ANTICANCER RESEARCH *40*: 6205-6212 (2020)
doi:10.21873/anticanres.14640

Protherapeutic Effects and Inactivation of Mammary Carcinoma Cells by a Medical Argon Plasma Device

MATTHIAS B. STOPE[1], RIM BENOUAHI[2], CAROLINE SANDER[2], LYUBOMIR HARALAMBIEV[2,3], ANDREAS NITSCH[2], EVA EGGER[1] and ALEXANDER MUSTEA[1]

[1]*Department of Gynecology and Gynecological Oncology, University Hospital Bonn, Bonn, Germany;*
[2]*Department of Trauma, Reconstructive Surgery and Rehabilitation Medicine, University Medicine Greifswald, Greifswald, Germany;*
[3]*Department of Trauma and Orthopaedic Surgery, BG Klinikum Unfallkrankenhaus Berlin gGmbH, Berlin, Germany*

Abstract. *Background/Aim: Mammary carcinoma (MC) remains one of the leading causes of morbidity and mortality in the female population worldwide. Cold physical plasma at atmospheric pressure (CAP) has an antioncogenic effect on tumor cells, and its anticancer properties may complement or even extend existing treatment options. In the present study, the efficacy of CAP was characterized on an MC in vitro cell culture system. Materials and Methods: MC cells (MCF-7, MDA-MB-231) were directly treated with CAP or incubated with CAP-treated cell culture medium. Cell growth, cell mobility and apoptosis were subsequently analyzed. Results: A single treatment of MC cells with CAP and CAP treated medium led to a treatment-time dependent reduction of cell growth. Furthermore, CAP exposure led to a loss of cellular motility and induced apoptosis. Conclusion: Due to its anticancer properties, CAP treatment is an innovative and promising physical approach to expand and complement the treatment options for MC. In particular, a combination of CAP application with surgical and/or chemotherapeutic interventions might significantly improve the therapeutic outcomes.*

Physical procedures, such as radiotherapy or electrosurgery entered medicine as early as the 1990s. The technical advancement of physical plasma led to the development of another physical therapy field, 'plasma oncology' (1). Cold physical plasma under atmospheric pressure (cold atmospheric plasma: CAP) is generated by applying high electrical voltage to two electrodes through which a carrier gas flows. In contact with the surrounding atmosphere, numerous excited and charged particles, free radicals, and electromagnetic radiation with high biological reactivity are generated (2, 3). In particular, reactive oxygen and nitrogen species (ROS, RNS) are formed, which play a major role in the biological effects of CAP treatment (4). It has been shown that after treatment of tumor cells with CAP, produced antiproliferative effects in varying tumor entities including carcinomas of the lung, ovaries, pancreas, and prostate, as well as osteosarcoma (5-9). In addition, CAP possesses antimicrobial, antiseptic, immunomodulating, anti-inflammatory and wound healing promoting properties (10, 11), which is why CAP has also been used in the treatment of body surfaces (skin diseases including skin tumours, tumours of the oral cavity) (12). Due to the short half-life of the reactive particles, CAP effects are temporally and locally limited. The intraoperative use of CAP in surgical oncology would be a further therapeutic alternative. Furthermore, the antimicrobial and wound healing promoting properties of CAP treatment may reduce postoperative complications.

The mammary carcinoma (MC) is one of the most common cancers in the female population and is the main cause of morbidity and mortality in women. Worldwide, MC is responsible for about 25% of all cancers and about 15% of all cancer-related deaths, although the incidence can vary considerably from country to country (13, 14). Five options are available for MC treatment: Hormonal therapy, radiotherapy, antibody therapy, surgical resection, and chemotherapy (frequently in combination with surgery). The choice of the appropriate therapeutic intervention depends on various factors, including subtype of breast cancer, size of the tumor, and spread.

To date, there is little data on the anticancer effectiveness of CAP on MC tumor cells. However, studies in other entities suggest that the application of CAP is very promising and that CAP treatment is also a promising complement to

Correspondence to: Matthias Stope, Department of Gynecology and Gynecological Oncology, University Hospital Bonn, Venusberg-Campus 1, 53127 Bonn, Germany. Tel: +49 22828711361, Fax: +49 22828716091, e-mail: matthias.stope@ukbonn.de

Key Words: Plasma oncology, plasma surgery, cold atmospheric plasma, mammary carcinoma, physical therapy.

MC therapy. The present study evaluated CAP efficacy on MC cells with respect to growth inhibitory effects on an MC cell culture model.

