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# The Effect of Cold Atmospheric Plasma on the Membrane Permeability of Human Osteosarcoma Cells

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**Abstract.** *Background: Cold atmospheric plasma (CAP) has a variety of anticancer effects on different cancer cell types. In osteosarcoma (OS) cells, CAP reduces growth and motility, induces apoptosis, and alters secretion of cellular factors. The influence of CAP on membrane integrity of OS cells is unknown. Materials and Methods: Two different OS cell lines (U-2 OS and MNNG-HOS) were treated with CAP. Proliferation assays for cell growth after treatment was performed. Alterations in membrane permeability and the associated translocation of low molecular weight particles through the cytoplasmic membrane of OS cells after CAP treatment were shown in fluorescein diacetate (FDA) assays. Results: FDA increasingly passed the membrane after CAP treatment and this effect depended on the duration of treatment. It was also shown that after CAP treatment, FDA was able to diffuse into the cells from the outside as well as out of the cell interior. These effects were observed when CAP-treated buffer was used and therefore no direct contact between cells and CAP occurred. Conclusion: The observations suggest that changes in membrane permeability and function may contribute to the antiproliferative effects of CAP.*

Osteosarcoma (OS) is the most common malignant tumor of the musculoskeletal system in children and adolescents, with incompletely explained etiology and pathogenesis. It is characterized by rapid progression and aggressive metastasis in about 20% of cases (1-3). Radical surgical treatment and neoadjuvant chemotherapy are currently the basic therapy for OS (4, 5). Overall survival has now increased to 75% through chemotherapy but there has been no further beneficial outcome to patient survival in the past 30 years (6). In addition, event-free 5-year survival is only possible for 30% of patients with OS (7). One of the reasons for the currently limited therapeutic success is the development of resistance to anticancer drugs (8). For the treatment of OS, it is therefore essential to identify innovative and effective treatment methods that supplement the existing therapy regimen (9).

In recent years, cold atmospheric plasma (CAP) seems to have become such a therapy option with increasing popularity (10). Due to its low temperature, CAP has excellent tissue tolerance and is already used in the treatment of acute and chronic wounds (11, 12). Its many anticancer effects have also been described in many cancer cell types, including prostate (13, 14), melanoma (15, 16), head and neck (17), colon (18), and lung cancer (19). At the cellular level, CAP treatment leads to various cellular mechanisms and finally to the induction of anti-oncogenic effects such as inhibition of growth and motility, apoptosis and changes in tumor–environment interactions. Those effects seem to be independent of the CAP device used (20). CAP treats the activation of redox signaling pathways that eventually induces p53-dependent apoptosis (21). In addition, there was first evidence that CAP also induces changes in secretion, *e.g.* of immunostimulatory factors (22). The influence of CAP on membrane integrity of OS cells and the possible influence of interaction with the microenvironment are unknown. The cytoplasmic membrane not only plays a central role in the connections between cells and their environment but is also essential for the physiological

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functions of the cell (23). The aim of the present study was therefore to investigate the effects of CAP treatment on the membrane permeability of OS cells in order to detect possible anti-oncological effects at this level as well.

## Materials and Methods

*Cell culture*. The human OS cell lines U-2 OS and MNNG-HOS (American Type Culture Collection, Manassas, VA, USA) were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 1.0 g/l glucose, 10% fetal bovine serum, 1 mM sodium pyruvate, and 1% penicillin/streptomycin (all PAN Biotech, Aidenbach, Germany) in a humidified atmosphere at 37˚C with 5% $CO_2$.

*Proliferation assay*. Cell growth was determined after 4, 24, 48, 72, 96 and 120 h using a CASY cell counter and analyzer model TT (Roche Applied Science, Mannheim, Germany) with a 150 μm capillary. For this purpose, $5\times10^4$ (U-2 OS) and $5\times10^4$ (MNNG-HOS) cells were suspended in 200 μl DMEM and treated with CAP or the carrier gas argon (control group) for 10 s, 30 s and 60 s. The atmospheric plasma beam kINPen MED (Neoplas Tools, Greifswald, Germany) was used for CAP treatment (carrier gas: argon, gas flow: 3 l/min; supply voltage=65 V DC; frequency: 1.1 MHz). After treatment, the cells were immediately transferred to 24-well cell culture plates coated with poly-L-lysine (PAN Biotech), 800 μl DMEM were added, and the plates were incubated in a humidified atmosphere at 37˚C with 5% $CO_2$.

