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Methylsulfonylmethane and mobilee prevent negative effect of IL-1β in human chondrocyte cultures via NF-κB signaling pathway

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ABSTRACT

Nutraceuticals are compounds that serve as nutrition with an easy accessibility and favourable safety profile. Recent studies showed their potential activity on osteoarthritis (OA) inflammation and cartilage metabolism.

We investigated the effect of methylsulfonylmethane (MSM) and mobilee in human OA chondrocyte cultures exposed to interleukin (IL)-1β.

OA cartilage was obtained from femoral heads of five patients undergoing total replacement surgery. Chondrocytes were incubated with mobilee (200 and 500 μM) and MSM (2000 and 6000 μM) in presence of IL-1β (10 ng/mL) and nuclear factor (NF)-κB inhibitor (BAY 11-7082, 1 μM), for 24 and 48 h. Viability and apoptosis were performed by MMT and flow cytometry. The metalloproteinase (MMP)-1,-3,-13 and type II collagen (Col2a1) were analyzed by qRT-PCR and ELISA, and NF-κB activation by immunofluorescence.

IL-1β stimulus determined a significant regulation of survival, apoptotic ratio, as well as of gene expression and serum levels of MMP-1,-3,-13 and Col2a1 in OA chondrocytes compared to baseline. Mobilee and MSM incubation significantly reversed the effect of IL-1β. IL-1β significantly induced NF-κB p50 nuclear translocation, which was significantly counteracted by the pre-treatment of OA chodrocytes with the tested compounds. BAY11-7082 significantly modulated MMPs and Col2a1 expression respectively to basal state. Co-treatment of IL-1β with mobilee, MSM and BAY11-7082 didn't cause changes of MMPs or Col2a1 beyond that caused by each single treatment.

We demonstrated that MSM and mobilee have a beneficial effect on OA chondrocytes metabolism, probably due to the modulation of NF-κB pathway, providing a powerful rationale for the use of these substances in OA treatment.

1. Introduction

Osteoarthritis (OA) is the most common degenerative joint disorder and the primary cause of chronic disability, impairment and reduced quality of life; its prevalence increases dramatically along with the rise of life expectancy [1]. OA is characterized by physical and chemical degenerative changes in the joint tissue, resulting in a progressive destruction of articular cartilage, remodeling of the underlying subchondral bone, and a state of chronic, low-grade inflammation, leading to the loss of function and pain [2].

Currently available treatment options for OA management focus on symptom relief and includes acetaminophen, traditional nonsteroidal anti-inflammatory drugs (*t*NSAIDs), selective cyclooxigenase-2 inhibitors (*coxibs*), opioids and duloxetine [3]. They only provide temporary and limited benefit, improving functionality and pain, but have potential safety concerns. Moreover, they do not impact on the progression of the disease nor have clinically relevant chondroprotective effects [4].

Nutraceuticals are compounds that serve as nutrition and showed potential symptomatic effects in OA [5,6]. Furthermore, nutraceuticals are easily accessible and are perceived by the patients as safe products in the chronic treatment too [7].

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Recent studies have shown the ability of some nutraceuticals to contribute in symptoms and modifying OA disease on the basis of their anti-inflammatory, anti-catabolic actions, and protective effects against oxidative stress [8,9]. A novel dietary supplement containing methylsulfonylmethane (MSM), mobilee® (60% hyaluronic acid sodium salt), myrrh, boswellia, group B vitamins, zinc, copper, selenium and vitamin C was authorized in Europe for inflammatory and degenerative joint diseases.

MSM is an organosulfur molecule that occurs naturally, in very low amounts, in grains, fruits, vegetables and in milk; *in vitro* and *in vivo* studies demonstrated its anti-inflammatory properties and its activity in free radicals scavenging [10–12]. MSM is generally used alone or in combination with other supplements for the treatment of OA [13,14]. Mobilee, is the commercial name of a rooster comb extract, rich in hyaluronic acid, polysaccharides and collagen, showing beneficial effects on joint and muscle function [15].

In the present *in vitro* study, we analyzed the effect of MSM and of mobilee in human OA chondrocyte cultures in presence of a negative stimulus such as interleukin (IL)-1β. The substances were tested alone or in combination. The cell viability and the ratio of apoptotic cells were evaluated; the main matrix degrading enzymes metalloproteinase (MMP)-1, MMP-3, MMP-13 and collagen type II alpha 1 chain (Col2a1) were analyzed at their expression levels as well as at their release in the supernatant. Finally, the possible activation of nuclear factor (NF)-κB by means of immunofluorescence and semi-quantitative analyses was detected.

2. Material and methods

2.1. Harvest of human osteoarthritic cartilage and isolation of chondrocytes

OA human articular cartilage was obtained from the femoral heads of five patients (two men and three women) with hip OA defined by clinical and radiological American College of Rheumatology criteria [16], undergoing total hip replacement surgery; OA grade ranged from moderate to severe and cartilage showed typical osteoarthritic changes such as the presence of chondrocyte clusters, loss of metachromasia, and fibrillation (Mankin degree 3–7) [17]. OA chondrocytes originated from the area adjacent to the OA lesion. The femoral heads were provided by Orthopaedics Surgery, University of Siena, Italy. The mean age of the patients was 69 years (range, 63–75 years). The ethics committee of the Azienda Ospedaliera Universitaria Senese/Siena University Hospital approved the use of human articular specimens (decision no. 726/07). The patients signed an informed consent.

Following surgery, the cartilage was aseptically dissected and minced into small pieces. The fragments were washed in Dulbecco's Modified Eagle's medium (DMEM) (Euroclone, Italy) with phenol red, containing 2% penicillin/streptomycin solution (Euroclone, Italy) and 0.2% amphotericin B (Euroclone, Italy). The chondrocytes were isolated from the articular cartilage using sequential enzymatic digestion: 30 min with 0.1% hyaluronidase (Sigma-Aldrich, Italy), 1 h with 0.5% pronase (Sigma-Aldrich, Italy), and 1 h with 0.2% collagenase (Sigma-Aldrich, Italy) at 37 °C in the wash solution (DMEM + penicillin/streptomycin solution + amphotericin B). The resulting cell suspension was filtered twice by using 70-μm nylon meshes, then washed and centrifuged for 5 min at 700*g*. The Trypan blue viability test pointed out a 90% to 95% cell survival. Cells were incubated, for 2 weeks, at 37 °C and 5% CO_2 in DMEM culture medium containing 10% fetal bovine serum (FBS) (Euroclone, Italy), 200 U/mL penicillin and 200 μg/mL streptomycin. The medium was changed three times per week. The cell morphology was examined daily with an inverted microscope (Olympus IMT-2, Japan) to avoid the dedifferentiation of expanded chondrocytes and to preserve their phenotypic stability.

For each single experiment, cells culture from a unique donor were used.

