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Comparison of Cold Atmospheric Plasma Devices’ Efficacy on Osteosarcoma and Fibroblastic In Vitro Cell Models

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Abstract. Background/Aim: Cold atmospheric plasma (CAP) attenuates tumor cell proliferation and induces apoptosis in various cell lines. While exerting marginal effects on non-neoplastic cells this unfolds promising applications in cancer therapy. The aim of the study was to analyse the effects of different CAP sources and application times on osteosarcoma (OS) cells and non-malignant fibroblast cell proliferation. Materials and Methods: U2-OS and 3-T-3 fibroblasts were treated with three different approved medical devices. Carrier gas-treated cells served as controls. Cell proliferation was determined by viable cell count at different time points after treatment. Results: Control exposed U2-OS and 3-T-3 cells exhibited characteristic cell growth. CAP application of U2-OS and 3-T-3 cells attenuated proliferation rates up to 98%. Attenuation rates varied between cell lines, plasma sources and application times. Conclusion: CAP treatment attenuates cell proliferation of OS cancer cells and fibroblasts in a treatment time-dependent manner, whereby U2-OS cells appeared more sensitive to CAP treatment as 3T3 fibroblasts after 10 sec of treatment.

*These Authors contributed equally to this study.

Osteosarcoma (OS), the most common primary malignant bone tumor in adolescents and young adults, is known to be highly aggressive and cause progressive bone destruction (1). Current OS treatment contains neoadjuvant multiagent chemotherapy regimens including doxorubicin, cisplatin, cyclophosphamide, ifosfamide and methotrexate (2). Subsequent complete surgical resection of the tumor is usually followed by postoperative, adjuvant chemotherapy (3). Although it occurs most frequently in the metaphyses of long bones, tumor localization in the pelvis, spine or skull may be challenging due to adjacent neurovascular structures. To date, despite a very limited number of active agents, a 5-year survival rate of up to 60-70% in non-metastatic disease has been achieved (4). However, limited treatment options especially in chemo-resistant or advanced cases and relapse have led to a stagnation in progress of systemic OS management for the last decades (5). The heterogeneous nature of OS further complicates treatment.

Cold atmospheric plasma (CAP), a highly reactive (partially) ionized physical state containing a dose controlled mixture of biologically-active agents has been shown to exert anticancer effects in vitro and in vivo (6-9). These active agents, generated by focused electrical discharge, include positive and negative ions, electrons, excited atoms and molecules, free radicals such as singlet oxygen, superoxide, reactive oxygen and nitrogen species (ROS and RNS), photons and electromagnetic radiation (10-12). Active plasma components can be adjusted through carrier gas composition (mostly argon and helium), gas flow, and electrical voltage and depends on environmental conditions, e.g. temperature and humidity (13, 14). The method of action is not completely understood. Tumor cell proliferation is attenuated by apoptotic cell death rather than by direct cytotoxic effects.
(15-20). In a balanced cell state, ROS are produced as a by-product of metabolic processes controlled with antioxidants. Prooxidant therapies are based on the rationale that a further oxidative stimulus produces oxidative stress in tumor cells, which cause the collapse of the antioxidant systems, leading to cell death (21). The mixture of ROS, RNS and their metabolites leads to an interaction with membrane structures producing oxidative stress in tumor cells (11). The tumor cell responses to these oxidative stimuli finally seem to result in apoptosis due to immature redox resistance (22). The antitumor efficacy may be triggered by the interaction of highly reactive H$_2$O$_2$ directly generated by CAP as well as possibly more importantly by an induction of intracellular generation of H$_2$O$_2$ by CAP, since H$_2$O$_2$ serves as an important signalling molecule in cancer cells (23, 24).

The purpose of this study was to evaluate the therapeutic potential of different commercially available and medically accredited CAP devices in the treatment of osteosarcoma.