Cell count was determined by suspending the cells by trypsin/EDTA treatment and diluting 100 μl cell suspension in $10^4$ μl CASYton (Roche Applied Science). The measurement was performed three times with 400 μl each of this dilution and was performed in triplicates. To discriminate against cell debris, dead cells and living cells, gates of 7.20 μm/13.95 μm (U-2 OS) and 7.20 μm/14.85 μm (MMNG-HOS) were used.

*Fluorescein diacetate (FDA) assays (24)*. U-2 OS and MNNG-HOS cells were harvested from confluent T75 cell culture flasks by trypsinization and diluted to approximately $1\times10^6$ cells per ml with stop solution [Dulbecco's phosphate-buffered saline (DPBS) with 10% fetal calf serum v/v]. The cell suspension was stored on ice until use. A dye solution containing 30 μg/ml ethidium bromide and 5 μg/ml FDA in DPBS was used for flow cytometric measurement. The ethidium bromide determination was used to discriminate against dead cells. CAP-treated or control cells (200 μl) were added to 200 μl ethidium bromide/FDA dye solution and incubated for 15 min in the dark on ice. After centrifugation (300 ×*g* for 5 min) the labelled cells were resuspended in DPBS buffer and analyzed in a FACSCanto™ flow cytometer (BD Biosciences, Heidelberg, Germany) with FACSDiva™ 6.0 Software (BD Biosciences) and evaluated with FlowJo Software Version 10 (Tree Star Inc, Ashland, OR, USA). The data of the CAP treated cells were normalized to the control cells.

An acetone stock solution with 10 mg/ml FDA was prepared for the detection of the bleeding of FDA from FDA-loaded cells and diluted to a concentration of 5 μl/ml with DPBS buffer for the measurement. The cells were loaded with FDA for 30 min in the dark on ice. FDA not incorporated into the cells was removed by washing three times (150 ×*g* for 3 min) and resuspended in 500 μl DPBS. In order to exclude direct effects of CAP on the FDA dye, the cells were treated indirectly. For this purpose, 200 μl DPBS buffer were treated with CAP for 60 s, added to the FDA-loaded cells and incubated on ice for 20 min in the dark. Subsequently, the cells were sedimented (150 ×*g* for 3 min) and 100 μl of the cell-free supernatant were analyzed in an Infinite m200 PRO multimode reader (Tecan, Männedorf, Switzerland) with i-control 1.9 software (Tecan) with an excitation wavelength of 300 nm and a wavelength of 525 nm. The CAP treatment measurements were normalized to the control measurements.

*Data analysis*. For data analysis and visualization, Microsoft Excel version 1903 (Microsoft Corp., Redmond, Washington, USA) and GraphPad Prism version 7.04 for Windows (GraphPad Software Inc., La Jolla, CA, USA) were used. As treated and control cells were harvested from the same cell culture flask, data were examined for significant differences with the paired *t*-test. The normal distribution of differences between groups was checked with the Shapiro–Wilk normality test. In case, of violation of the preconditions for the paired *t*-test, the Wilcoxon matched-pairs test was used ($p<0.05$).

## Results

*CAP treatment inhibited the proliferation of OS cells in vitro*. Both OS cell lines demonstrated significant growth inhibition after CAP treatment compared to control cells treated with carrier gas (Figure 1). The inhibitory effect of CAP treatment depended on the duration of CAP exposure. However, both cell lines differed in their sensitivity to CAP treatment. While MMNG-HOS cells showed a significant growth reduction after 10 s treatment (Figure 1A), the U-2 OS cells had to be treated for at least 30 s to achieve a comparable inhibitory effect (Figure 1B). Treatment for 60 s significantly suppressed cell proliferation of both OS cell lines.