2.2. Pharmacological treatment of chondrocyte cultures

In the first passage OA human chondrocytes were seeded in 6-well dishes at a starting density of 6×10^6 cells/well until they became confluent. Mobilee and MSM were purchased from Laborest s.r.l., Italy. The powders of mobilee and MSM were first dissolved in the culture medium and in water respectively, and then they were diluted in culture medium immediately before treatment to achieve the final concentration required. The cells were treated for 24 h and 48 h with mobilee (200 μM and 500 μM) (Laborest s.r.l., Italy) and MSM (2000 μM and 6000 μM) (Laborest s.r.l., Italy) in presence or in absence of IL-1β (10 ng/mL) (Sigma-Aldrich, Italy); the treatment was also performed analyzing the combination of both substances at the two tested concentration for 24 h and 48 h in presence or in absence of IL-1β.

After the treatment the media were removed, cleared through centrifugation, and stored at −80 °C to subsequently perform a sandwich enzyme-linked immunosorbent (ELISA) assay for MMP-1, MMP-3, MMP-13 and Col2a1; the chondrocytes were immediately processed to evaluate their viability, the ratio of apoptosis, the gene expression of MMP-1, MMP-3, MMP-13 and Col2a1, as well as the activation of NF-κB signaling pathway.

Afterwards, the cells were pre-incubated for 2 h with 1 μM BAY 11-7082 (NF-κB inhibitor, Sigma-Aldrich, Italy) and then treated 24 h with the tested concentrations of mobilee and MSM in presence of IL-1β. Subsequently, the gene expression of MMP-1, MMP-3, MMP-13 and Col2a1 was evaluated.

2.3. MTT assay

Immediately after the treatment, cell viability was evaluated by MTT assay. Chondrocytes were incubated for 3 h at 37 °C in a culture medium containing 10% of 5 mg/mL MTT (3-[4,4-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide) (Sigma-Aldrich, Italy). The medium was then discarded and 0.2 mL of Dimethyl sulfoxide (DMSO) (Rottapharm Biotech, Italy) was added to each well to solubilize the formazan crystals. The absorbance was measured at 570 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Blank measurement was performed using a well without cells.

The percentage of cell survival was calculated as follows:

$$\%\text{Survival} = (\text{Absorbance of test})/(\text{Absorbance of control}) \times 100$$

The experiments were carried out on pre-confluent cell cultures to prevent contact inhibition influencing the results. Data were expressed as OD units per 10^4 adherent cells.

2.4. Detection of apoptotic cells

OA chondrocyte cultures were plated in 12-well plates (8×10^4 cells per well) during 24 h in DMEM with 10% FBS. Then, the medium was discarded and the cells were cultured in DMEM with 2% FBS normally used for the treatment. The positive control was incubated in presence of staurosporine, at 0.2 μM (Sigma-Aldrich, Italy) during 2 h to induce apoptosis. Afterward, the cells were washed and harvested with trypsin, collected into cytometry tubes and centrifuged at 1500 rpm for 10 min. The supernatant was removed and the pellet was resuspended in 100 μL of 1 × Annexin-binding buffer; 5 μL of Alexa Fluor 488 Annexin-V conjugated to fluorescein (green fluorescence) and 1 μL of 100 μg/mL propidium iodide (PI) working solution were added to each 100 μL of cell suspension (ThermoFisher Scientific, Italy). Cells were incubated at room temperature for 15 min in the dark. After incubation, 600 μL of 1 × Annexin-binding buffer was added before being analyzed by flow cytometry. A total of 1×10^4 cells per assay (10,000 events) were measured at a flow cytometer. The obtained data were analyzed with Cell Quest software (Becton Dickinson). The evaluation of apoptosis was performed considering staining cells simultaneously with Alexa Fluor 488 Annexin-V and PI, allowing the

discrimination of intact cells (Alexa Fluor Annexin-V and PI negative), early apoptotic state (Alexa Fluor Annexin-V positive and PI negative) and late apoptosis state (Alexa Fluor-Annexin-V and PI positives) [18]. Results were expressed as percentage of positive cells to each dye (total apoptosis) and represented the mean of three independent experiments (mean ± standard deviation, SD).

2.5. RNA isolation and RT-qPCR analysis

Total RNA was extracted using TriPure Isolation Reagent according to the manufacturer's instructions (Roche Diagnostics GmbH, Germany) and was stored at −80 °C. The concentration, purity and integrity of RNA were evaluated by measuring the OD at 260 nm and the 260/280 and 260/230 ratios by Nanodrop-1000 (Celbio, Italy). The quality of RNA was also checked through electrophoresis on agarose gel (FlashGel System, Lonza, Rockland, ME, USA). Reverse transcription for target genes was performed by using QuantiTect Reverse Transcription Kit (Qiagen, Hilde, Germany) according to the manufacturer's instructions.

Target genes were analyzed by real-time PCR using QuantiFast SYBR Green PCR (Qiagen, Hilde, Germany) kit. All qPCR reactions were performed in glass capillaries using a LightCycler 1.0 (Roche Molecular Biochemicals) with LightCycler Software Version 3.5. The reaction procedure for target genes amplification consisted in: 5′ at 95 °C, 40 cycles of 15 s at 95 °C and 30 s at 60 °C. In the last step of both protocols, the temperature was raised from 60 °C to 95 °C at 0.1 °C/step to plot the melting curve. To further analyze the dissociation curves, we visualized the amplicons length in an agarose gel to confirm the correct amplification of the resulting PCR products [19,20].

For data analysis, the C_t values in each sample and the efficiencies of the primer set were calculated using LinReg Software [21] and then converted into relative quantities (RQ) and normalized according to the Pfaffl model [22].

Normalization was carried out considering as housekeeping genes, HPRT-1. These genes were chosen by software geNorm [23] version 3.5. The primers are listed in Table 1.

2.6. MMP-1, MMP-3, MMP-13 and Col2a1 ELISA assay

MMP-1, MMP-3, MMP-13 and Col2a1 were assayed using an ELISA assay (Boster Biological Technology, Pleasanton, CA). Monoclonal antibodies from mouse specific for MMP-1, MMP-3, MMP-13 and Col2a1, respectively, were adsorbed on microwells. Human MMP-1, MMP-3, MMP-13 and Col2a1 present in the sample bound to antibodies adsorbed to the microwells; biotinylated detection polyclonal antibodies from goat specific for MMP-1, MMP-3, MMP-13 and Col2a1 were subsequently added. Avidin-Biotin-Peroxidase Complex was added and unbound conjugates; HRP substrate TMB was used to visualize HRP enzymatic reaction. The substrate turnover was determined colorimetrically by measuring the absorbance, resulting proportional to the MMP-1, MMP-3, MMP-13 and Col2a1 concentrations. The sensitivity of the method was estimated to be < 10 pg/mL for MMP-1 and MMP-3, < 5 pg/mL for MMP-13 and < 0.375 ng/mL for Col2a1.

Table 1
Primers for RT-qPCR.

