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Methylsulfonylmethane (organic sulfur) induces apoptosis and decreases invasiveness of prostate cancer cells

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ABSTRACT

A major challenge in the management of prostate cancer (PC) is to limit tumor growth and metastases. Targeted therapies applying natural compounds might be potentially useful in PC treatment. Methylsulfonylmethane (MSM), also known as organic sulfur, is a dietary supplement used for various clinical purposes, mostly known for its anti-inflammatory properties. Therefore, we decided to evaluate the effect of MSM on PC cells LNCaP, PC3 and DU-145 which represent different *in vitro* models of PC. We observed that MSM decreases the viability and invasiveness of PC cells through the induction of apoptosis and cell cycle arrest in the G0/G1 cell cycle phase. Moreover, MSM in a low dose (200 mM) is able to reduce the migration and invasion of PC cells. Considering the low overall body toxicity and insignificant side effects of MSM, its apoptosis-inducing properties might be used in PC treatment in the future.

1. Introduction

Prostate cancer (PC) is one of the most common cancers in men worldwide and a leading cause of cancer-related deaths in Europe and the United States (Di et al., 2018). PC affects mainly men aged 65 or older and is considered as a hormone-dependent cancer due to the known involvement of hormones in its proliferation and progression (Snaterse et al., 2017). A great number of research studies have been carried out on the molecular mechanisms of PC, however, it still has not been examined in detail. Many preclinical and observational studies have revealed that the progression, mortality and the whole disease can be modulated by diet, exercises and lifestyle interventions involving supplementation with vitamins and other natural products (Ballon-Landa and Parsons, 2018).

Methylsulfonylmethane (MSM), also known as organic sulfur, $DMSO_2$, methyl sulfone or dimethyl sulfone, occurs naturally in plants and food (Sousa-Lima et al., 2016). It is known as a polar solvent, similar to the well-known and commonly used dimethyl sulfoxide (DMSO) (Clark et al., 2008). MSM is also available as a natural supplement, categorized by the Food and Drug Administration (FDA) as "generally recognized as safe" and has low toxicity in human studies (Sousa-Lima et al., 2016). MSM is believed to be a natural source of sulfur for the production of the amino-acids methionine and cysteine (Amirshahrokhi and Khalili, 2017). Its membrane penetrability, as well as its antioxidant, anti-inflammatory and anticholinesterase properties, triggered a great number of studies evaluating the effect of MSM on human health (Butawan et al., 2017). Most studies involving MSM have confirmed its anti-inflammatory and antioxidant role, especially in arthritis, and in muscle and cartilage function, as well as physical functioning of the body mainly exerting an impact on hair and nails condition (Butawan et al., 2017). Recent research indicates that anti-inflammatory and antioxidant properties of MSM might be useful in cancer treatment, especially MSM is reported to modulate cell cycle and induce apoptosis in breast (NS et al., 2017), stomach (Jafari et al., 2012), skin (Caron et al., 2010) and colon (Karabay et al., 2016) cancers. Interestingly, the idea of combining well-known inhibitors of cancer cell proliferation and natural compounds that could be used synergistically triggered various studies which found the MSM suppressed bladder tumor (Joung et al., 2014) and breast cancer growth (SP et al., 2015). Hence, it might be essential to evaluate the potential of MSM to induce apoptosis in cancer cells, especially taking into consideration its low toxicity to the human body (Sousa-Lima et al.,

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2016).

The process of carcinogenesis in PC is mainly associated with uncontrolled proliferation of cells, lack of apoptosis and increased migration and invasiveness of cells (Kowalska and Piastowska-Ciesielska, 2016). In order to evaluate the effect of MSM on PC cell viability, apoptosis and invasiveness, three PC cell lines with different hormone sensitivities and invasiveness potentials (LNCaP, PC3 and DU-145) were treated with MSM.

2. Materials and methods

2.1. Cell culture

Metastatic human prostate adenocarcinoma cell lines LNCaP and PC3 were obtained from the European Collection of Authenticated Cell Cultures (ECACC) (Sigma Aldrich), whereas the DU-145 cells were obtained from the American Type Culture Collection (ATCC). The cell lines were cultured in RPMI (LNCaP, PC3) or Dulbecco's modified Eagle's medium (DMEM) (DU-145) (Thermo Fisher Scientific) with 10% heat-inactivated foetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES) and antibiotics (penicillin, 50 U/ml; streptomycin, 50 µg/ml; and neomycin, 100 µg/ml) (Thermo Fisher Scientific), in a humidified atmosphere of 5% CO_2 at 37 °C. Cell culture medium without phenol red, serum and antibiotics was used for the experiments. MSM (Sigma Aldrich) stock solution was dissolved in RPMI/DMEM experimental medium and filtered (0.22 µm PVDF) (Merck Millipore) before use. Cells were treated with MSM for 24 h. Non-treated cells were used as a control. Cells treated with 0.1µM staurosporine (AppliChem) were used as a positive control.

2.2. Cell viability

The viability of cells was determined with AlamarBlue® reagent. 7×10^4 cells were seeded on 96-well plates and incubated at standard conditions to reach 90% confluence (two to three days). Next, the cells were treated with experimental medium containing 10–800 mM MSM for 20 h. Then 10 µl of AlamarBlue® reagent were added to wells and incubated for additional four hours at 37 °C. Absorbance was measured at 570 nm with ELX808IU plate reader (Biotek). Results were expressed as a percentage of control cells of six replicates. IC_{50} value was calculated with GraphPad Prism 5.0 (GraphPad software).

2.3. Mitochondrial transmembrane potential

The changes in mitochondrial transmembrane potential (ΔΨm) caused by MSM were evaluated with Muse™ MitoPotential Assay (Merck Millipore). Briefly, 3×10^5 of cells were seeded on 6-well plates and incubated at standard conditions to reach 90% of confluence. Then, the cells were treated with experimental media in concentrations of 200–500 mM MSM for 24 h. Next, the cells were detached with TrypLE reagent (Thermo Fischer Scientific) and MitoPotential Assay was performed as recommended by the manufacturer. Probes were measured with a Muse™ Cell Analyzer (Merck Millipore). The results were expressed as a percentage of the total depolarized cells. The experiment was conducted in triplicate.

2.4. Determination of apoptosis

Apoptosis was estimated with Muse™ Annexin V & Dead Cell Kit (Merck Millipore). The cells were seeded on 6-well plates at a density of 3×10^5 cells/well and incubated at standard conditions to reach 90% confluence. Then cells were treated with 200–500 mM MSM for 24 h and the assays were performed according to the manufacturer's recommendations. Probes were measured on Muse™ Cell Analyzer. Both positive and negative control were used. The experiment was done in triplicate.

PathScan® Multi-Target Sandwich ELISA Kit #7105 (Cell Signalling) was used to analyse the expression of key signalling proteins associated with apoptosis, according to the manufacturer's recommendations. The concentrations of protein used is experiment were 0.1 mg/ml, 0.6 mg/ml and 0.3 mg/ml for PC3, LNCaP and DU-145, respectively.

DNA fragmentation was detected on 2% agarose (Eurogentec) gel. Briefly, cells were seeded on 10 mm Petri dishes at a density of 2×10^6 and incubated at standard conditions to reach confluence. Then cells were treated with 200–500 mM MSM, a positive and negative control for 24 h. DNA was isolated with TRIzol Reagent (Thermo Fischer Scientific) reagent according to the manufacturer's recommendations. DNA concentration was measured on BioDrop µLittle (BioDrop). Following this, 4 µg of DNA was separated on agarose gel (80 V) on ice. DNA fragmentation was visualized using UV light and photographed.