*CAP treatment reduced the intracellular concentration of membrane-permeable fluorescent dye in OS cells*. To investigate possible effects of CAP exposure on membrane integrity, OS cells were incubated with FDA and ethidium bromide immediately after CAP treatment and the mean fluorescence intensity (MFI) of FDA in living single cells was analyzed by flow cytometry (Figure 2).

The study showed that the intracellular MFI of individual cells decreased with increasing duration of exposure CAP (Figure 3). Similar to the growth-inhibitory effect, U-2 OS required longer CAP treatment times than did MNNG-HOS cells to achieve comparable effects.

*Indirect CAP treatment of FDA-loaded OS cells lad to liberation of FDA into the extracellular cell culture supernatant*. After the increase of the intracellular FDA concentration during incubation with FDA solution, the increased leakage of FDA into the cell culture supernatant should be proven in a *vice versa* approach. Since direct CAP treatment may lead to photochemical modifications of the dye, OS cells were loaded with FDA and exposed to DPBS buffer treated with CAP or carrier gas argon. After this indirect treatment, the cells were incubated for 20 min before the extracellular dye concentration in the supernatant was analyzed

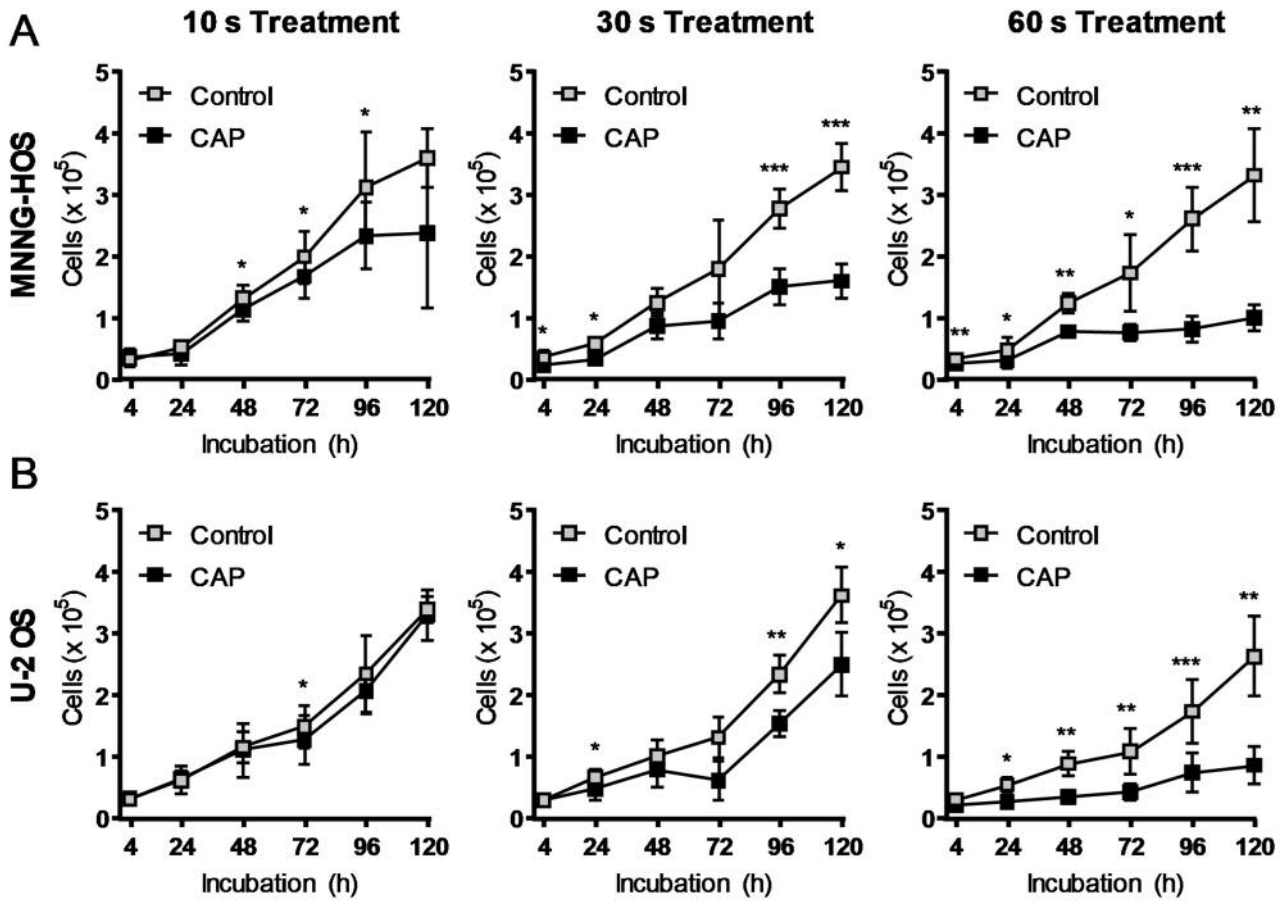


Figure 1. *Growth inhibition of osteosarcoma cells by cold atmospheric plasma (CAP) treatment. MNNG HOS (A) and U 2 OS (B) cells were treated with CAP for 10, 30 and 60 s. Control cells were treated with carrier gas for corresponding times. The number of living cells was counted with a CASY Cell Counter at the indicated time points. Data are presented as mean±SD (n=5, each). Means were tested for significant differences with a paired t-test or a Wilcoxon matched-pair test: *p<0.05, **p<0.01 and ***p<0.001.*