Target gene	Cat. No. (Qiagen)
MMP-1	QT00014581
MMP-3	QT00060025
MMP-13	QT00001764
Col2a1	QT00049518
Housekeeping candidate genes primers	
HPRT-1	QT00564771
HLA-G	QT01839726
HMBS	QT00014462
ACTB	QT01680476

2.7. Immunocytochemistry

Immunocytochemical investigations of NF-κB localization were performed in chondrocytes grown on coverslips in DMEM with 10% FBS, 200 U/mL penicillin, 200 U/mL streptomycin, and 2 mM glutamine, at a density of 1×10^5 cell/mL. After the treatment, the cells were washed in PBS and fixed in methanol for 20 min and in acetone for 5 min at −20 °C. Afterwards, the samples were permeabilized and blocked for 20 min at room temperature with PBS–BSA (1%) containing NGS (5%) and incubated overnight at 4 °C with the primary antibody diluted 1:50 (anti p50 subunit, Santa Cruz, CA) in PBS/0.1% BSA/1% NGS. The reaction was revealed by a goat anti-mouse IgG-Texas Red conjugated antibody diluted 1:100 in PBS/0.1% BSA/1% NGS, for 1 h at room temperature. Finally, the coverslips were washed three times in PBS and submitted to nuclear counterstain by 4,6-diamidino-2-phenylindole (DAPI). The coverslips were mounted on glass slides with Vectashield. Fluorescence was examined under a AxioPlan (Zeiss, Oberkochen, Germany) light microscope equipped with epi-fluorescence, at 400 × magnification. The negative controls were produced by omitting the primary antibody. Immunoreactivity of p50 was semi-quantified as the mean densitometric area of p50 signal (red) into the nucleus and into the cytoplasm, by AxioVision 4.6 software measure program. At least 100 chondrocytes from each group were evaluated.

2.8. Statistical analysis

Three independent experiments were performed and the results were expressed as the mean ± standard deviation of triplicate values for each experiment. Between-treatment comparisons were performed using generalized linear models (GLM) with Gaussian family and logarithmic link [24]. The GLM used the continuous variable of interest as response and time (discrete), treatment (discrete) and a treatmentXtime interaction (discreteXdiscrete) as predictors. Time was coded as 0 = 24 h; 1 = 48 h. The coding of treatment varied according to the experimental setting, e.g. 1 = IL (reference value); 2 = MOB200 + IL; 3 = MOB200 & MSM2000 + IL, 4 = MOB200 & MSM6000 + IL; 5 = MOB500 + IL; 6 = MOB500 & MSM2000 + IL; 7 = MOB500 & MSM6000 + IL; 8 = MSM2000 + IL; 9 = MSM6000 + IL for the PCR setting (MMP1, MMP3, MMP13 and COL2A1). Because of the different coding of treatment, also the coding of the treatmentXtime interaction varied according to the experimental setting. Cluster confidence intervals were used by all the GLM to account for within-patient clustering of the cells used for experiments. Effect sizes are reported as means and differences versus the reference group as estimated from the GLM. The clustered 95% confidence intervals of the differences are corrected for the number of multiple comparisons using Bonferroni correction, e.g. 8 comparisons for the PCR setting described above. Statistical analysis was performed using Stata 15.1 (Stata Corporation, College Station, TX, USA).

For the immunofluorescence staining, mean densitometric labelling intensities were submitted to analysis of variance (ANOVA), by GraphPad 6.1 software.

A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Cell viability

In Figs. 1 and 2 are reported the results on cell viability evaluated by MTT assay. The chondrocytes exposed to the negative stimulus of IL-1β showed a significant reduction ($p < 0.001$) in the percentage of survival cells in comparison to basal conditions, after 24 h and 48 h (Fig. 1A). The incubation with mobilee (200 μM and 500 μM) and MSM (2000 μM and 6000 μM), tested alone or in combination, induced a slight increase of cell viability in comparison to basal state (Supplementary Fig. 1A); the better results were obtained with the combination of the drugs after 24 h of treatment. Both concentrations of the studied

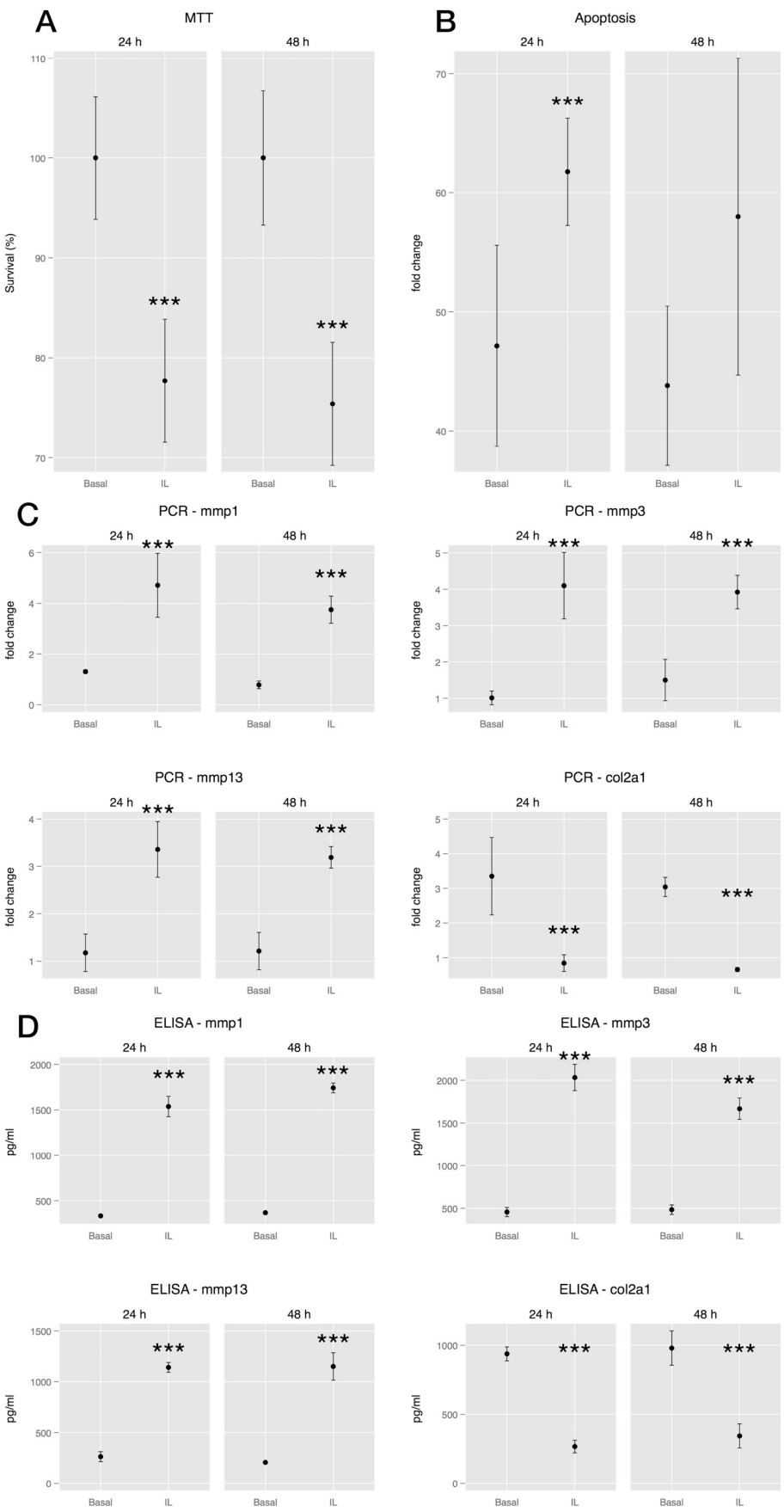


Fig. 1. (A) Evaluation of cell viability by MTT assay. Data were expressed as percentage of cell viability in the studied conditions. The value of basal conditions was reported equal to 100. (B) Apoptosis was measured with Annexin Alexa fluor 488 assay. Data were expressed as percentage of positive cells for Annexin-V and propidium iodide (PI) in the studied conditions. (C) Gene expression levels were evaluated for MMP-1,-3,-13 and Col2a1 and were expressed as fold change. (D) Total amount of MMP-1,-3,-13 and Col2a1 was measured in the cultured medium and expressed in pg/mL.