DAPI staining was conducted to evaluate the morphological changes in the cell nuclei. The cells were cultured as described previously. After 24 h incubation, wells were washed with PBS twice and stained with 4% paraformaldehyde (PFA, Sigma Aldrich) for 20 min. Then washed once again and stained with 0.0001 mg/ml DAPI (Sigma Aldrich) solution in PBS for five minutes. Next, once again washed with PBS and observed in FLoid®Cell Imaging Station (Thermo Fisher Scientific).

2.5. Cell cycle

The cell cycle was determined by a Muse™ Millipore Cell Cycle Kit (Merck Millipore) according to the manufacturer's instructions. The cells were cultured on six-well plates at a density of 3×10^5cells/well. After reaching confluence, the cells were exposed to MSM for 24 h. Then, cells were harvested with Trypsin-EDTA 0.05% solution (Thermo Fisher Scientific) and processed according to the instruction. The probes were measured on a Muse™ Cell Analyzer. The experiment was performed in triplicate.

2.6. Cell adhesion assay

The cells were seeded on 6-well plates at the density of 5×10^5/well and incubated to reach 90% confluence. Then medium was exchanged for experimental media: 100–200 mM MSM. After 24 h cells were detached with Trypsin/EDTA solution (Thermo Fisher Scientific Inc.) and counted on automated Cell Counter (Thermo Fisher Scientific). 1×10^5 of cells were plated on 24-well collagen type I, collagen type IV, laminin and fibronectin coated (Diag-med) plates. The plates were then incubated in standard culture conditions for 1.5 h. After incubation, the medium was removed and wells were washed three times with PBS to remove unattached cells. Then cells were stained with 0.1% crystal violet (Sigma Aldrich) for 10 min, washed three times with water and dried. Next, representable pictures were taken before dissolving in 10% acetic acid. The solute mixture was transferred on 96-well plate (200 µl/well) and absorbance was measured at 550 nm on a ELX U808IU plate reader. The experiment was conducted in three replicates.

2.7. Monolayer wound migration assay

The cells were seeded on 6-well plates and left to reach 100% confluence. Cell monolayers were 'wounded' as described previously (Dominska et al., 2016) and the medium was exchanged for an experimental one (100–200 mM MSM) supplemented with 0.5 mM solution of hydroxyurea (Sigma Aldrich). The cells were photographed with Olympus DP20 camera (Olympus), magnitude 40x after 0 and 24 h. Migration was calculated as a difference between the area of "wound" after 24 h and 0 h. The experiment was conducted in triplicate.

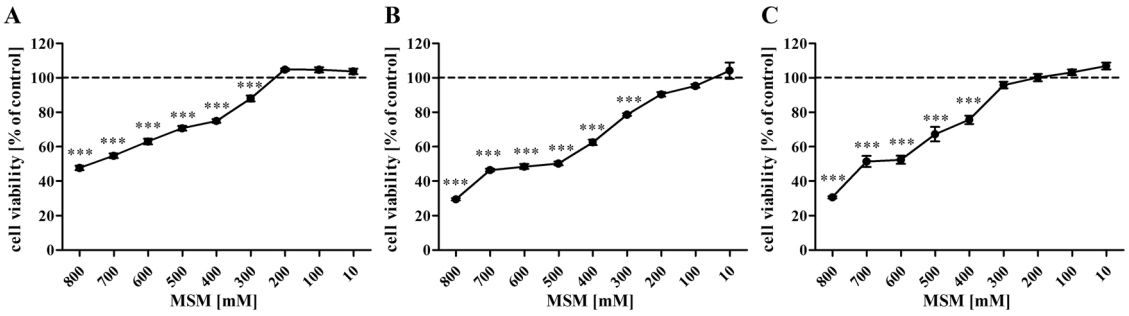

Fig. 1. MSM decreases viability of prostate cancer cells: LNCaP, PC3 and DU-145. Viability of LNCaP (A), PC3 (B) and DU-145 (C) was determined in AlamarBlue® assay. The results are expressed as a percentage rate of control (non-treated) cells. $p < 0.05$ was considered as statistically significant, $***p < 0.001$ as compared to control cells; MSM-methylsulfonylmethane.

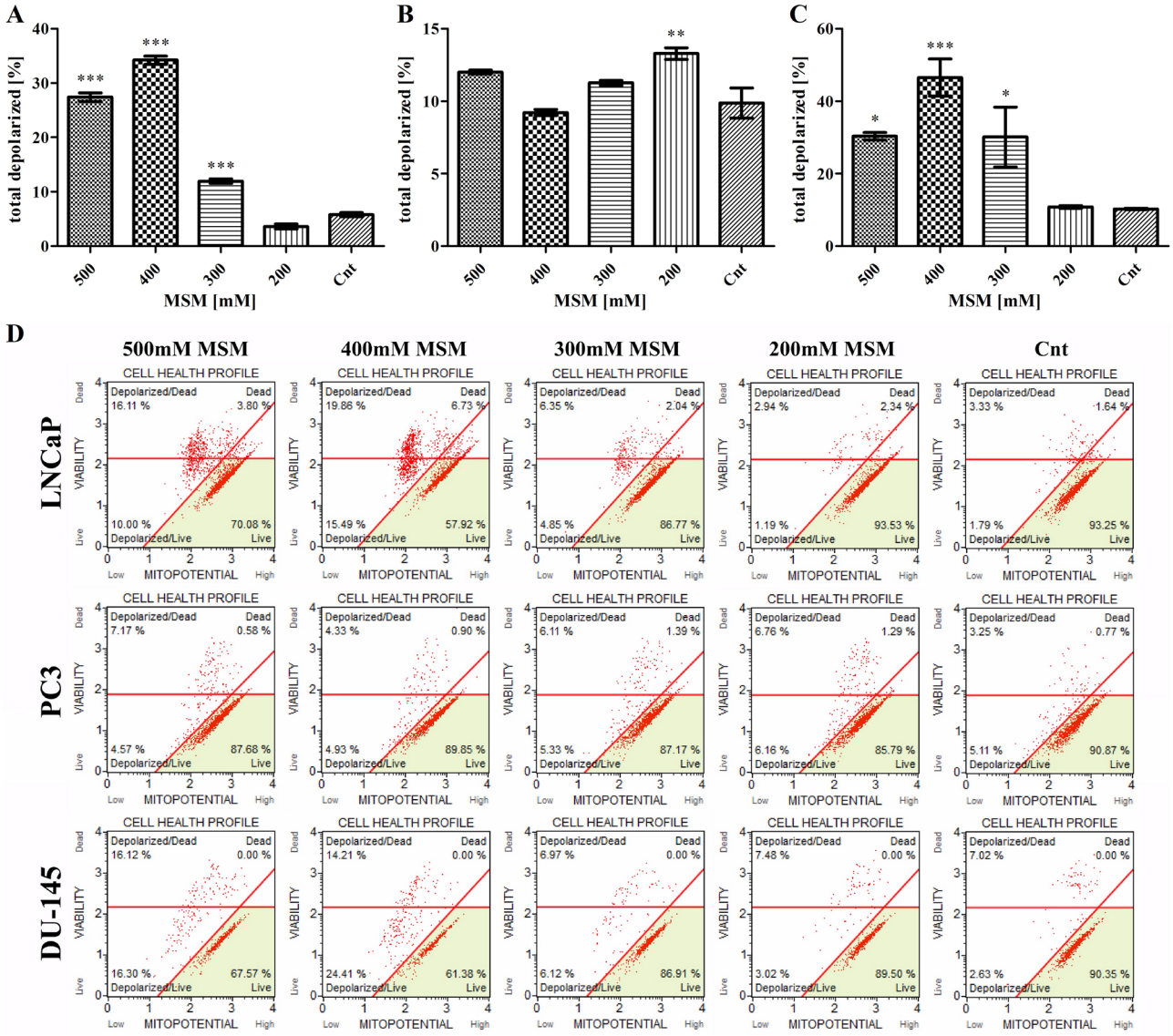

Fig. 2. MSM modulates mitopotential of prostate cancer cells. The changes in mitochondrial potential after MSM treatment in LNCaP (A), PC3 (B) and DU-145 (C) cells were expressed as a percentage rate of total depolarized cells and compared to control (non-treated) cells. $p < 0.05$ was considered as statistically significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ as compared to control cells. D- representative results of mitopotential assay, MSM- methylsulfonylmethane, Cnt- control.