Materials and Methods

Cell culture. MC cell lines MCF-7 and MDA-MB-231 (both received from Cell Line Service, Eppelheim, Germany) were propagated in DMEM/F12 medium (PAN Biotech, Aidenbach, Germany) containing 10% fetal bovine serum, and 0.125% gentamycin (both from PAN Biotech). The cells were passed twice a week and cultivated at 37˚C, 5% CO_2, and saturated humidity.

CAP treatment. Due to extensive drying effects of CAP treatment of confluent cell layers, MC cells were treated in suspension. A cell suspension of 45,000 MCF-7 cells/200 µl medium and 30,000 MDA-MB-231 cells/200 µl medium were treated with CAP (kINPen MED plasma jet, Neoplas tools, Greifswald, Germany) in the wells of a 24-well cell culture plate for 5 s, 20 s, and 60 s. The treated cell suspension was then transferred to a 24-well cell culture plate in which 800 µl of fresh untreated medium was provided and cells were incubated until they were analyzed. For indirect treatment, untreated cells were incubated with CAP-treated medium. For this procedure, 1,000 µl medium was treated with CAP for 60 s. Afterwards, the medium was removed from 24 h previously seeded untreated cells and replaced by CAP-treated medium.

Cell growth assay. Cell growth of CAP treated cells was assessed by cell counting using a CASY Cell Counter and Analyzer Model TT (Roche Applied Science, Mannheim, Germany) and compared to control cells. For this purpose, 45,000 MCF-7 and 30,000 MDA-MB-231 cells per well were treated as indicated and seeded in a 24-well cell culture plate. The number of living cells was determined after trypsin/ethylene diaminetetraacetic acid treatment in suspension. For this purpose, 100 µl of the cell suspension was diluted in 10 ml CASYton (Roche Applied Science). For analysis, 400 µl of this dilution were aspirated through a 150 µm capillary into the device and measured. This process was repeated three times. For differentiation of living cells, dead cells, and cell debris, gate settings of 7.60 nm/14.55 nm (MCF-7) and 6.90 nm/12.00 nm (MDA-MB-231) were utilized.

Cell motility assay. A total of 45,000 MCF-7 and 30,000 MDA-MB-231 cells were treated with CAP, seeded in a 24-well cell culture plate and incubated for 24 h. The cell layer was then scratched with a sterile 200 µl pipette tip and incubated for 96 h in a Life Cell Imaging Setup at 37˚C and 5% CO_2. For the evaluation of cell motility, the cell culture medium that was used contained 2% fetal bovine serum. Microscopic analysis was performed with an Axio Observer Z1 microscope (Carl Zeiss, Oberkochen, Germany) every 6 h. The microscopic images were analyzed with the open source ImageJ software (National Institutes of Health, Bethesda, MD, USA) with 'MRI wound healing tool' plugin. Cell motility was expressed as a relative decrease of the cell-free area of the scratch compared to t=0.

Caspase 3/7 assay. A total of 80,000 (24 h incubation) and 40,000 (48 h incubation) MCF-7 cells and 60,000 (24 h incubation) and 30,000 (48 h incubation) MDA-MB-231 cells were treated with CAP and incubated for 24 h and 48 h. To determine caspase-3 and caspase-7 activity, the used medium was then removed and the cells were incubated with 100 µl Caspase 3/7 Detection Solution (CellEvent Caspase 3/7 Green Detection Reagent; Thermo Fisher Scientific, Waltham, MA, USA) for 45 min. The fluorescence (535 nm) was measured using a Infinite M200 Pro plate reader (Tecan, Männedorf, Switzerland). Fluorescence intensity was normalized to the cell number. Therefore, a parallel cell culture plate was carried along to determine the cell count. As a quality control, one sample without fluorescence-labeled substrate (negative control) and another with apoptosis-inducing staurosporine (positive control) was carried along.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. 50,000 (24 h incubation) and 25,000 cells (48 h incubation) were treated with CAP, transferred to a 96-well cell culture plate, and incubated for 24 h and 48 h. The colorimetric apoptosis detection kit TiterTACS (Trevigen, Gaithersburg, MD, USA) was applied according to the manufacturer's instructions. Absorption (450 nm) was measured using a Infinite M200 Pro plate reader (Tecan). Absorption intensity was normalized to the cell number. Therefore, a parallel cell culture plate was carried along to determine the cell count. As a quality control, one sample lacking the transferase enzyme (negative control) and another incubated with a DNA nuclease (positive control) was carried along.