Cells were evaluated at basal conditions and after 24 and 48 h of stimulus of IL-1β (10 ng/mL).

GLM with Gaussian family, log link, cluster 95% CI and Bonferroni correction for multiple comparisons. Values are means and 95% CI estimated from the GLM.

$^{***}p < 0.001$ versus basal conditions.

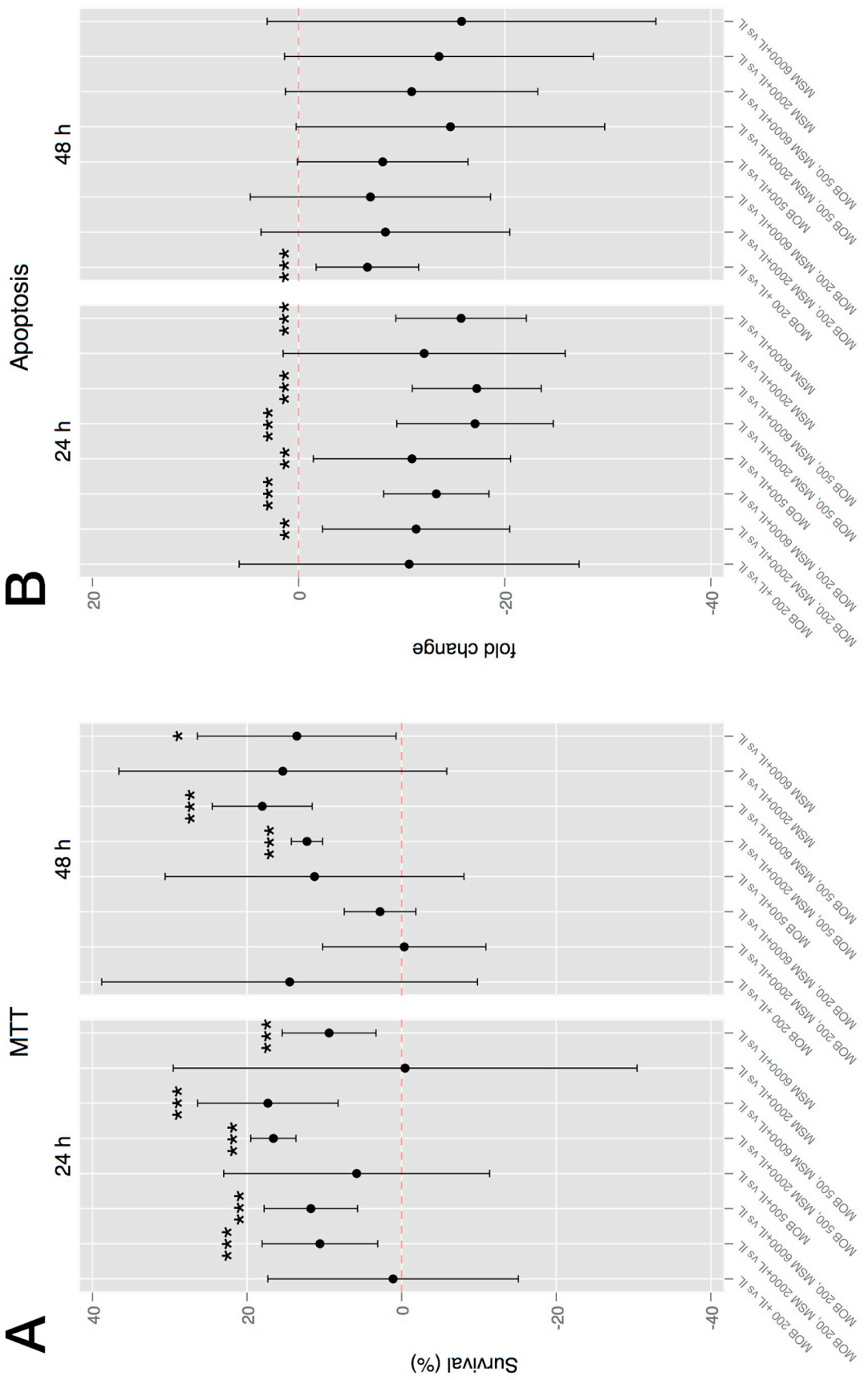


Fig. 2. (A) Evaluation of cell viability by MTT assay. Data were expressed as percentage of cell viability in the studied conditions. The percentage was referenced to the ratio of the value of interest and IL-1β stimulus. The value of IL-1β was reported equal to 0. (B) Apoptosis was measured with Annexin Alexa fluor 488 assay. Data were expressed as percentage of positive cells for Annexin-V and propidium iodide (PI) in the studied conditions. The ratio of apoptosis was referenced to the ratio of the value of interest and IL-1β stimulus. The value of IL-1β was reported equal to 0. Cells were evaluated after 24 and 48 h of treatment with mobilee (200 μM and 500 μM) and MSM (2000 μM and 6000 μM) in presence of IL-1β (10 ng/mL). GLM with Gaussian family, log link, cluster 95% CI and Bonferroni correction for multiple comparisons. Values are means and 95%CI estimated from the GLM. $^{**}p < 0.01$, $^{***}p < 0.001$ versus IL-1β. Mob = mobilee.

compounds showed a significant trend ($p < 0.001$) in restoring the cell survival in IL-1β-stimulated chondrocytes at both 24 h and 48 h (Fig. 2A).

The data was confirmed by Trypan Blue test (data not shown).

3.2. Detection of apoptotic cells

Data concerning chondrocyte apoptosis obtained by flow cytometry assay is shown in Figs. 1 and 2. The stimulus of chondrocytes with IL-1β induced an increase in apoptotic cell ratio in comparison to basal time at both analyzed time points, in a significant manner at 24 h ($p < 0.001$) (Fig. 1B). The incubation of our cultures with the two concentrations of mobilee and MSM alone or combined significantly reduced ($p < 0.001$) the apoptosis ratio at 24 h and 48 h with respect to basal conditions (Supplementary Fig. 1B). This reduction was also maintained when the compounds were co-incubated with IL-1β at all the tested concentrations, especially after 24 h of treatment ($p < 0.001$) (Fig. 2B).