2.8. Cell invasion assay

The cells were pretreated with 100 and 200 mM MSM for 24 h. Then they were harvested and 1.5×10^5 of cells in 600 µl of the experimental media were seeded on 8 µm pore transwell 12-well inserts coated with 100 µl Geltrex™ (Thermo Fisher Scientific) in a final concentration of 200 µg/ml (modified Boyden chamber assay) and incubated for 24 h on companion plates filled with 2 ml of the experimental media. Next, cell inserts and companion plates were washed three times with PBS, fixed with 4% PFA, washed once again with PBS and stained with 0.1%

crystal violet for 10 min. The non-invasive cells that remained on the upper surface of the filter were removed with cotton wool. Stained invasive cells on inserts were photographed with Olympus DP20 camera, then dissolved in 200 µl of 10% acetic acid and transferred on 96-well plate. The absorbance was measured at 550 nm on ELX U808IU plate reader. The experiment was conducted in triplicate.

2.9. Gelatin zymography

Firstly, 5×10^5 cells were seeded on 6-well plates and incubated

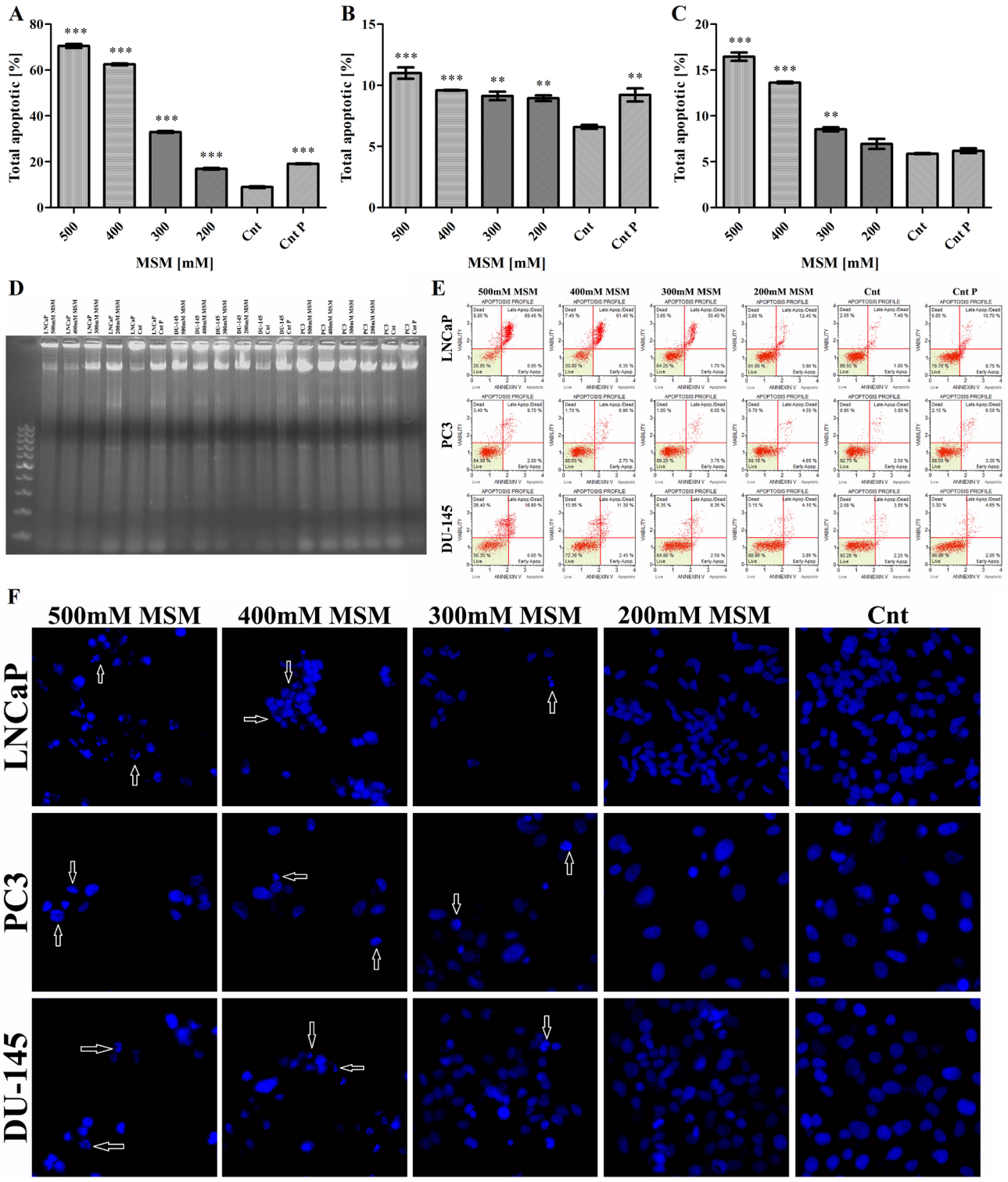

Fig. 3. MSM induces apoptosis in prostate cancer cells. The number of apoptotic cell was expressed as a percentage rate of total apoptotic cells for LNCaP (A), PC3 (B) and DU-145 (C) cell lines. Fig. 3F shows representative results. Fragmentation of DNA was visualized on agarose gel (E) as well as with DAPI staining (F). Arrows present representative changes in the nuclei of cells. The number of cells in sub G0/G1, G0/G1, S and G2/M cell cycle phase were presented in the Fig. 3 for LNCaP (G), PC3 (H) and DU-145 (I) as well as representative results of cell cycle measurement (J). $p < 0.05$ was considered as statistically significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ as compared to control cells. MSM-methylsulfonylmethane, Cnt-control.