Statistics. The data are given as mean value±standard deviation (SD). Statistical analysis was performed using the unpaired Student's *t*-test. $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***) were defined as significant.

Results

The biological effect of a cytostatic agent is primarily determined by its concentration. No concentrations can be given for CAP treatment, but the biological effect can be modulated by the duration of CAP treatment. Therefore, different treatment times of 5 s, 20 s, and 60 s were tested for MCF-7 and MDA-MB-231 cells (Figure 1). Both MC cells did not show significant effects on cell growth after 5 s treatment during the 120-h incubation (Figure 1A and D). These effects only occurred after a 20 s treatment (Figure 1B and E). After 48 h (MCF-7) and 72 h (MDA-MB-231) of incubation the growth of CAP treated cells was significantly reduced compared to control cells. This CAP effect was even more pronounced after treatment for 60 s (Figure 1C and F). Already after 24 h (MCF-7) and 4 h (MDA-MB-231), CAP treated cells grew significantly slower than controls. After 60 s of treatment, growth of MCF-7 cells was reduced by 4.3-fold from 4 h to 120 h of incubation (Ctrl: 10.3-fold increase in cell count; CAP: 2.4-fold increase in cell count). Similarly, treatment of MDA-MB-231 cells resulted in a 4.5-fold reduction in growth (Ctrl: 16.2-fold increase in cell count; CAP: 3.6-fold increase in cell count).

Due to the formation of ROS and RNS in the CAP flame, CAP has a strong biochemical reactivity in biological solvents such as cell culture medium. Depending on the chemical stability of these components, the biological effects of CAP