with a fluorescence plate reader. After incubation with both CAP-treated DPBS and control-treated DPBS, cells liberated FDA during the incubation (Figure 4). However, the FDA concentration of the cell culture supernatant of cells indirectly treated with CAP was significantly higher than that of control-treated cells. This effect was observed in both OS cell lines.

## Discussion

Growth-inhibitory effects of CAP on OS cells have already been demonstrated (25) and were confirmed in the initial experiments for appropriate treatment times. The underlying cell biological mechanisms in OS cells are presumably multifaceted. So far, redox effects and apoptotic processes have been identified which lead to apoptosis and thus to a strongly reduced growth rate of CAP-treated OS cells (21, 26).

Membrane functionality has not yet been investigated. However, this might be disturbed by contact with CAP and CAP-derived reactive molecules and may contribute to the CAP-mediated anticancer effect. The effect of CAP components on the bacterial cell membrane and its integrity is responsible for the bactericidal activity of CAP (27, 28). This effect has also been demonstrated in keratinocyte membranes, whose structure and membrane potential were altered by CAP treatment (29). In the context of a potential anticancer strategy, in cervical HeLa cells treated with CAP, changes in the cytoplasmic membrane were observed. CAP finally led to the collapse of the membrane architecture by increased electrical conductivity and increased lipid oxidation (30).

The data of the presented study indicate another CAP-mediated membrane effect, the loss of membrane permeability and the associated increase in unregulated diffusion of low-molecular-weight compounds through the membrane. After CAP treatment, the fluorescent dye FDA was able to penetrate increasingly into the cells. After CAP treatment, conversely, FDA also increasingly leaked from cells loaded with FDA and