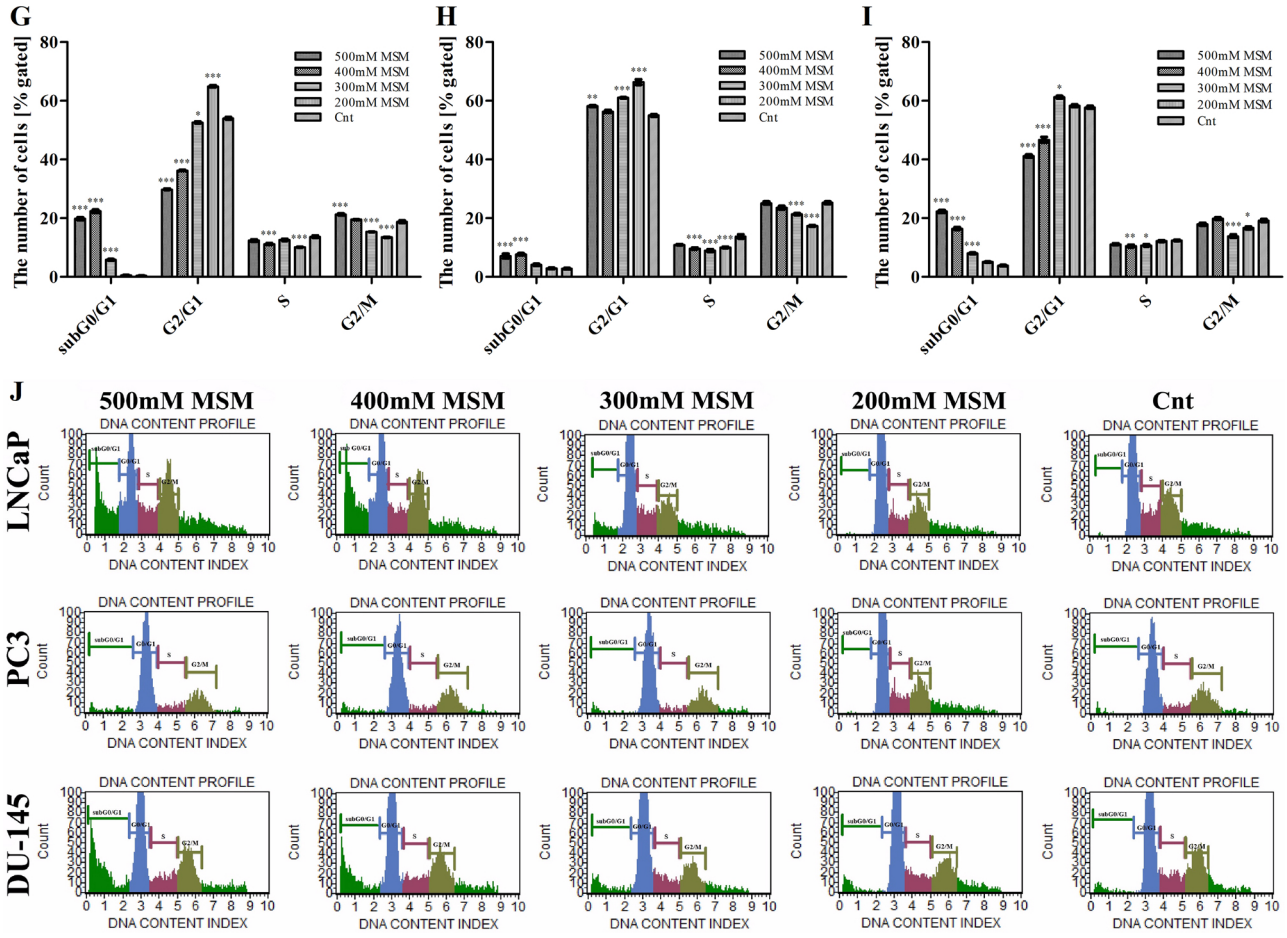

Fig. 3. (*continued*)

until 90% confluence was reached. Next, cells were treated with the experimental media for 24 h, as previously described. After the incubation time, the media were collected. Concentration of protein content was calculated with Qubit Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's recommendations. 7 µg of protein was loaded on 10% gelatin zymography gels and subjected to electrophoresis (120 V, on ice) and then incubated in 2.5% Triton X-100 (Sigma Aldrich) two times for 30 min. Next gels were incubated for 72 h in developing buffer in 37 °C to enable the determination of total proteolytic MMP activity. The gels were stained with Coomassie brilliant blue (Sigma Aldrich) and destained with 50% methanol and 20% acetic acid. Areas of enzymatic activity appeared as clear bands over the dark blue background. The gels were scanned and the intensity of bands was calculated in an inverted picture in ImageJ software. The experiment was done in triplicate.

2.10. Real time quantitative polymerase chain reaction (RTqPCR)

For RNA isolation, 5×10^5 cells were cultured on six-well plates and treated as described above. RNA was isolated with TRIzol Reagent according to the manufacturer's protocol. Isolated RNA was diluted in 50 µl of sterile deionized water. The concentration of RNA was measured with a BioDrop spectrophotometer. RealTime Ready Custom Panel (Roche) was used to analyse the expression of genes associated with the cell cycle and apoptosis. For this assay, the reaction mixture was prepared using a LightCycler® 480 Probes Master Kit (10 ng of cDNA per well) according to the manufacturer's instructions. The mixture was added to a RealTime ready Custom Panel with pre-plated genes: *CCNE1*, *CCND1*, *SOD2*. *CDK2*, *CDK4*, *CDKN1B*, *BAX*, *CYBB*. *RPLP0*, *RN18S1* and *RPL13A* were used as reference genes. The reaction was carried out using Light Cycler® 480 II (Roche) according to the manufacturer's instructions. The results from RT-qPCR arrays are presented as fold change as compared to control (non-treated) cells.

2.11. Statistical analysis

The results were expressed as the mean ± SE. The statistically significant differences were calculated based on the one-way ANOVA test. $P < 0.05$ was considered as statistically significant. All statistical analyses were carried out with GraphPad Prism software.

3. Results

3.1. MSM decreases viability of prostate cancer cells

The influence of MSM on viability of the cells was evaluated with AlamarBlue® reagent. We observed that MSM caused a dose-dependent decrease in viability of LNCaP, PC3 and DU-145 prostate cancer cells (Fig. 1). A statistically significant decrease in the viability of LNCaP cells, as compared to control cells, was observed in concentrations ranging from 300 to 800 mM (***$p < 0.001$) (Fig. 1A). A similar effect was observed in the case of the PC3 cell line treated with 300–800 mM MSM (***$p < 0.001$) (Fig. 1B). The DU-145 cells were less sensitive to MSM and a decrease in cell viability, as compared to control cells, was observed for 400–800 mM MSM (***$p < 0.001$) (Fig. 1C). The IC_{50} values calculated for LNCaP, PC3 and DU-145 were 901.1 mM, 633.7 mM and 722.5 mM, respectively. Thus, the range of MSM concentrations 500-200 mM has been chosen for the rest of experiments. Those results indicated that various prostate cancer cell lines with different hormonal sensitivity might differently respond to MSM.

Table 1
MSM modulates the expression of proteins associated with apoptosis. The results of ELISA are presented as a percentage rate of control- non- treated cells. $p < 0.05$ was considered as statistically significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ as compared to control cells. MSM- methylsulfonylmethane, p53- tumor protein p53, PARP- Poly [ADP-ribose] polymerase 1, Bad- Bcl-2-associated death promoter.