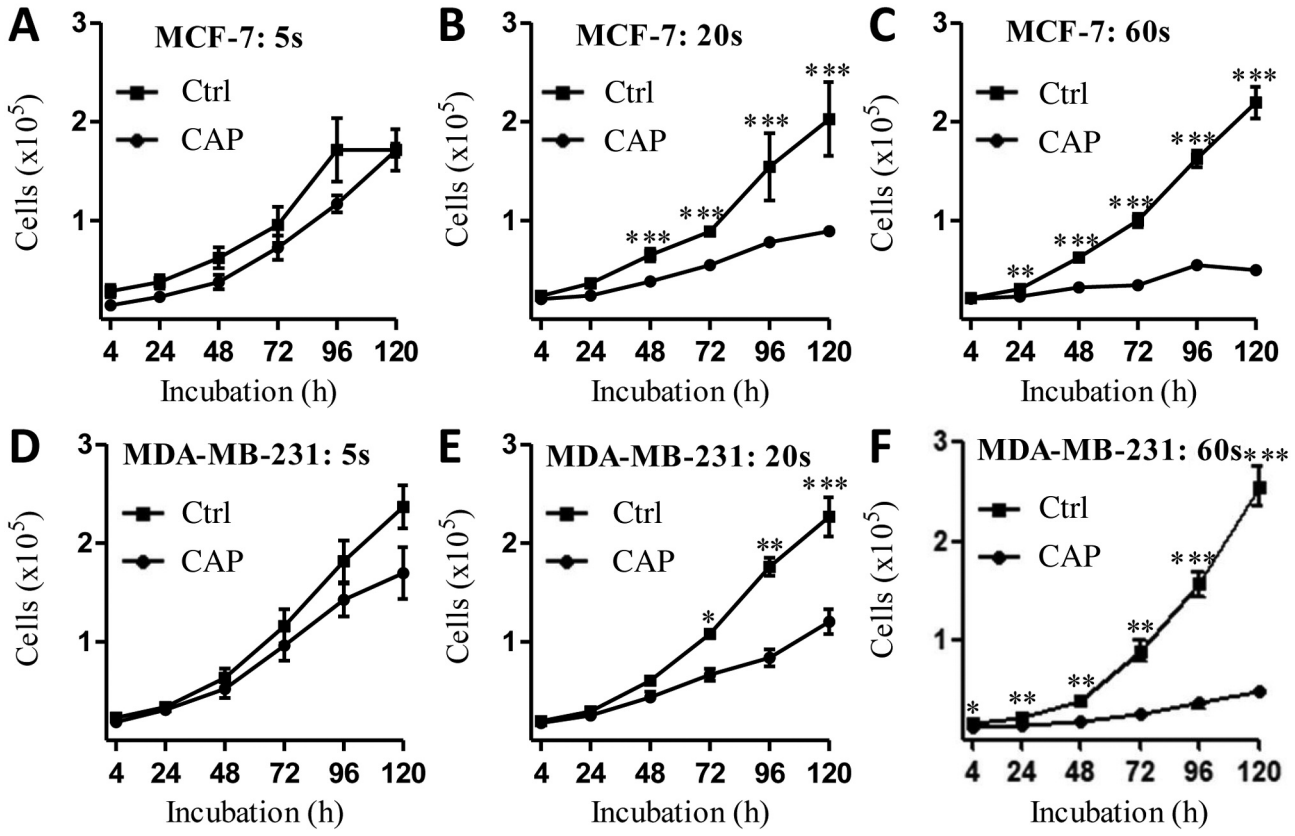

Figure 1. *Growth kinetics of MCF-7 (A-C) and MDA-MB-231 cells (D-F) directly treated with CAP. Cells were treated for 5 s (A and D), 20 s (B and E), and 60 s (C and F) and incubated for 120 h. After 4 h, 24 h, 48 h, 72 h, 96 h, and 120 h the cell number was determined. Depicted are the mean values ±SD. p≤0.05 (*), p≤0.01 (**), and p≤0.001 (***) were defined as statistically significant.*

can be transferred indirectly to cells that have not been in direct contact with CAP (5). This indirect treatment was performed for 20 s and 60 s, which also led to significant changes in cell growth when MC cells were treated directly. The inhibitory effects of CAP treated cell culture medium on untreated MCF-7 and MDA-MB-231 cells were similar to those of direct CAP treatment, but somewhat more moderate (Figure 2). At least 48 h after incubation, the growth of indirectly CAP treated cells differed significantly from the control cells at all conditions. The indirect treatment of MCF-7 cells with medium treated for 60 s led to a 2.4-fold reduction of cell growth within the incubation period (Ctrl: 9.6-fold increase in cell count; CAP: 4.0-fold increase in cell count). Also, MDA-MB-231 cells indirectly treated with CAP showed a slightly lower anti-proliferative potential compared to direct treatment. Cell growth from 4 h to 120 h incubation was reduced by 3.0-fold in the presence of CAP treated cell culture medium (Ctrl: 12.7-fold increase in cell count; CAP: 4.3-fold increase in cell count).

In addition to malignant cell growth, the invasive and metatstatic capacity of cancer cells is also an important clinical characteristic. Initial data suggest that treatment with CAP may also inhibit cell motility (5, 15). To characterize the impact of CAP on MC cell motility, scratch assays were performed and the percentage of cell-free area was determined. CAP treated MCF-7 cells tended to show lower motility than controls over the entire observation period (Figure 3A). The results were above the controls at all times during the 96-h incubation, but these differences were not statistically significant at any time. In both CAP and controls, MCF-7 cells had completely colonized the cell-free areas after 84 h. MDA-MB-231 cells showed markedly higher cell motility. After 18 h, control cells had already completely colonized the cell-free area (Figure 3B). CAP treated MCF-7 cells, however, reached this only after 48 h incubation. These differences were statistically significant 6 h (Ctrl=49.7%; CAP=76.2%; p=0.0045), 12 h (Crtl=15.4%; CAP=47.0%; p=0.0028), and 18 h (Ctrl=2.8%; CAP=23.6%; p=0.0028) after the beginning of incubation.

Ideally, the therapeutic deactivation of tumor cells is achieved by apoptosis, which minimizes the release of