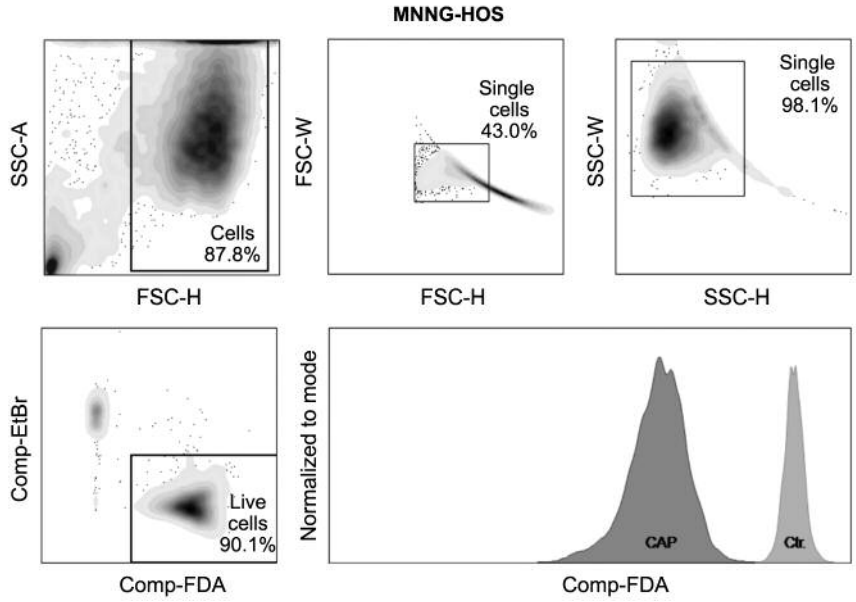


Figure 2. *Gating strategy. A representative example of analysis of the mean fluorescence intensity of fluorescein diacetate (FDA) in single living cells (MNNG-HOS) by flow cytometry. Comp: Compensated; FSC-H/-W: forward scatter height/width; SSC-A/-W: side scatter area/width.*

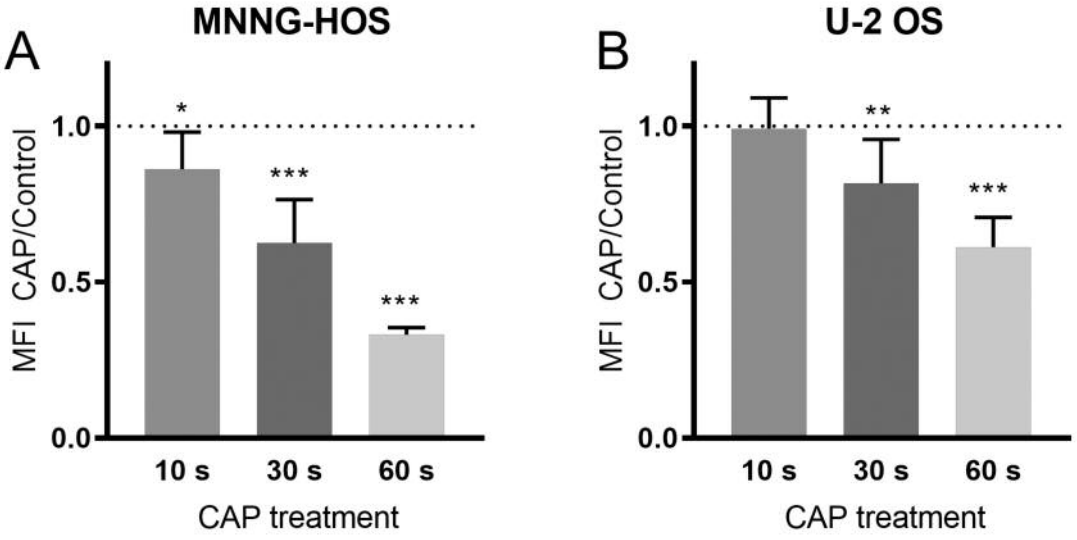


Figure 3. *Reduction of the intracellular mean fluorescence intensity (MFI) of fluorescein diacetate (FDA) after cold atmospheric plasma (CAP) treatment. Osteosarcoma cells were treated for 10, 30 and 60 s and incubated immediately with a solution of FDA (5 μg/ml) and etbrethidium bromide (30 μg/ml) for 15 min. The cells were then washed and the MFI of FDA was analyzed by flow cytometry. Control cells were treated with carrier gas. Data are given as mean±SD MFI normalized to control cells (n=8, MNNG-HOS; n=9, U-2 OS). Means were tested for significant differences with a paired t-test: *p<0.05, **p<0.01 and ***p<0.001.*

washed with buffer. This effect was detectable in both OS lines, although somewhat more prominent in MNNG-HOS cells. The uncontrolled uptake of FDA also depended on the treatment time. The longer the effect of the CAP components on the membrane, the more fluorescent dye was able to penetrate into the cell. The fact that FDA increasingly passed the membrane in both directions after CAP treatment indicates a rather nonspecific mechanism, possibly independent of transport systems.