	LNCaP			
	500 mM MSM	400 mM MSM	300 mM MSM	200 mM MSM
phospho-p53	302.5% ***	230.0% ***	120.9% ***	102.5%
p53	82.2% ***	39.2% ***	20.8% ***	33.9% ***
cleaved caspase 3	172.6% ***	177.1% ***	99.2%	55.5% ***
cleaved PARP	287.8% ***	288.0% ***	242.5% ***	100%
phospho-Bad	131.3% ***	108.8% ***	113.1% ***	110.0% ***
Bad	102.0% ***	73.2% ***	93.1% ***	103.3% ***
	PC3			
	500 mM MSM	400 mM MSM	300 mM MSM	200 mM MSM
phospho-p53	125.1% *	103.5%	103.5%	101.2%
p53	97.4%	96.5%	87.9%	78.6% **
cleaved caspase 3	557.9% ***	139.2% ***	78.2% ***	74.5% ***
cleaved PARP	648.4% ***	764.2% ***	154.2% ***	176.1% ***
phospho-Bad	99.4%	181.9% ***	198.6% ***	93.6%
Bad	77.0% ***	32.3% ***	93.4% ***	105.6% ***
	DU-145			
	500 mM MSM	400 mM MSM	300 mM MSM	200 mM MSM
phospho-p53	69.7% ***	104.1% ***	19.8% ***	35.3% ***
p53	5.4% ***	13.2% ***	39.6% ***	61.8% ***
cleaved caspase 3	38.0% ***	82.2% ***	295.1% ***	333.4% ***
cleaved PARP	238.9% ***	264.6% ***	219.5% ***	394.2% ***
phospho-Bad	55.6% ***	68.5% ***	85.9% ***	78.2% ***
Bad	52.1% ***	37.4% ***	98.8% ***	103.0% ***

Table 2
MSM modulates the expression of genes associated with the cell cycle control and apoptosis: *CCNE1, CCND1, CDK2, CDK1B, BAX* and *SOD2*. The results are expressed as a fold change of gene expression as compared to control (non-treated) cells measured by RT-qPCR. MSM-methylsulfonylmethane, *CCNE1*- cyclin E1, *CCND1*- cyclin D1, *CDK2*- cyclin-dependent kinase 2, *CDKN1B*- cyclin-dependent kinase inhibitor 1B, *BAX*- Bcl-2 associated X protein, *SOD2*- superoxide dismutase 2.

Gene	Cell line	300 mM MSM	400 mM MSM
CCNE1	LNCaP	−3.15	−14.5
	PC3	−1.02	−1.13
	DU-145	1.61	−1.13
CCND1	LNCaP	−3.39	−8.6
	PC3	−3.51	−3.65
	DU-145	−1.39	1.14
CDK2	LNCaP	−4.12	−4.72
	PC3	−2.7	−5.36
	DU-145	1.04	−1.65
CDKN1B	LNCaP	1.56	2.93
	PC3	1.88	−1.75
	DU-145	1.35	5.09
BAX	LNCaP	−3.14	1.88
	PC3	−1.85	1.46
	DU-145	−2.59	1.21
SOD2	LNCaP	−1.59	−2.5
	PC3	−1.9	−4.36
	DU-145	1.29	−1.61

3.2. MSM modulates mitochondrial potential of prostate cancer cells

The next stage of the study established whether MSM could modulate changes in the mitochondrial potential associated with the identified decrease in viability. It was observed that MSM caused a significant increase in the mitochondrial potential in LNCaP cells ($***p < 0.001$), although the most remarkable one was observed for 400 mM MSM ($***p < 0.001$) (Fig. 2A). For 500 mM MSM, an increase was also observed as compared to control cells, however it was lower than 400 mM, which could be associated with a lower number of living cells. A similar effect was observed for the DU-145 cell line ($*p < 0.05$; $***p < 0.001$) (Fig. 1C). PC3 cells showed no significant decrease in the number of depolarized cells for 300–500 mM MSM as compared to control cells (Fig. 1B). Only in the case of the lowest tested concentration, 200 mM MSM, a significant increase was observed ($**p < 0.01$). These results suggest that PC3 cells might be less sensitive to MSM and the effect observed in other cell lines was not detected at the same time point (after 24 h). The representative results are shown in Fig. 2D.

3.3. MSM induces apoptosis in prostate cancer cells

The previously observed changes in the mitopotential of cells as well as the decrease in cell viability might be associated with programmed cell death- apoptosis. Thus, the induction of apoptosis by MSM was verified by counting the number of apoptotic cells by the Muse™ Cell Analyzer, evaluating of the expression of apoptosis-associated proteins (p53, Bad, cleaved caspase 3 and PARP) and detecting morphological changes in nuclei by DAPI staining. In the LNCaP and DU-145 cell lines, MSM caused a significant dose-dependent increase in the number of apoptotic cells ($*p < 0.05$) (Fig. 3A and C). Although MSM also caused an increase in the number of apoptotic cells in PC3 cell line (Fig. 3B), the effect was not as remarkable as for other cell lines ($*p < 0.05$). In all cases, MSM was found to have a more powerful effect than the staurosporine used as a positive control. In addition, the morphology of the cell nuclei was also changed: visible apoptotic cells are marked in Fig. 3F, and the fragmentation of DNA visible on agarose gel is given in Fig. 3E.

To confirm that apoptosis was induced by MSM, the expression of a key apoptosis protein was verified. The expression of phospho-p53 was found to be elevated with increasing MSM concentration in all cell lines, although the highest expression in DU-145 was reported in the case of 400 mM MSM (Table 1). The expression of p53 increased with MSM concentration in LNCaP and PC3 cells, but decreased in DU-145 cells. A similar effect was observed for cleaved caspase 3; the expression increased with MSM level in LNCaP and PC3 cell lines but decreased in DU-145. Cleaved-PARP expression was the highest in the treatment of PC3 cells with 500 mM MSM, LNCaP cells with 400 mM MSM and 200 mM MSM in the case of DU-145 cell line. The phospho-Bad expression was decreasing with MSM concentration in DU-145 cell line, whereas in the case of LNCaP and PC3 this effect was not observed. The highest expression was observed for 300 mM MSM in PC3 cells and 500 mM in LNCaP cells. Bad expression also decreased with MSM concentration in DU-145 cell line, although the lowest expression was observed for 400 mM MSM. A similar effect was observed for the PC3 cells and LNCaP; however, in these cell lines, the decrease was much smaller than in DU-145 cell line.

The induction of apoptosis was also visible during evaluation of the cell cycle (Fig. 3G–J). A statistically significant increase in the number of cells in the sub G0/G1 cell cycle phase was observed after treatment with MSM for LNCaP (300–500 mM MSM, $***p < 0.001$) (Fig. 3G), PC3 (400–500 mM MSM, $***p < 0.001$) (Fig. 3H) and DU-145

