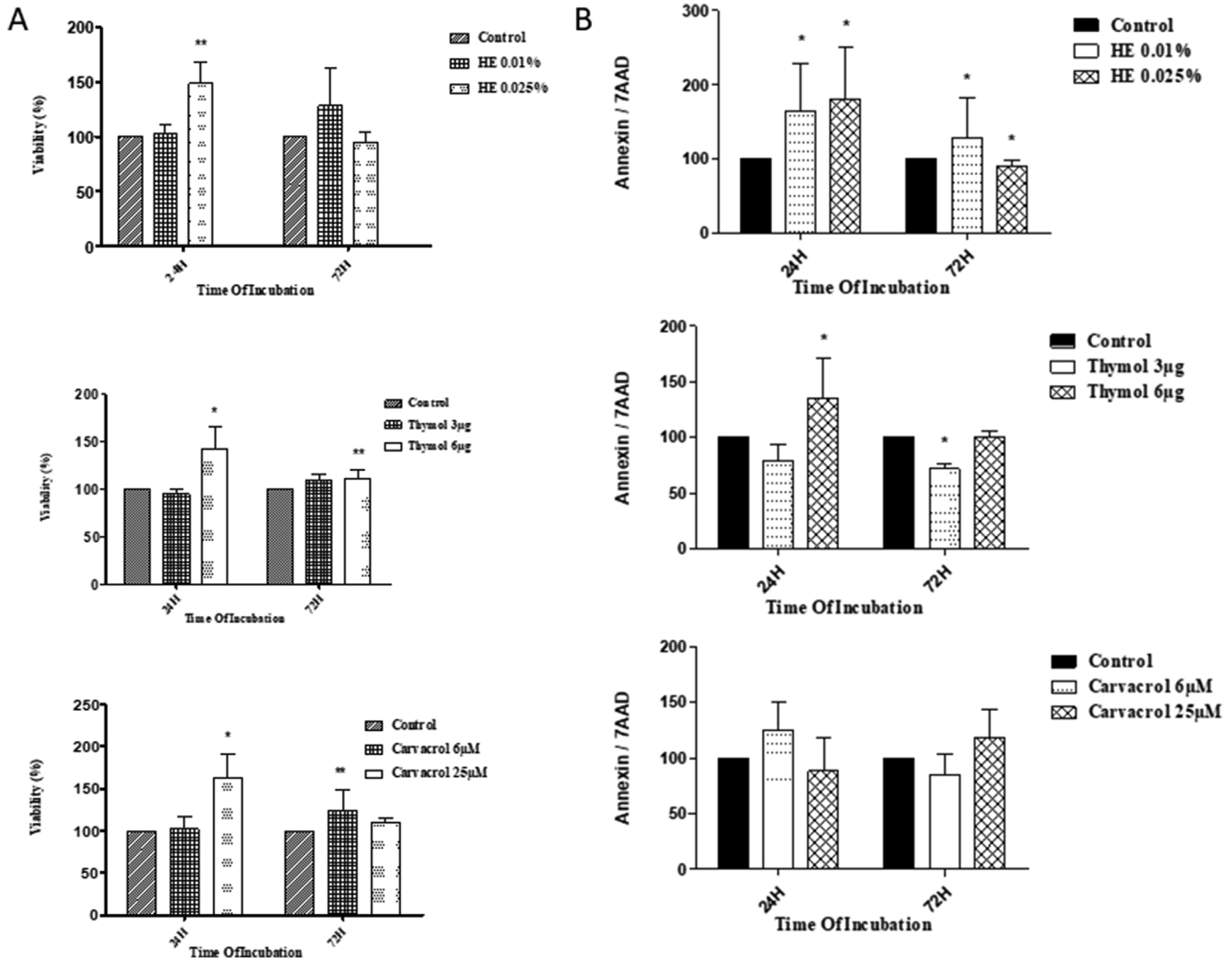

Fig. 2. Viability of MSCs. MSCs were cultivated, under the indicated time, alone or in the presence of different concentrations of Essential Oil, Thymol and Carvacrol. The viability of MSCs was measured by trypan blue exclusion assay (A) and by Annexin/7 amino-actinomycin (7-AAD) staining assay (B). The data are presented as the mean ± SEM.