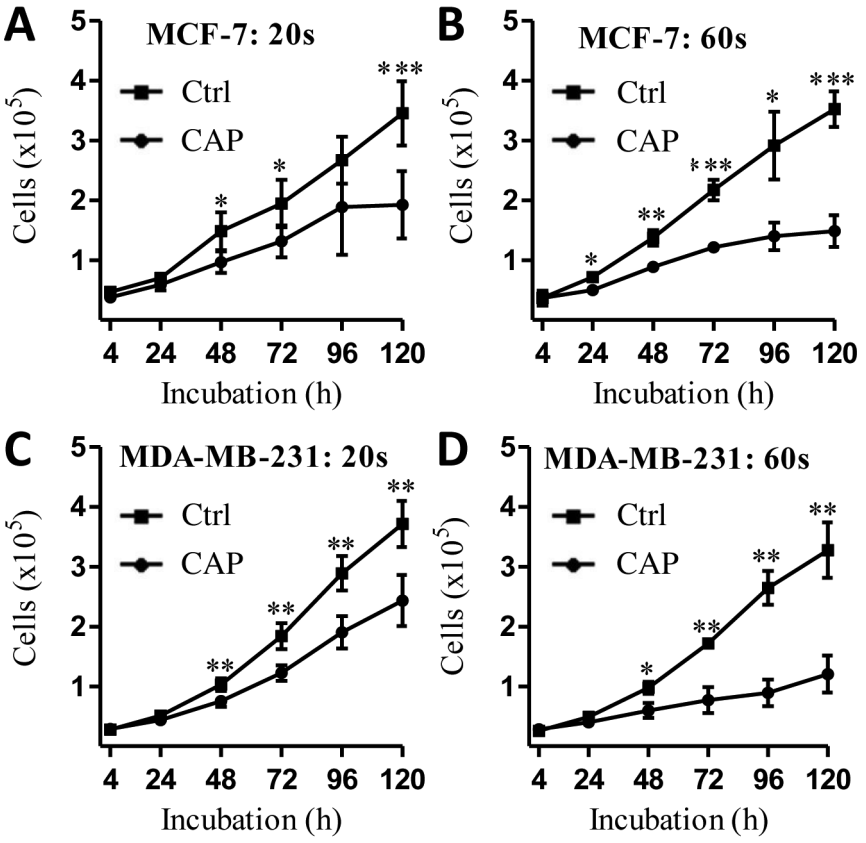

Figure 2. *Growth kinetics of MCF-7 (A and B) and MDA-MB-231 cells (C and D) indirectly treated with CAP. Cell culture medium was treated for 60 s, transferred to untreated cells, and incubated for 120 h. After 4 h, 24 h, 48 h, 72 h, 96 h, and 120 h the cell number was determined. Depicted are the mean values ±SD. p≤0.05 (*), p≤0.01 (**), and p≤0.001 (***) were defined as statistically significant.*

inflammatory factors with subsequent local or systemic responses. Therefore, two independent assays were performed to demonstrate the induction of apoptotic mechanisms after CAP treatment. The activation of pro-apoptotic caspases (Caspase-3/7 assay) occurs in a rather early phase of apoptosis, whereas DNA fragmentation (TUNEL assay) is more likely to be observed towards the end of apoptotic cascades.

In both MCF-7 and MDA-MB-231 cells, significant increases in caspase-3/7 activity were detectable after 24 h and 48 h incubation (Figure 4A and B). Comparable values were obtained in both MC cell lines (MCF-7 - 24 h: 2.3-fold, p=0.0271; 48 h: 2.4-fold, p=0.0389; MDA-MB-231 - 24 h: 2.1-fold, p=0.0016; 48 h: 3.2-fold, p=0.0036). Additional controls included cells that were not labelled with fluorescent substrate (negative control) and cells incubated with staurosporine (positive control).

Using the TUNEL assay, apoptosis induction after CAP treatment was confirmed (Figure 4C and D). In MCF-7 cells (24 h: 2.1-fold, p=0.0051; 48 h: 2.8-fold, p=0.0240) as well as in MDA-MB-231 cells (24 h: 1.6-fold, p=0.0417; 48 h: 2.6-fold, p=0.0330) the TUNEL signal increased after CAP treatment. In this assay unlabelled cells (negative control) and nuclease incubated cells (positive control) were used as controls.

Discussion

Despite the advances made in recent years, the treatment of the different subtypes of MC is still an oncological challenge. CAP treatment is a completely new treatment method for cancer and seems to be feasible as an innovative anticancer procedure for all entities investigated so far (4).

The presented study shows a significant reduction in the growth of MCF-7 and MDA-MB-231 MC cells after treatment with CAP. In addition, indirect treatment of cancer cells with CAP-treated cell culture medium was similarly effective, which has been confirmed by other studies involving these cell lines (16, 17). The cellular and molecular processes underlying CAP-induced proliferation inhibition are still largely unexplained. It has been shown