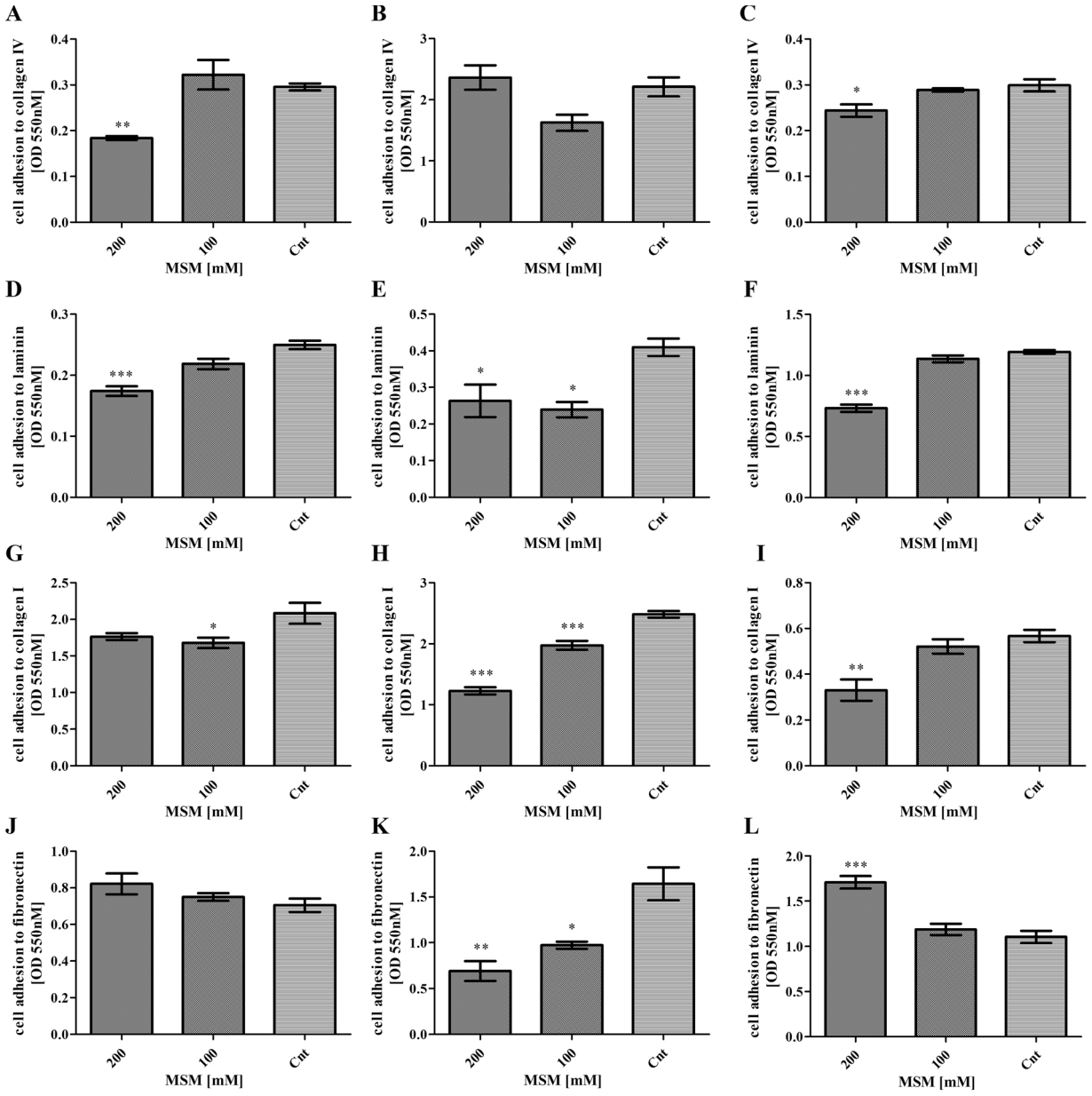

Fig. 4. MSM modulates adhesion of LNCaP, PC3 and DU-145 prostate cancer cells. A, D, G, J- adhesion of LNCaP cells, B, E, H, K- adhesion of PC3 cells, C, F, I, L- adhesion of DU-145 cells. $p < 0.05$ was considered as statistically significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. MSM- methylsulfonylmethane, Cnt- control.

(300–500 mM MSM, $***p < 0.001$) (Fig. 3I). In addition, a concentration-dependent decrease in the number of cells in G0/G1 was revealed. Moreover, a significant decrease in the number of LNCaP cells in S phase was observed after treatment with 200 and 400 mM MSM ($***p < 0.001$), PC3 cells after treatment with 200–400 mM MSM ($***p < 0.001$) and DU-145 cells after treatment with 300 and 400 mM MSM ($*p < 0.05, ***p < 0.001$). The changes in the number of cells in G2/M phase of the cell cycle were not concentration dependent. The number of LNCaP cells in G2/M phase increased following 500 mM MSM treatment and decreased with lower doses (i.e. 200 and 300 mM MSM) ($***p < 0.001$). For the same doses of MSM, a decrease was also observed in PC3 and DU-145 cells.

The observed increase in G0/G1 cell cycle phase was also confirmed by gene expression analysis in RT-qPCR (Table 2). The procedure evaluated the expression of genes associated with transition of cells from G1 to S cell cycle phase: *CCNE1*, *CCND1*, *CDK2*, *CDKN1B* as well as *SOD2* and *BAX* expression. A dose-dependent decrease in cyclin E (*CCNE1*) expression was observed in the LNCaP, PC3 and DU-145 cell lines. A similar effect was observed in the case of cyclin D (*CCND1*) expression for LNCaP and PC3 cells, whereas DU-145 treated with 400 mM MSM showed an increase in expression. *CDK2* demonstrated a decrease for all cell lines in a dose-dependent manner. Moreover, *CDKN1B* (p27) expression was found to increase after treatment with MSM in the LNCaP and DU-145 cell lines, whereas an increase in *CDKN1B* expression was observed only for 300 mM MSM in PC3 cells. *BAX* expression decreased after 300 mM of MSM and increased after 400 mM MSM treatment in all evaluated cell lines. *SOD2* expression decreased in all cell lines in a dose-dependent manner.

3.4. MSM decreases cell invasiveness and migration

The next stage of the experiment examined whether lower concentrations of MSM (100 mM and 200 mM) might modulate the invasiveness and migration of PC cells. Lower concentrations were used where the percentage rate of apoptotic cells was not so high, thus allowing a reliable invasion and migration test to be carried out.