based therapy, is the maintaining of a proliferative and non-cytotoxic cell-product. ***Proliferation and cytotoxicity of MSCs after the drug combination:*** The MTS assay was used to determine the number of proliferating cells and their cytotoxicity state. The drug combination differentially affected the MTS assay in a dose- and time-depending manner (Fig. 3). During the time, both Essential Oil and Thymol concentrations have increased MSC proliferation rate without any sign of cytotoxicity. In contrast, Carvacrol after 48H induced a decrease in the proliferative capacity of MSCs while the effect was reversed after 72H by obtaining an increase in the cell number. In this context, the stem cell yield after culture is of utmost importance (Najar et al., 2014). Indeed, ex vivo cell amplification is essential for exploration of clinical applications of MSCs. Expansion to a quantity of more than 10^8 cells is therefore a minimal prerequisite for therapeutic use. In general, such compounds have potent cytotoxic effect against cancer cells (Slamenova and Horvathova, 2013). Interestingly, MSCs have not shown cytotoxicity after exposure to P. verticillata extracts. In contrast, Carvacrol and Thymol from oregano essential oils have caused cellular damages and alterations on the intestinal cells line Caco-2 (Llana-Ruiz-Cabello et al., 2014). Cell-based therapies show some limitations due, at least in part, to the death of transplanted cells following a combination of environmental, cellular, and host factors (Baldari et al., 2017). Therefore, it is important to prevent BM-MSCs from apoptosis. ***Mitochondrial membrane potential of MSCs after the drug combination:*** The assessment of the mitochondrial membrane potential in intact cells is important considering the role of mitochondria during apoptosis (Salvioli et al., 1997). Flow cytometric analysis of MSCs stained with DiOC6(3) indicated that their mitochondrial membrane potential was slightly modulated by the drug in a time- and dose-dependent manner (Fig. 4). After 24H, the mitochondrial membrane potential was not significantly altered independently of Essential Oil, Carvacrol and Thymol concentrations. After 72H, the effect tends to decrease the mitochondrial membrane potential of MSCs in a drug-type dependent manner. Both low (3 µg/ml) and high (6 µg/ml) concentration of Thymol reduced the mitochondrial membrane potential as shown by the decrease in DiOC6(3) MFI. Essential Oil and Carvacrol had contrasted effects as low concentrations slightly reduced DiOC6(3) MFI whereas high concentrations tended to increase it. The beneficial effect of cell therapy ultimately depends on the number of administered cells reaching the target tissue, their viability, and their therapeutic functions. Therefore, strategies aiming at improving viable cell engraftment are crucial for cell-therapy. It has been found that BM-MSCs undergo apoptosis in ischemic and hypoxic environment affecting thus their therapeutic effects (Chen et al., 2018). The proportion of apoptotic and