Materials and Methods

**Cell culture.** Cells of the OS cell line U2-OS (purchased from the American Type Culture Collection, Manassas, VA, USA) were propagated in DMEM medium containing 1.0 g/l glucose supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, and 1% penicillin/streptomycin (all from PAN Biotech, Aidenbach, Germany) in a humidified atmosphere at 5% CO$_2$ and 37°C. 3-T-3 fibroblasts were propagated in DMEM medium containing 4.5 g/l glucose supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, and 1% penicillin/streptomycin (all from PAN Biotech, Aidenbach, Germany) in a humidified atmosphere at 5% CO$_2$ and 37°C. A total of 4×10$^6$ U2-OS cells or 2×10$^6$ 3-T-3 cells were seeded on an uncoated cell culture plate. After 4 days cells were washed twice in phosphate buffered saline and detached using 0.1% trypsin/0.04% ethyldiaminetetraacetic acid (EDTA) prior to resuspension in DMEM medium.

**CAP treatment.** For comparative analysis of CAP treatment three different plasma sources were used: The following atmospheric pressure plasma jets (APPJ) were used for the experiments: Maxium® (KLS Martin, Tuttingen, Germany) using the following settings: Carrier gas: Argon, gas flow: 2.6 l/min; 25 W.

Cold Plasma Coagulator 1000 (Söiring GmbH, Quickborn, Germany) using the following settings: Carrier gas: Helium, gas flow: 2.6 l/min; 25 W

kINPen® MED (Neoplas Tools, Greifswald, Germany) using the following settings: Carrier gas: Argon, gas flow: 4 l/min; pulsed plasma (2.5 kHz), supply voltage=65 V DC; frequency: 1.0 MHz.

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Significant differences between two groups were determined using the unpaired Student’s t-test. p-Values of ps≤0.05 (*), ps≤0.01 (**) and ps≤0.001 (***) were considered statistically significant. Data in the figures are shown as mean±SD of at least three independent experiments for each device and application time.

**Results**

**Antiproliferative efficacy of Maxium®.** Over a period of 120 h, argon treated U2-OS cells (controls) exhibited characteristic cell growth. In contrast, CAP treated U2-OS cells showed significantly attenuated cell proliferation 120 h after treatment depending on application time. While 120 h after CAP treatment over 5 sec viable cell count was 6.5×10$^5±4.3×10^4$ compared to 7.7×10$^5±3.6×10^5$ (15% reduction), 10 sec of treatment led to a reduced cell count by 53% (3.5×10$^5±9.4×10^4$ compared to 7.4×10$^5±8.0×10^4$ ps≤0.01 (Figure 1A and B). An 84% reduction was observed 120 h after CAP treatment over 30s (9.8×10$^5±8.4×10^4$ compared to 6.2×10$^5±7.0×10^4$ ps≤0.01) (Figure 1C).
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Figure 1. Cell proliferation of U2-OS in an OS in vitro cell culture model. Viable cell counts of U2-OS cells after CAP treatment using Maxium® (KLS Martin) over 5 sec (A), 10s (B) and 30 sec (C). Data are given as the mean±SD of cell count. *p≤0.05; **p≤0.01 and ***p≤0.001 vs. control, as determined by Student’s t-test.

Figure 2. Cell proliferation of U2-OS in an OS in vitro cell culture model. Viable cell counts of U2-OS cells after CAP treatment using Cold Plasma Coagulator 1000 (Söring GmbH) over 5 sec (A), 10 sec (B) and 30s (C). Data are given as the mean±SD of cell count. *p≤0.05; **p≤0.01 and ***p≤0.001 vs. control, as determined by Student’s t-test.
Figure 3. Cell proliferation of U2-OS in an OS in vitro cell culture model. Viable cell counts of U2-OS cells after CAP treatment using kINPen MED (Neoplas tools) over 5 sec (A), 10 sec (B) and 30 sec (C). Data are given as the mean±SD of cell count. *p≤0.05; **p≤0.01 and ***p≤0.001 vs. control, as determined by Student’s t-test.