When evaluating the data, it has to be taken into consideration that membrane-permeable FDA is converted by

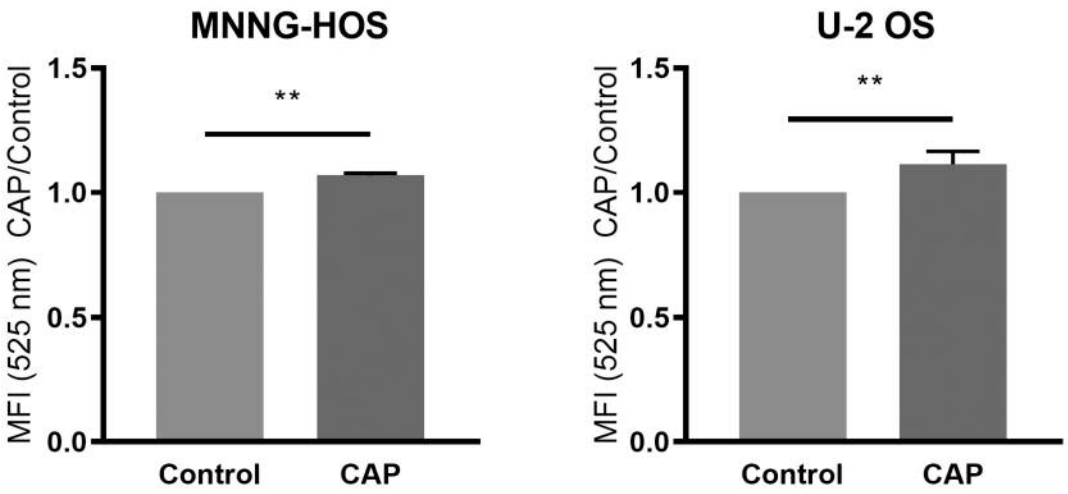


Figure 4. *Loss of intracellular fluorescein diacetate (FDA) by indirect cold atmospheric plasma (CAP) treatment. Osteosarcoma cells were loaded with FDA for 30 min, washed, and resuspended in Dulbecco's phosphate-buffered saline buffer which was previously treated with CAP or carrier gas argon for 60 s. After incubation of 20 min, the cell culture supernatant was transferred into a microtiter plate and the mean fluorescence intensity (MFI) of FDA was determined. Results are expressed as mean±SD MFI normalized to control cells (MNNG-HOS: n=3; U-2 OS: n=5). Means were tested for significant differences with a paired t-test: **p<0.01.*

enzymatic hydrolysis in the cell to the nonpermeable fluorescein (31). If active components of CAP interact with the corresponding enzymes, the hydrolysis of FDA molecules might be altered. If CAP interacts with FDA outside the cell and hydrolyses it, uptake might be prevented and any apparent increase in release might be an artifact. In addition, redox processes can also alter the state of excitation of fluorochromes, which may further induce inaccuracy in the translocation of FDA dye. However, indirect CAP treatment by the incubation of cells with CAP-treated buffer without direct contact between CAP and cells, to some extent excluded this.

The molecular processes involved in facilitating the passage of FDA through the cell membrane are still unknown. Physicochemical modifications of membrane building elements, *e.g.* lipid and protein oxidation that disturb membrane dynamics, are just as conceivable as the activation or inhibition of physiological membrane transport systems. However, that changes in membrane permeability are part of the antiproliferative and anticancer efficacy of CAP treatment can also be considered proven for OS cells.

## Conflicts of Interest

The Authors have declared that no competing interests exist.

## Authors' Contributions

LH, AN, DG, MBS prepared and edited the article; LH, DG and MBS conceived the study design. LH, AN, RE and NG performed the experiments; AN, RE, DOM and MBS performed data analysis; and MB, MZ and AK supervised the project.

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