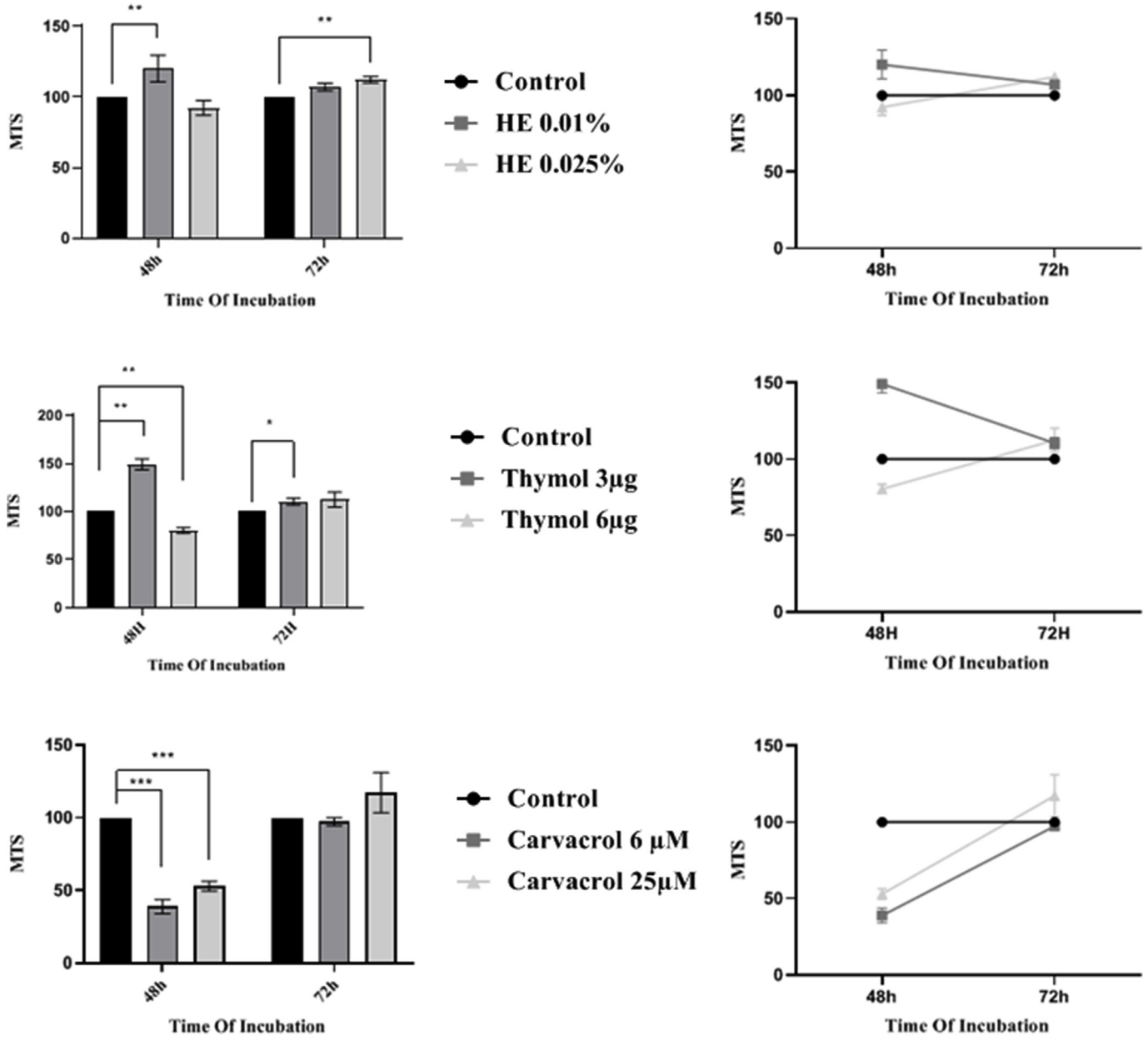

Fig. 3. Proliferation and cytotoxicity of MSCs. MSCs were cultivated, under the indicated time, alone or in the presence of different concentrations of Essential Oil, Thymol and Carvacrol. The proliferation and cytotoxicity of MSCs were measured by MTS assay. The data are presented as the mean ± SEM.

senescent cells increases during cryopreservation in comparison with fresh live culture. Not only cell viability but also alteration of MSC characteristics is to be considered during cell-therapy (Tomoki, 2017). Although the suppressive effect of MSCs can take place independent of their viability (functionally compromised, apoptotic, or dead MSCs) through their phagocytosis by immune cells (efferocytosis), there are limitations to such process (Galipeau and Sensebe, 2018). Considering that the therapeutic efficacy of apoptotic MSCs is substantially less than that of live MSCs suggests that, in addition to efferocytotic clearance, biological fitness of MSCs and their functionalities play an important role in immunomodulation. Of importance, MSCs are environmentally sensitive to the signals present in their neighborhood and may thus adapt their profile and functions to ensure the right response (Murphy et al., 2013). As discussed previously, the poor viability of the transplanted MSCs is a major challenge of stem cell-based therapy. Excessive stressors may increase the concentration of reactive oxygen species (ROS), causing lipid peroxidation and oxidative damage to cellular membranes. The lack of sufficient antioxidants to eliminate ROS will lead to oxidative damage and trigger inflammation (Lee et al., 2017). Therefore, determining the impact of oxidative stress to MSC biology is required as they may lead to loss of transplanted MSCs from the injured sites. ***Oxidative response of MSCs after the drug combination:*** The generation of ROS was measured by DCF-DA staining. Although MSCs were able to respond to oxidative stress by increasing the generation of ROS, Essential Oil, Carvacrol and Thymol, independently of the drug time and dose, have not accentuated this ROS generation in comparison to DMSO (Fig. 5). ROS are generated during mitochondrial oxidative metabolism as well as in cellular response to damage/injury stimuli. The oxidative stress occurs when ROS overwhelm the cellular anti-oxidant defense system inducing therefore damage of nucleic acids, proteins, and lipids which is implicated in various disease (Ray et al., 2012). Following infusion, the survival rate of transplanted MSCs is very low. Indeed, in the injured tissue, impoverished blood supply, low oxygen pressure, and inflammation may generate oxidative stress, as