Figure 4. Cell proliferation of 3T3 fibroblasts in an OS in vitro cell culture model. Viable cell counts of 3T3 fibroblast cells after CAP treatment using Maxium® (KLS Martin) over 5 sec (A), 10 sec (B) and 30 sec (C). Data are given as the mean±SD of cell count. *p≤0.05; **p≤0.01 and ***p≤0.001 vs. control, as determined by Student’s t-test.
Figure 5. Cell proliferation of 3T3 fibroblasts in an OS in vitro cell culture model. Viable cell counts of 3T3 fibroblast cells after CAP treatment using Cold Plasma Coagulator 1000 (Söring GmbH) over 5 sec (A), 10 sec (B) and 30 sec (C). Data are given as the mean±SD of cell count. *p≤0.05; **p≤0.01 and ***p≤0.001 vs. control, as determined by Student’s t-test.

Figure 6. Cell proliferation of 3T3 fibroblasts in an OS in vitro cell culture model. Viable cell counts of 3T3 fibroblast cells after CAP treatment using kINPen MED (Neoplas tools) over 5 sec (A), 10 sec (B) and 30 sec (C). Data are given as the mean±SD of cell count. *p≤0.05; **p≤0.01 and ***p≤0.001 vs. control, as determined by Student’s t-test.
In parallel, 3-T-3-fibroblasts showed characteristic cell growth following argon control treatment. Following CAP treatment over 5 sec, cell count after 120 h of incubation was 
\[9.4 \times 10^5 \pm 2.7 \times 10^4 \] compared to 
\[1.1 \times 10^6 \pm 8.1 \times 10^2 \] , thus resulting in a 17% reduction. 10 sec of CAP treatment resulted in a 51% reduced cell number (3.5\times10^5\pm4.6\times10^4 compared to \[7.4\times10^5\pm8.0\times10^4 \], p≤0.01), while 30 sec led to an 81% decrease in cell number (9.8\times10^4\pm8.4\times10^4 compared to \[6.2\times10^5\pm7.0\times10^4 \], p≤0.01) (Figure 2A-C).

Antiproliferative efficacy of Cold Plasma Coagulator 1000. Helium treated U2-OS cells (controls) exhibited characteristic cell growth. Cell proliferation was significantly altered following CAP treatment of U2-OS cells depending on application time. Following CAP treatment over 5 sec viable cell count was \[1.5\times10^5\pm1.6\times10^4 \] compared to \[6.7\times10^4\pm4.4\times10^3 \] (78% reduction) after 120 h of incubation (Figure 3A). 10s of treatment led to a reduced cell count by 74% (3.5\times10^5\pm9.4\times10^4 compared to \[7.4\times10^5\pm2.7\times10^5 \], p≤0.05 and 90% reduction was observed 120h after CAP treatment over 30s (4.4\times10^5\pm5.0\times10^4 compared to \[4.3\times10^5\pm1.5\times10^5 \], p≤0.01) (Figure 3B and C).

In parallel, 3-T-3-fibroblasts showed characteristic cell growth following helium control treatment. Following CAP treatment over 5 sec cell count after 120 h of incubation was \[2.0\times10^5\pm1.9\times10^5 \] compared to \[8.9\times10^4\pm2.1\times10^4 \], which corresponds to a reduction of 77%. 10s of treatment led to a 82% reduced cell number (1.3\times10^5\pm9.0\times10^4 compared to \[7.4\times10^5\pm2.7\times10^5 \], p≤0.05) while 30 sec led to a 98% decrease in cell number (1.4\times10^4\pm8.9\times10^3 compared to \[7.5\times10^5\pm6.2\times10^4 \], p≤0.001) (Figure 4A-C).

Antiproliferative efficacy of kINPen MED. Argon treated U2-OS cells (controls) exhibited characteristic cell growth. Cell proliferation was significantly altered following CAP treatment of U2-OS cells depending on application time. After 120 h of incubation following CAP treatment over 5 sec viable cell count was diminished by 7% (9.2\times10^5\pm1.7\times10^4 compared to \[9.9\times10^5\pm2.0\times10^4 \], p≤0.05) (Figure 5A). 10s of treatment led to a reduced cell count by 40% (6.6\times10^5\pm3.0\times10^4 compared to \[1.1\times10^5\pm6.4\times10^4 \], p≤0.05 and 72% reduction was observed 120 h after CAP treatment over 30s (2.5\times10^5\pm4.6\times10^4 compared to \[9.0\times10^4\pm8.4\times10^4 \], p≤0.001) (Figure 5B and C).