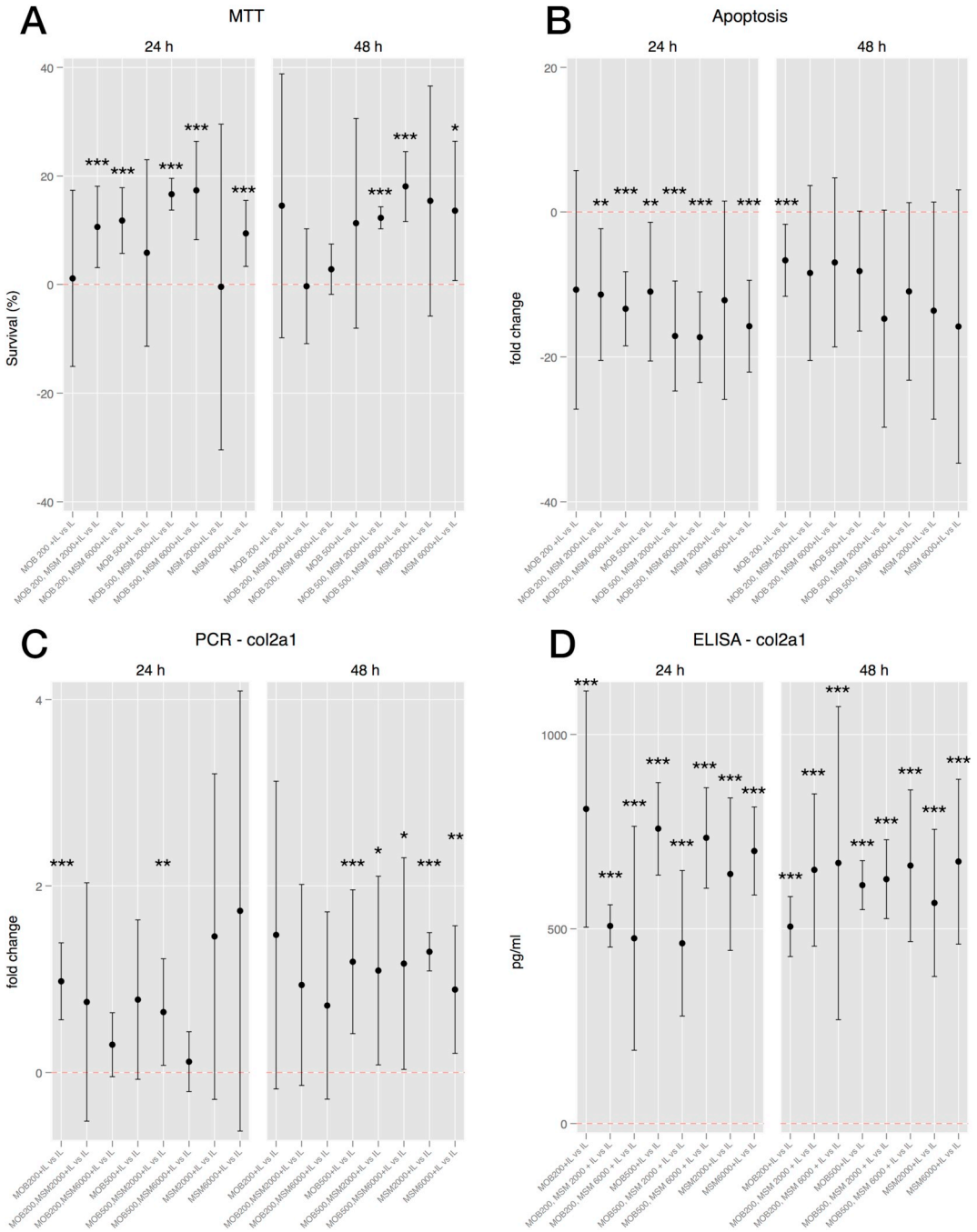


Fig. 3. Evaluation of gene expression levels of MMP-1 (A) and Col2a1 (C) by real time PCR. The gene expression was referenced to the ratio of the value of interest and IL-1β stimulus. The value of IL-1β was reported equal to 0.
Measure of total amount of MMP-1 (B) and Col2a1 (D) released in the conditioned medium (pg/mL) by ELISA assay. The values were referenced to the ratio of the value of interest and IL-1β stimulus. The value of IL-1β was reported equal to 0.
Cells were evaluated after 24 and 48 h of treatment with mobilee (200 μM and 500 μM) and MSM (2000 μM and 6000 μM) in presence of IL-1β (10 ng/mL).
GLM with Gaussian family, log link, cluster 95% CI and Bonferroni correction for multiple comparisons. Values are means and 95% CI estimated from the GLM.
$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus IL-1β. Mob = mobilee.

3.3. Modifications of gene expression of factors involved in cartilage turnover

As reported in Fig. 1C, the expression levels of MMP-1, MMP-3 and MMP-13 were significantly increased ($p < 0.001$) and the gene expression of Col2a1 was statistically reduced ($p < 0.001$) after 24 h and 48 h of stimulus with IL-1β in comparison to basal time. No significant changes were detected after the incubation of OA chondrocytes with the different concentrations of mobilee and MSM alone and in combination compared to basal state (Supplementary Fig. 2). The negative modulation of IL-1β on MMP-1, MMP-3, MMP-13 and Col2a1 gene expression was significantly counteracted ($p < 0.05$, $p < 0.01$, $p < 0.001$) when our cultures were pre-treated with mobilee and MSM, with a particular exacerbation in a combination of them both at 24 h and 48 h (Fig. 3A for MMP-1 and Fig. 3C for Col2a1; Supplementary Fig. 4A and B for MMP-3 and MMP-13).

3.4. Evaluation of MMP-1, MMP-3, MMP-13 and Col2a1 release

The total amount of MMP-1, MMP-3, MMP-13 and Col2a1 released in the culture medium of OA chondrocytes after our treatment were performed by an ELISA assay. After 24 h and 48 h of IL-1β stimulus a significant increase ($p < 0.001$) of MMP-1, MMP-3 and MMP-13 and a significant reduction ($p < 0.001$) of Col2a1 levels, in comparison to basal conditions, were observed (Fig. 1D). The treatment of the cells with mobilee and MSM alone or in a combination didn't show any detectable modification compared to basal time (Supplementary Fig. 3).

The negative stimulus of IL-1β on MMP-1, MMP-3, MMP-13 and Col2a1 levels was significantly counteracted ($p < 0.001$) by the pre-treatment of the OA chondrocytes with the both tested concentrations of mobilee and MSM (alone or combined) at 24 h and 48 h (Fig. 3B for MMP-1 and Fig. 3D for Col2a1; Supplementary Fig. 4C and D for MMP-3 and MMP-13).

3.5. Immunofluorescence analysis and NF-κB activation

As reported in Fig. 4A–C, the signal of p50 NF-κB subunit was consistently detected in the cytoplasm and in the nucleus of OA chondrocytes at basal conditions. Mobilee and MSM tested in combination significantly reduced ($p < 0.001$) p50 levels in the cytoplasm and in the nucleus of the cells in comparison to baseline. Stimulus of IL-1β significantly induced NF-κB cytoplasmic upregulation and activation, as indicated by nuclear translocation ($p < 0.01$), which were significantly ($p < 0.001$) counteracted by the pre-treatment of OA chondrocytes with the studied compounds tested alone or in combination.

3.6. Regulation of gene expression after NF-κB inhibition

Considering the optimum results obtained, we selected the combination of mobilee and MSM for the evaluation of gene expression of MMP-1, -3, -13 and Col2a1 after the inhibition of NF-κB signaling pathway by BAY11-7082 (Fig. 5).