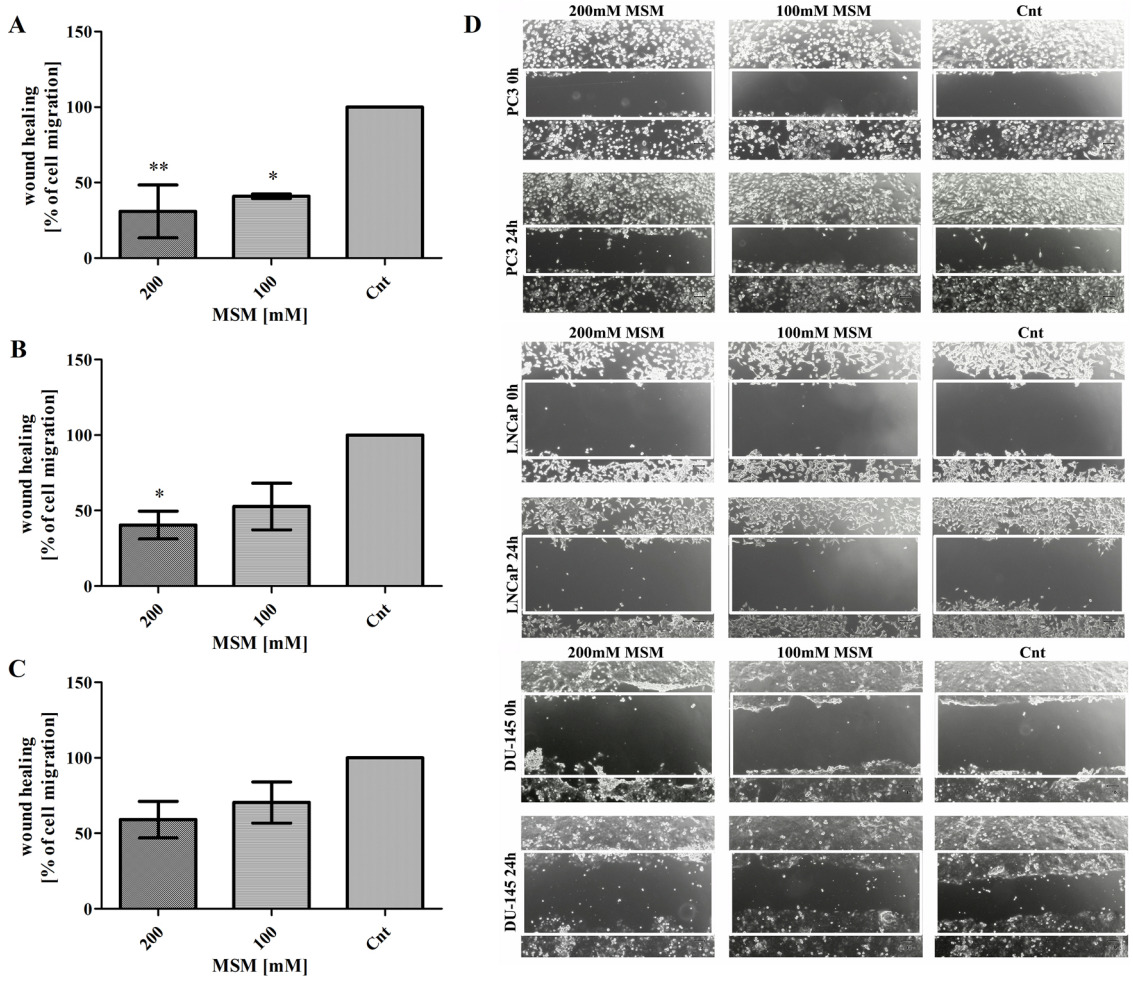

Fig. 5. MSM influences invasion of prostate cancer cells. The results from the scratch assay are presented for LNCaP (A), PC3 (B) and DU-145 (C) as a percentage rate of cell migration, as compared to control cells. Representative results are presented in the Fig. 5D. The results of the invasion assay are presented for LNCaP (E), PC3 (F), DU-145 (G) as well as representative images of stained cells (H). The activity of MMP-2 and MMP-9 was measured with zymography assay for LNCaP (I, L), PC3 (J, M) and DU-145 (K, N) cells. The representative results of zymography of MMP-9 activity (O) and MMP-2 (P) were presented. $p < 0.05$ was considered as statistically significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, as compared to control cells. MSM- methylsulfonylmethane, Cnt- control, MMP-2- metalloproteinase 2, MMP-9- metalloproteinase 9, OD- optical density.

First, we evaluated the effect of MSM on adhesion of cells to selected ECM proteins (Fig. 4). 200 mM of MSM caused a statistically significant decrease in adhesion of LNCaP cells to collagen IV and laminin ($**p < 0.01$,$***p < 0.001$, respectively) (Fig. 4A). The adhesion of PC3 to collagen IV was not significantly different from control cells ($p > 0.05$) (Fig. 4B). In the case of DU-145, a significant decrease was observed in adhesion to collagen IV after treatment with 200 mM MSM as compared to control cells ($*p < 0.05$) (Fig. 4C). The adhesion to laminin was significantly decreased after treatment with 200 mM MSM in all the cell lines (Fig. 4D–F). For PC3 cells a statistically significant decrease was also observed in the case of 100 mM MSM ($*p < 0.05$). As for collagen I, decreased adhesion was observed in LNCaP and PC3 cells following treatment with 100 mM MSM ($*p < 0.05$, $***p < 0.001$) as well as in PC3 and DU-145 cells following treatment with 200 mM MSM ($***p < 0.001$, $**p < 0.01$) (Fig. 4G–I). MSM treatment caused an increase in adhesion to fibronectin in LNCaP and DU-145 cells, which was significant only for DU-145 cells ($***p < 0.001$) (Fig. 4J and L). MSM caused a dose-dependent and significant decrease in adhesion to fibronectin in PC3 cells ($*p < 0.05$, $**p < 0.01$) (Fig. 4K).

Changes in the migration of cells caused by MSM were then evaluated. We observed that in all three PC cell lines MSM caused a dose-dependent decrease in cell migration, which was significant for 200 mM for LNCaP and PC3 ($**p < 0.01$, $*p < 0.05$) (Fig. 5A–D). Moreover, the results showed that 200 mM MSM significantly reduced cell invasion for LNCaP and DU-145 cells compared to control cells ($**p < 0.01$, $***p < 0.001$, respectively) (Fig. 5E–H). For PC3 cells the invasion of cells was slightly, but not significantly increased. MSM did not cause any significant decrease in MMP-9 or MMP-2 activity in LNCaP or PC3 cells, and no changes in MMP-9 and MMP-2 activity were observed in DU-145 cells (Fig. 5I–N).

4. Discussion

PC is considered to be a hormone-dependent cancer, since steroid hormones regulate its development and progression (Di et al., 2018). Initially, PC is androgen-sensitive, but during disease progression, it develops into castration- resistant PC (CRPC) which is androgen insensitive and associated with poor prognosis (Ballon-Landa and Parsons, 2018; Di et al., 2018). Nowadays many preventive therapies are focused on nutrition, physical activity and an overall healthy lifestyle (Ballon-Landa and Parsons, 2018). Moreover, these trends are also visible in cancer therapies, where a number of research studies focus on natural compounds which also serve as sensitizers of known cytotoxic drugs (Joung et al., 2014). The main advantage of using natural cytotoxic drugs is their low overall toxicity as well as the fact that they cause less severe side effects than well-known and used cancer