In parallel, 3-T-3-fibroblasts showed characteristic cell growth following argon control treatment. Following CAP treatment over 5 sec cell count after 120 h of incubation was \[5.4\times10^5\pm6.4\times10^4 \] compared to \[7.2\times10^5\pm2.6\times10^4 \], which corresponds to a reduction of 24%. 10 sec of CAP treatment resulted in a by 57% reduced cell number (3.5\times10^5\pm6.3\times10^4 compared to \[8.2\times10^5\pm6.1\times10^4 \], p≤0.001) while 30 sec led to a 72% decrease in cell number (1.9\times10^5\pm4.2\times10^4 compared to \[7.0\times10^5\pm2.8\times10^4 \], p≤0.001) (Figure 6A-C).

Discussion

CAP has been shown to exert antitumor effects in a variety of cancer entities including prostate, pancreatic, head and neck squamous cell carcinoma, melanoma, myeloma and osteosarcoma (7, 15, 16, 25-28). Here, CAP effects on tumor cell proliferation involved the induction of apoptosis rather than direct cytotoxic effects (29). More recently, a potential involvement of reactive species like ROS and RNS leading to DNA damage, lipid peroxidation or mitochondrial dysfunction has been reported as active agents in oncotherapy (30). Both, reactive species composition as well as the pattern of these species, is primarily determined by the used CAP device (31). Beside environmental conditions constructional and physical properties of the plasma devices are responsible for physical CAP characteristics and thus for biological efficacy. Device-dependent effects of different plasma sources on cells, however, remain elusive.

In the study presented, epithelial osteosarcoma cells (U2-OS) and non-malignant fibroblasts (3-T-3) were applied as cellular model system to assess anti-cancer efficacy of three approved CAP devices in Europe. The Cold Plasma Coagulator 1000, the Maxium® and the kINPen MED, are plasma jets. Within this setup, CAP is generated by high voltage applied between two electrodes. In medical approaches, human skin or tissue represents one of them. The design principle is based on two inbuilt electrodes with a direct evacuation of the target structure by a non-oxygen carrier gas (argon/helium). The originally low concentration of CAP compounds compared to DBD devices is effectively compensated by bundling of the gas flow and results in high concentrations of reactive species (32).

As expected, our data showed both a cell type-specific as well as a treatment time-dependent effect on epithelial and fibroblastic cell proliferation. Applying Maxium® and Cold Plasma Coagulator, epithelial U2-OS cells appeared more sensitive to CAP treatment than 3-T-3 fibroblasts depending on treatment time. A single kINPen Med treatment of over 30 sec on epithelial U2-OS cells tended to a stronger attenuation of cell growth compared to fibroblastic 3-T-3 cells.

Furthermore, comparable experiments applying epithelial prostate cancer cells to a kINPen MED device demonstrated a stronger anti-proliferative effect on epithelial prostate cancer cells than to epithelial U2-OS cells (16), emphasizing cell type-specific CAP efficacy.

Furthermore, the anti-proliferative cell response increased with increasing times of CAP treatment for all three devices. CAP treatment represents a single, short-time application of CAP and thus treatment time corresponds to drug
concentration and dose response of chemical compounds in conventional chemotherapy. Due to the physical state of CAP and due to the dependency on different environmental variables during CAP generation, it is currently not feasible to define a parameter corresponding to the ‘CAP concentration’. Consequently, examination of the biological impact is the only strategy to specify CAP’s biological efficacy.

Besides cell type and treatment time, the CAP impact on cellular growth strongly depends on the CAP device utilized in the experiments. On the other hand, the individual biological impact of CAP devices should be examined and considered in every single case of medical approach.

In summary, CAP treatment using quite different plasma sources and application times individually attenuates cell proliferation of OS and fibroblast cells in a dose-dependent manner, whereby tumor cells tend to be more susceptible to CAP therefore making it a promising new treatment option. Further studies, however, should examine the effects of CAP treatment on more complex malignant tissues evaluating effects on tumor microenvironment and penetration depth to determine optimal tumor specific application sources and dosage.

Conflicts of Interest

None of the Authors stated a conflict of interest. No funding was received for the study.

References