The expression levels of MMP-1 (A), MMP-3 (B) and MMP-13 (C) were significantly increased ($p < 0.001$) and those of Col2a1 (D) were statistically reduced ($p < 0.001$) after 24 h of stimulus with IL-1β in

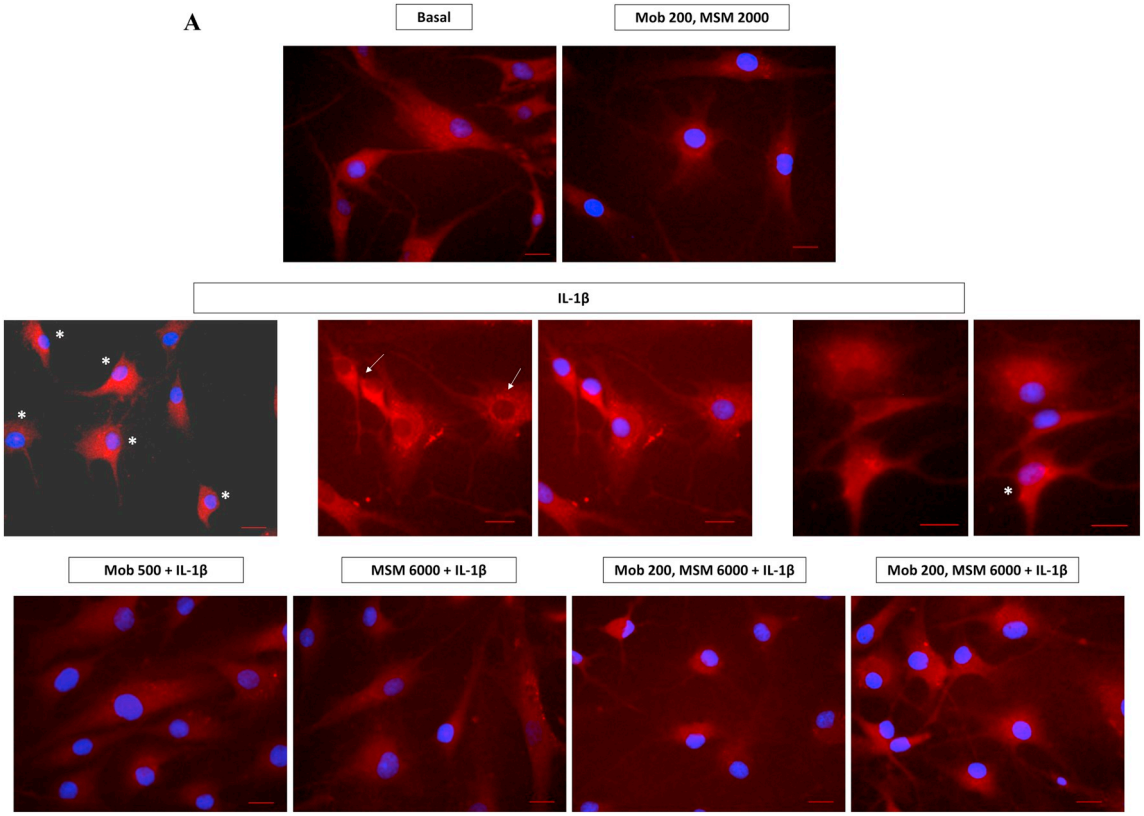
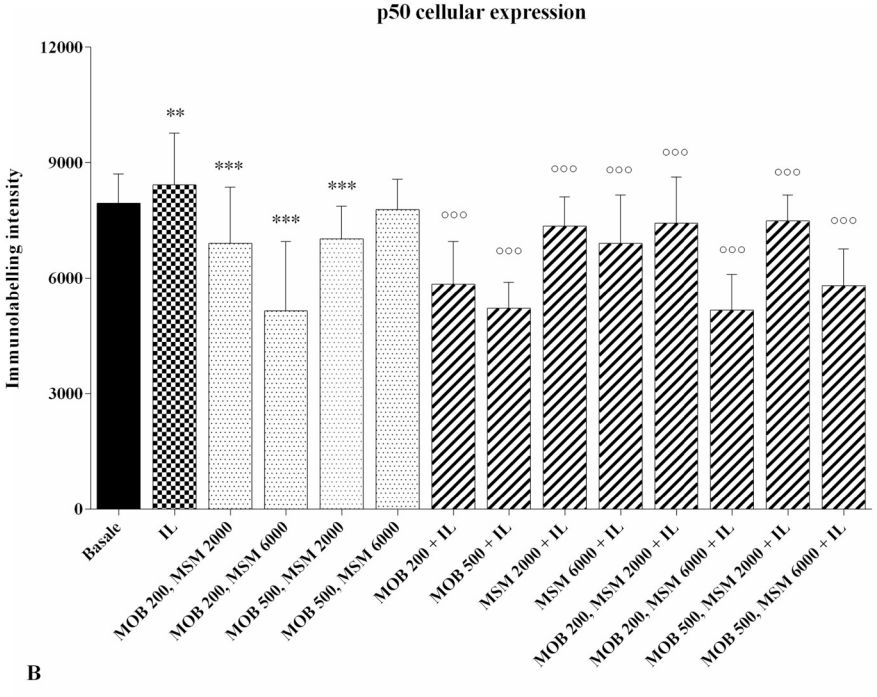


Fig. 4. Immunofluorescence labelling of p50 NF-κB subunit localization of OA chondrocytes analyzed after 24 h of pre-treatment with mobilee (200 μM and 500 μM) and MSM (2000 μM and 6000 μM) and, then, 1 h of stimulus with IL-1β (10 ng/mL). (A) Representative immunocytochemical images of the cells showing localization of p50 NF-κB (red); nuclei were stained with DAPI (blue). Original Magnification 400×. Scale bar: 20 μm. In IL-1β stimulated chondrocytes, p50 nuclear translocation was highlighted by merge (asterisks) and p50 up-regulation with distinctive granular perinuclear localization was observed (arrows). (B,C) Immunolabelling intensity was plotted for p50 subunit nuclear and cytoplasm expression.
$^{**}p < 0.01$, $^{***}p < 0.001$ versus basal conditions; $^{°°°}p < 0.001$ versus IL-1β. Mob = mobilee. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