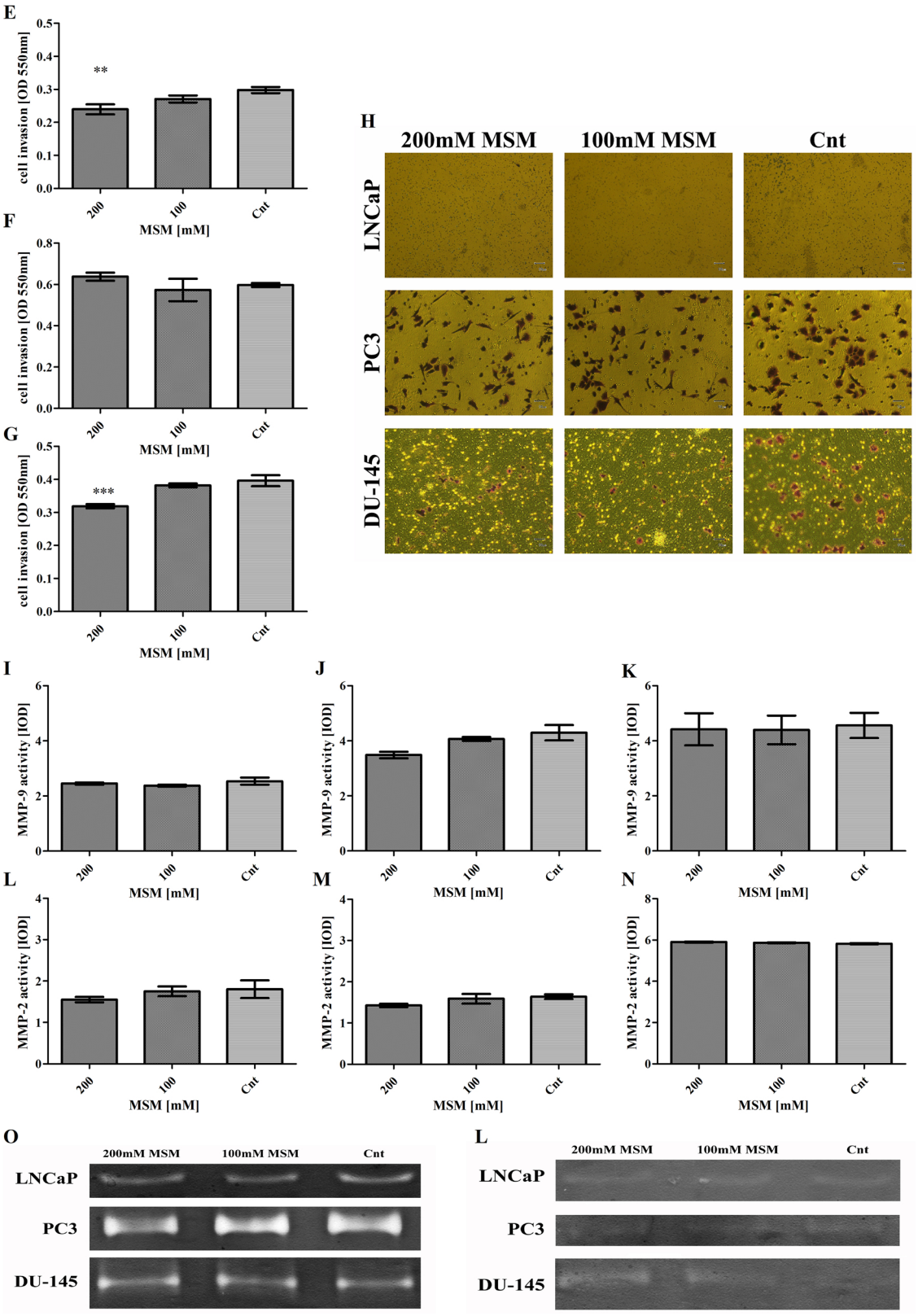

Fig. 5. (*continued*)

therapies (NS et al., 2017). The previously-reported cell cycle inhibition, induction of apoptosis and decrease in cell invasiveness induced by MSM (Butawan et al., 2017) may be also potentially useful in PC therapy.

Our results prove that MSM induces a concentration-dependent decrease in PC cell viability. Interestingly, the same doses of MSM appeared to cause different effects in LNCaP, PC3 and DU-145 cells. LNCaP cells seem to be less sensitive to MSM than PC3 and DU-145, even though they are reported to be androgen sensitive, like PC3 cells (Dominska et al., 2017), and are considered to have low metastatic

potential (Kowalska et al., 2016). We also noticed that PC cells are less sensitive to MSM than e.g. breast cancer cells SK-BR3 (Kang et al., 2016), murine breast cancer cells 66cl-4 (Caron et al., 2013) or gingival cancer cells YD-38 (NS et al., 2017).

The observed reduction in cell viability of PC cells was associated with induction of apoptosis. MSM was found to induce apoptosis of cells, as noted in previous reports (Lim et al., 2012; SP et al., 2015; Jafari et al., 2012), as well as changes in the mitopotential of cells and changes in the expression of key regulators of apoptosis. Interestingly, changes in the expression of p53 occurred only in LNCaP and PC3 cells, and no increase in phospho-p53 expression was present in DU-145 cells. p-53 independent induction of apoptosis was also observed by Karabay et al. in colon cancer cells (Karabay et al., 2016). In contrast, an increase in phospho-p53 expression was observed in LNCaP and PC3 cells, which indicated p-53-dependent apoptosis. These facts might be associated with the metastatic potential of the DU-145 cell line, which is reported as highly metastatic and androgen independent (Dominska et al., 2017). Moreover, these results indicate that MSM might induce apoptosis differently in hormone-dependent and independent cell lines, but this thesis requires further studies to be confirmed.

Similarly to other researchers, we also observed that MSM induces cell cycle arrest in the G0/G1 cell cycle phase, with a characteristic sub G0/G1 peak in Annexin-V staining (NS et al., 2017). The cell cycle arrest associated with apoptosis was also visible in the expression of genes associated with cell-cycle control. The decrease in the expression of cyclin D and cyclin E indicated a lack of progression from phase G1 to S (Qie and Diehl, 2016). An increase in *BAX* expression was also observed in the case of higher dose of MSM (400 mM). Bax is a pro-apoptotic protein that acts as a pore factor in mitochondria and plays a crucial role in apoptosis (NS et al., 2017). Similarly to gingival cancer cells, MSM in PC cells also caused an increase in Bax expression (NS et al., 2017). Moreover, fragmentation of DNA and morphological changes in nuclei induced by MSM were also observed, which confirms that MSM induces apoptosis in PC cells. The induction of mitopotential might be associated with induction of apoptosis, and seems to be evident in the expression of *SOD2*- a regulator of mitochondrial oxidants, specifically $O2^{\bullet -}$ and H_2O_2 (Kim et al., 2017).

The mortality in cancer is mainly associated with metastases in which cell migration and invasion are crucial (SP et al., 2015). Hence, the natural compounds which decrease cell migration and invasion might be beneficial in PC treatment. It appeared that 200 mM MSM might significantly decrease migration in LNCaP and PC3 cells, whereas an insignificant decrease was observed in DU-145. The decrease caused by MSM in cell migration was also visible in cell invasion where 200 mM MSM caused a significant decrease in LNCaP and DU-145 cells. Interestingly, no decrease in PC3 cell invasion was observed. This fact might be associated with the observed previously lower induction of apoptosis in PC3 cells. This, in turn, may suggest that PC3 cells are less sensitive to MSM. Among all MMPs MMP-2 and MMP-9 are overexpressed in various types of cancer and participate in invasion of cancer cells (Folgueras et al., 2004). Our present findings indicate no statistically significant decrease in MMP-2 and MMP-9 activity after treatment with 200 mM MSM, evaluated by zymography. A similar effect after MSM treatment was observed by SP et al. (SP et al., 2015) in combination with tamoxifen in breast cancer as well as in combination with AG490 in lung cancer (Joung et al., 2014). In these experimental studies, MSM was used as a sensitizer to some other well-known drug, which in PC might be useful too. MSM was also reported to modulate migration of other cell lines: breast (Caron et al., 2013; Caron and Caron, 2015) as well as melanoma (Caron et al., 2010). Interestingly, the reported ability of MSM to decrease the level of HIF-1α in response to oxidative stress (Caron and Caron, 2015) with a decrease in cell invasion and migration might be beneficial in potential application of MSM in cancer treatment. Moreover, the administration of MSM before induction of cancer in animals resulted in reduction of the tumor (McCabe et al., 1986). The migration of cells is also regulated by adhesion of cells to each other and the extracellular matrix proteins (ECM) (Dominska et al., 2016). Therefore, we also attempted to establish whether MSM may modulate adhesion of PC cells to collagen I, collagen IV, laminin and fibronectin. Various cell lines were found to differently adhere to ECM proteins following MSM treatment. Nevertheless, in most cases MSM decreases the adhesion of cells to ECM proteins, which confirms the previously observed decrease in cell migration. The increase in adhesion of LNCaP and DU-145 cells to fibronectin, as well as PC3 cells to collagen IV, might be triggered by the fact that cancer cells differently adhere to various different ECM proteins, as reported previously (Moro et al., 2006).

5. Conclusions

To sum up, our results demonstrate that MSM may decrease the viability and invasiveness of PC cells, that this effect is concentration dependent and is associated with induction of apoptosis in cells. We also observed that differences in hormonal sensitivity and migration potential might determine different responses to MSM, but further studies are required to confirm this. The induction of apoptosis caused by MSM in PC cells might also be useful in currently-applied PC drugs where MSM may sensitize and synergistically decrease the viability and invasiveness of PC cells.

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Contributions

KK and AP-C conceptualized the study. KK, DH-G and KU conducted the research. KK, KD and AP-C analysed the results. KK and DH-G wrote the manuscript. All authors read and approved the final version of manuscript.

Transparency document

The Transparency document associated with this article can be found in the online version.

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