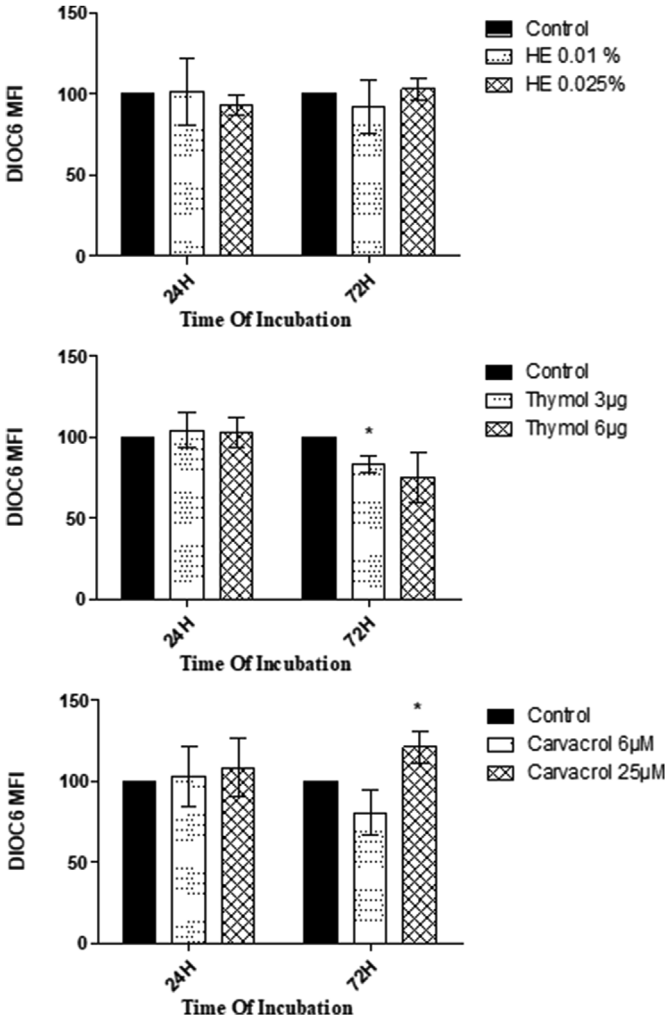

Fig. 4. Mitochondrial membrane potential of MSCs. MSCs were cultivated, under the indicated time, alone or in the presence of different concentrations of Essential Oil, Thymol and Carvacrol. The Mitochondrial membrane potential was determined by the DiOC6(3) labelling. The data are presented as the mean ± SEM.