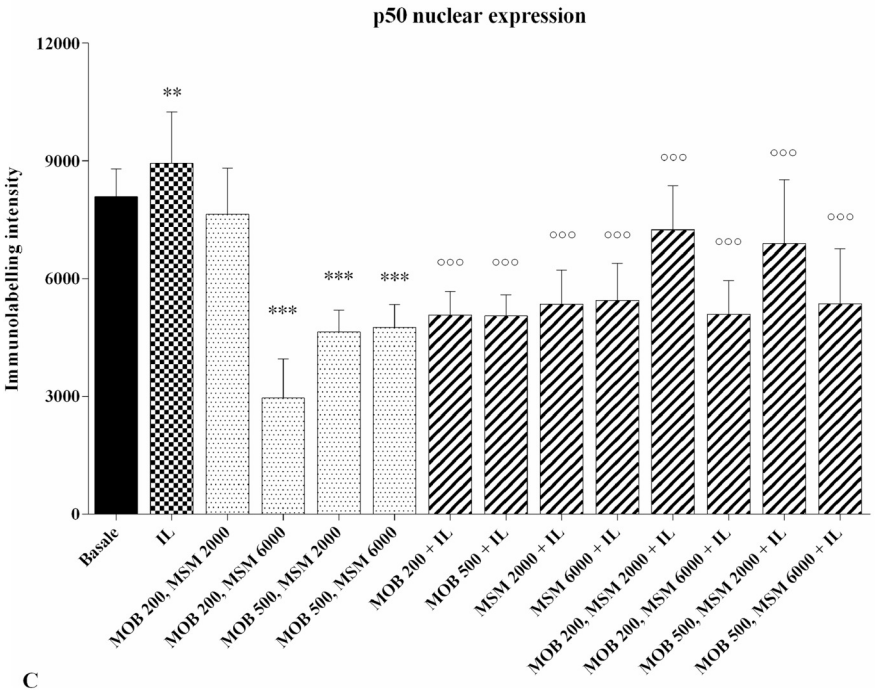


Fig. 4. (*continued*)

comparison to basal time. The negative modulation of IL-1β on these factors was significantly counteracted ($p < 0.001$) when our cultures were pre-treated with mobilee, MSM and BAY11-7082.

The incubation with BAY11-7082 significantly reduced MMP-1 ($p < 0.05$), MMP-3 ($p < 0.05$) and MMP-13 ($p < 0.01$), and significantly increased Col2a1 ($p < 0.05$) gene expression respectively to basal conditions.

Chondrocytes co-treated with mobilee, MSM plus BAY11-7082 did not exhibit enhanced effects in MMP-1, -3, -13 and Col2a1 expression levels compared to cells treated with mobilee, MSM or BAY11-7082 alone. Moreover, co-treatment of IL-1β-stimulated chondrocytes with mobilee, MSM and BAY11-7082 did not cause further changes of MMP-1, -3, -13 or Col2a1 expression beyond that caused by treatment with mobilee, MSM or BAY11-7082 alone.

4. Discussion

The term nutraceutical was coined in 1989, as a combination of “nutrition” and “pharmaceutical”, for any natural or food-derived product with a potential health benefit, including botanicals, vitamins, and different kinds of extracts [25]. Nutraceuticals are differently classified in different countries. In the United States, they are not defined by law, but are regulated as dietary supplements under the Dietary Supplement Health and Education Act (DSHEA) of 1994 [26].

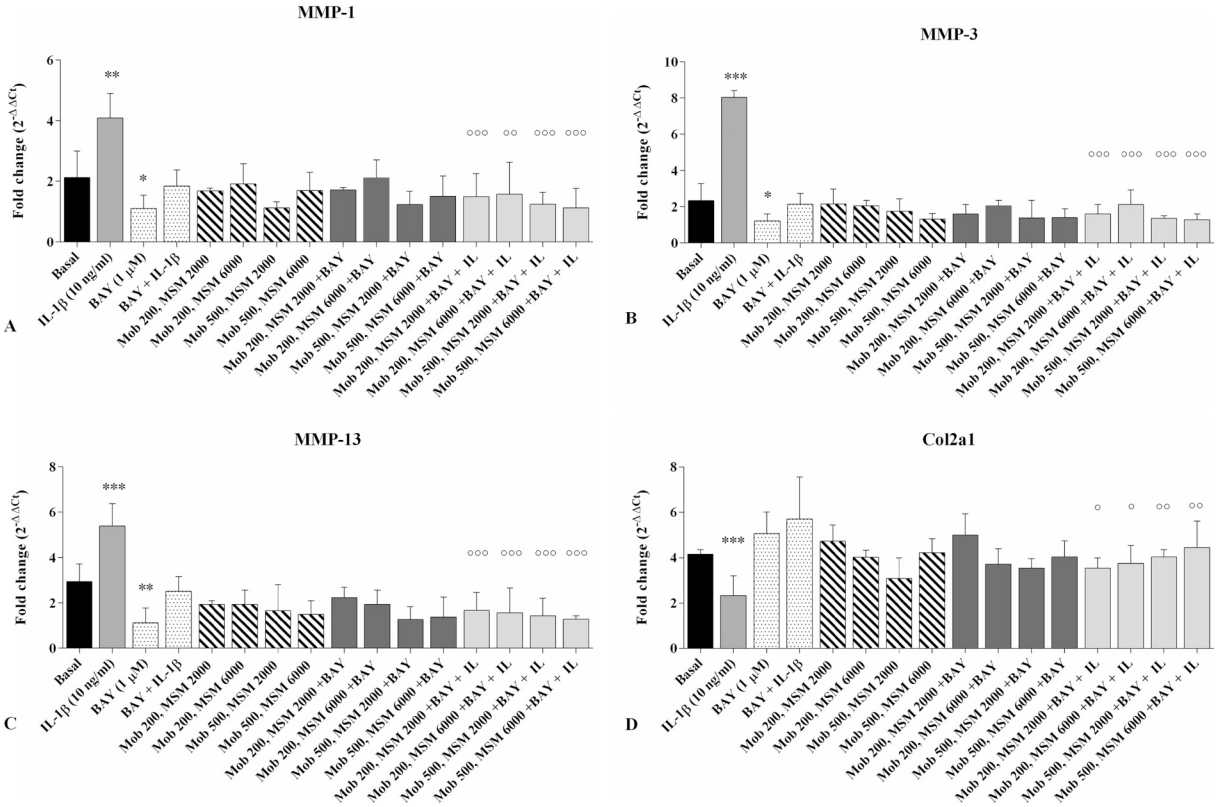


Fig. 5. Evaluation of gene expression levels of MMP-1 (A), MMP-3 (B), MMP-13 (C) and Col2a1 (C) by real time PCR after pre-incubation of the cells for 2 h with 1 μM BAY 11-7082 and 24 h treatment with the tested concentration of mobilee (200 μM and 500 μM) and MSM (2000 μM and 6000 μM) in presence of IL-1β (10 ng/mL). *p < 0.05, **p < 0.01, ***p < 0.001 versus basal conditions; °p < 0.05, °°p < 0.01, °°°p < 0.001 versus IL-1β.

In EU countries, Canada and Australia they are known and classified as complementary medicines [27,28]. Nutraceuticals are suggested to have a role in decreasing pain, improving function and reducing joint progression in OA [12]. Results from randomized controlled studies reported an improvement of pain relief, quality of life and muscle strength in patients with knee OA and mild joint discomfort treated with dietary supplementation of mobilee and MSM [14,29–31]. Furthermore, intake of MSM for 13 weeks decreased degeneration of cartilage at the joint surface in the knee joints of STR/Ort OA mouse model in a dose dependent manner [11]. However, there are debates on their efficacy and the international recommendations highlight that the use of nutraceuticals in OA remains controversial [32].