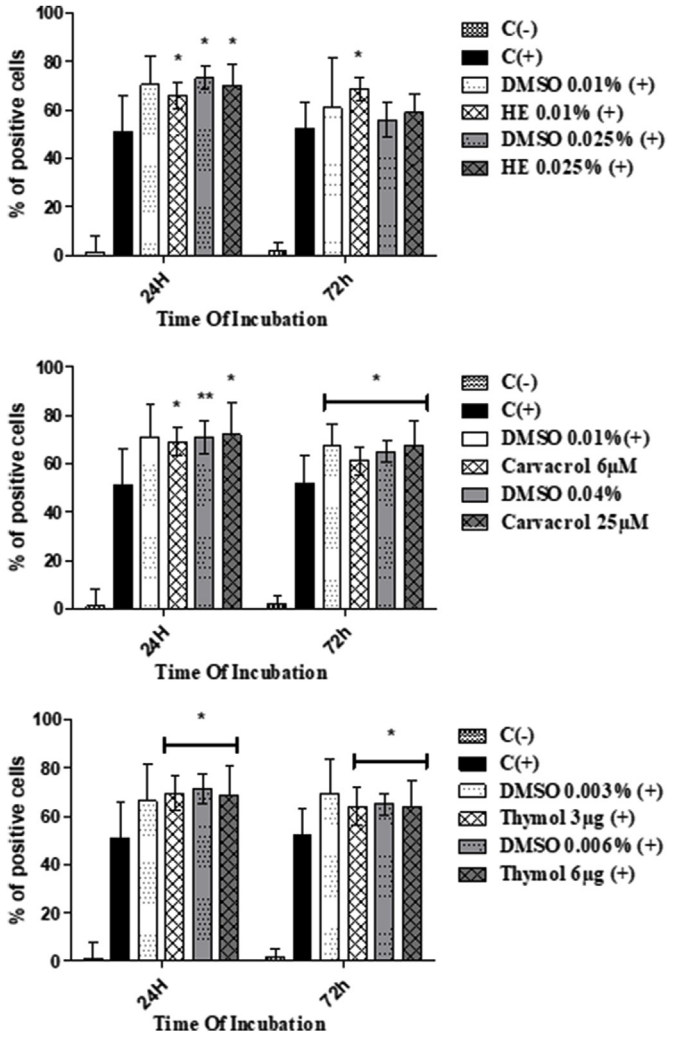

Fig. 5. Oxidative response of MSCs. MSCs were cultivated, under the indicated time, alone or in the presence of different concentrations of Essential Oil, Thymol and Carvacrol. The Oxidative response of MSCs was evaluated by the DCF-DA labelling. The data are presented as the mean ± SEM.

well as cytotoxic radicals and proteins. Such a pro-apoptotic microenvironment may cause the death of transplanted cells through several different mechanisms, including apoptosis. Herein, P. verticillata derived compounds have not induced oxidative stress in MSCs. Indeed, the solvent extracts and essential oil of P. verticillata were found to be effective antioxidants (Horvathova et al., 2006). Carvacrol and Thymol were reported to reduce the level of DNA lesions induced by H_2O_2 which can be associated with their antioxidative activity (El Ouariachi el et al., 2011).

Collectively, we think that combining MSCs and P. verticillata derived compounds particularly Thymol and Carvacrol, will provide a synergistic and more efficient therapeutic effect. We observed that such compounds have not negatively interfered with MSC biology. Thymol and Carvacrol preserved the morphology of MSCs, sustained their viability, were pro-proliferative, not cytotoxic and not inducer of oxidative stress. The combined administration of P. verticillata extract and MSCs may represent a new approach to enhance the therapeutic effects of MSCs. Specifically, by reducing T-cell immune response (Gholijani and Amirghofran, 2016) and oxidative stress (Calio et al., 2014), Carvacrol and Thymol may promote survival, anti-apoptotic, cytoprotective, and immunomodulatory effects that will improve healing and repair of injured tissue by MSCs. However, the variability and complexity of bioactive constituents present in herbal formulations represent a real challenging for the field. Using different solvents and methods to prepare phytochemicals may also influence the quality and the quantity of bioactive constituents, which may lead to different outcomes and undesirable side effects. Accordingly, standardized protocols and regulated characterization of for plant-derived products are needed to ensure quality, efficacy, safety, and reproducibility of their therapeutic effects.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank Karlien Pieters for her technical assistance. This study was supported in Belgium by 'Le Fonds National de la Recherche Scientifique (FNRS)', 'Le Télévie', 'Les Amis de l'Institut Jules Bordet'; in Morocco by 'Le Centre National pour la Recherche Scientifique et Technique (CNRST)' and 'L'Agence Nationale des Plantes Médicinales et Aromatiques (ANPMA)'.

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