The goal of this study was to examine the effect of MSM and of mobilee, two natural components contained in a single preparation, tested alone or in combination, in human OA chondrocyte cultures in presence of the negative stimulus of IL-1β. We assessed the cell viability and the percentage of apoptosis, as well as the modulation of the main extracellular matrix degrading enzymes and of the Col2a1; the identification of the possible mechanism of action of the studied compounds was also evaluated by testing the activation of the NF-kB signaling pathway.

The concentrations of MSM and of mobilee used in our *in vitro* study were similar to those found *in vivo* and comparable with those tested by other Authors [33,34]. The experiments were performed in presence of the cytokine IL-1β, generally used in *in vitro* models to mimic the circumstances driving to *in vivo* cartilage degradation [35–37].

Apoptosis is a highly-regulated, active process of cell death involved in development, homeostasis and aging processes. Dysregulation of apoptosis leads to pathological states, onset of abnormalities and degenerative diseases [38]. Apoptosis clearly occurs in OA cartilage, however, its relative contribution in the pathogenesis of the disease is difficult to evaluate; it is not clear whether chondrocyte apoptosis is the inducer of cartilage degeneration or a product of cartilage destruction [38]. Some Authors observed the increase of apoptosis percentage in human OA chondrocyte cultures after the stimulus of IL-1β at different concentrations, tested at 24 and 48 h [39,40]. These data are in agreement with our results where IL-1β induced a decrease of percentage of survival cells and a concomitant increase of their apoptotic ratio. On the contrary, MSM and mobilee tested alone had not a cytotoxic effect on chondrocytes at either of the tested concentrations and, more interestingly, we reported for the first time their anti-apoptotic effect in IL-1β-stimulated OA cells. Our results seem to be consistent with the study of Karabay et al. [41], demonstrating the ability of MSM to revert the apoptosis induced by lipopolysaccharide (LPS) and interferon-gamma in RAW 264.7 macrophage-like cells.

It is well known that MMPs are the main factors implicated in cartilage turnover, playing a significant role in the regulation of cartilage extracellular matrix (ECM) degradation that occur during OA damage [42]. MMP-1 and MMP-3 may indirectly contribute to promote cartilage breakdown, by the activation of other MMPs, inducing the degradation of proteoglycans and Col2a1, the main component of articular ECM and a major structural protein in articular cartilage [43]. MMP-13 represent one of the most common downstream targets, up-regulated or inappropriately activated at different times during OA processes by stress or pro-inflammatory signals [44]. Some studies demonstrated that many pro-inflammatory cytokines, such as IL-1β, induce the activation of MMP-1, MMP-3, and MMP-13 and the consequent degradation of Col2a1 in OA articular cartilage [45,46]. Our data appeared in agreement with the current literature showing a significant increase of MMP-1, -3, -13 and reduction of Col2a1 in IL-1β-stimulated chondrocytes.

MSM is often combined with other supplements for the treatment of OA [12]; however, few reports investigated its possible role in chondrocytes metabolism [47–49]. Our data demonstrated that the

incubation of the cells with MSM and mobilee counteract the negative effect of IL-1β stimulus on MMP-1, -3, -13 and Col2a1. This finding was observed at gene expression level as well as at their release in the supernatant, suggesting a possible anti-catabolic effect of the tested compounds. Interestingly, the treatment appeared more efficacious when the substances were used in combination. Similarly, studies performed on fibroblast cultures and on peripheral blood cells demonstrated the ability of mobilee to reduce the MMP-1 levels and MMP23B gene expression, respectively, induced by IL-1β [15,34]. Furthermore, the treatment of mucosa samples, derived from a mice model of gastric ulcer, with MSM revealed a significant reduction of MMP-9 levels induced by ethanol injury [50].

It is accepted that NF-κB represents a key regulator of tissue inflammation, since it controls the transcription of a number of pro-inflammatory genes that regulate the synthesis of cytokines, chemokines and adhesion molecule [51]. Under physiological conditions, NF-κB is present in inactive form in cytoplasm as a heterodimer of the p50 and p65 subunits. Many pro-inflammatory cytokines initiate signaling cascades, inducing the phosphorylation and the subsequent degradation of NF-κB inhibitor which results in the activation and nuclear translocation of p50 and p65 subunits [51]. The NF-κB signaling pathway is a central inflammation pathway involved in cartilage degradation and inflammatory response in the pathogenesis of OA [52]. Once activated by IL-1β, NF-κB triggers the up-regulation of various inflammation-related genes as MMPs, prostaglandins, ILs and tumor necrosis factor (TNF)-α, which contribute to the degradation of Col2a1 and proteoglycans and to the synthesis of catabolic factors [52]. Therefore, the inhibition of NF-κB pathway should be effective for the treatment of OA.

In this report we evaluated NF-κB p50 subunit localization in the cells to assess the involvement of MSM and mobilee in the regulation of the pathway activation and subsequently identify their possible mechanism of action in OA conditions. We demonstrated a significant increase of the expression, activation and in turn of nuclear translocation of NF-κB p50 subunit after the stimulus of OA chondrocytes with IL-1β in comparison to basal time; this result is confirmed by previous data from the literature [45,53–55]. Some *in vitro* studies indicated that MSM was able to inhibit transcriptional activity of NF-κB by blocking its translocation into the nucleus and preventing the degradation of the NF-κB inhibitor, in LPS-stimulated murine RAW264.7 cells and in bone marrow derived macrophages [48,56]. No previous information on mobilee and NF-κB is reported. To our knowledge, this is the first study showing the ability of MSM and mobilee to reduce the NF-κB p50 subunit activation in OA chondrocytes stimulated with IL-1β. We further demonstrated the direct interaction between NF-κB and the studied compounds by the inactivation of NF-κB signaling pathway through a specific NF-κB inhibitor, BAY11-7082, as reported by other Authors [48]. Our results were indicative of the fact that BAY11-7082, down-regulating NF-κB activation, reduced the activity of mobilee and MSM on MMP-1, MMP-3, MMP-13 and Col2a1 expression. The present data showed that mobilee and MSM modulate the expression of MMPs and Col2a1 in IL-1β-stimulated chondrocytes similarly to NF-κB inhibitor. This evidence suggests that the regulation of chondrocytes by mobilee, MSM and BAY11-7082 follows the same molecular pathway, supporting the hypothesis that the studied compounds can be effective through the inhibition of NF-κB signaling.

The results of the present study showed for the first time that MSM and mobilee, two ingredients of a complex preparation, have a beneficial effect on the metabolism of human OA chondrocytes. In particular, both compounds are able to exert a significant protection against the catabolic damage induced by IL-1β stimulus; indeed, a protective role by inhibiting the apoptosis and the production of some factors involved in cartilage turnover was observed. Interestingly, the combination of the substances seems to be more efficacious especially in the modulation of MMPs and Col2a1 underlining that the combined use of MSM and mobilee is more effective overall than the single compound.

The observed regulation of OA chondrocytes metabolism is due to the modulation of NF-κB signaling pathway.

The data of the current study are limited by the lack of the same experiments on normal chondrocytes and on the evaluation of the tested compounds on chondrocyte anabolism.

Taken together, our findings provide a powerful rationale for the use of MSM and mobilee in the treatment of OA, even if, more results are needed to confirm their effect *in vivo*.

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Authors' contributions

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Competing interest statement

The authors affirm that there are no conflict of interest.

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