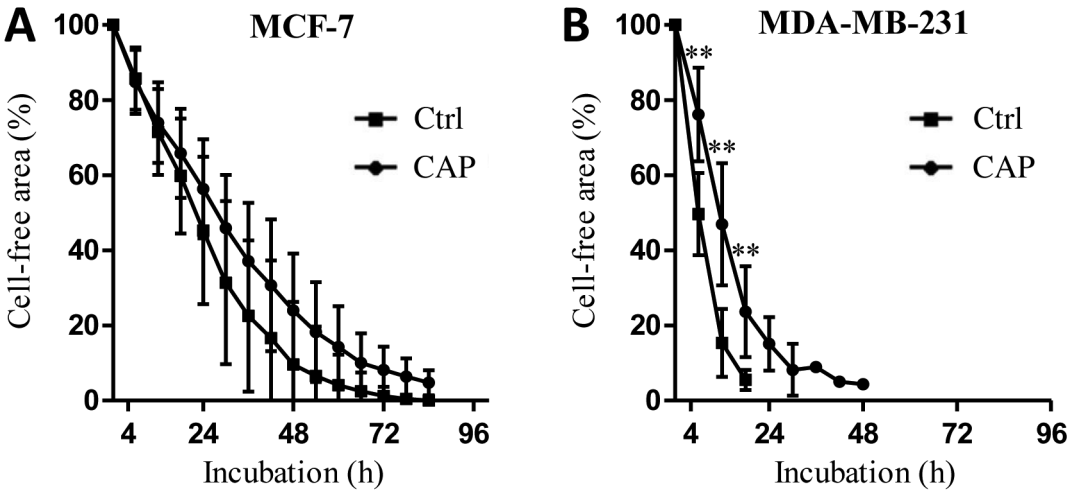

Figure 3. *Cell mobility assay with MCF-7 (A) and MDA-MB-231 cells (B). Cells were treated with CAP, seeded and incubated for 24 h. Subsequently, a sterile 200 µl pipette tip was used to scrape the cell layer and cells were incubated for another 96 h in a live cell imaging setup. The evaluation of the cell-free area was performed by image analysis and is shown as a relative decrease of the cell-free area in relation to the start time t=0. Depicted are the mean values ±SD. p≤0.05 (*), p≤0.01 (**), and p≤0.001 (***) were defined as statistically significant.*

that after CAP treatment the expression of the regulatory microRNA miR-19a-3p in MCF-7 cells was suppressed and consequently cell growth was reduced. In MCF-7 cells, oncogenic miR-19a-3p inhibited the expression of the ATP-binding cassette transporter ABCA1, which regulates numerous metabolic processes, and the tumor suppressor phosphatase and tensin homolog (PTEN) (18).

A general effect of CAP on the signaling cascades of MC cells is a change in the methylation status of DNA (18, 19). Pathway analyses have shown that this also affects proliferative and apoptotic signaling pathways in MCF-7 and MDA-MB-231 cells (19). There are no data on the impact of CAP on signaling pathways controlling invasiveness and metastasis in MC cells. However, the available data show that a single CAP treatment can inhibit the motility of MCF-7 and MDA-MB-231 cells, as has been shown in other MC cells (20). The present findings also verify that MDA-MB-231 cells are significantly more aggressive and mobile than MCF-7 cells (21).

The main cause of CAP-mediated growth inhibition is the formation of reactive oxygen species (ROS). This has also been shown for the MC cell lines MDA-MB-231, MDA-MB-453, and MDA-MB-468 (9, 22-24). Investigations on the formation of OH radicals during CAP treatment have also provided experimental evidence for the induction of apoptotic mechanisms in MC cells (24). However, these were based on rather unspecific tests for Annexin V binding and chromatin condensation after DAPI staining. However, the data of the present study confirm CAP-induced apoptosis by detecting apoptosis-specific caspase-activation and by TUNEL assay. Antiproliferative and proapoptotic effects of CAP have also been demonstrated in two other MC cell lines (BrSK, SK-BR3) (25-27). However, necrosis seems to be hardly induced following CAP treatment of MC cells (25).

Remarkably, non-malignant MC cells are also sensitive to CAP treatment and respond with growth inhibition and apoptosis (23, 28, 29). In one study, non-malignant MCF10A cells reacted more sensitively to CAP than malignant MDA-MB-231 and MDA-MB-435 cells (30). This challenges the widely supported hypothesis that CAP specifically affects tumor cells and that adjacent non-malignant cells remain unaffected. A comparison of CAP-treated MDA-MB-231 with non-malignant primary MC cells showed only minor differences (31). However, these comparisons were mainly based on non-malignant and malignant cells of different origin, so that here probably predominantly individual differences between the various patients were considered, less the differences between malignant and non-malignant cells of the same organ. Such (sometimes even considerable) individual differences can already be detected when comparing different cell lines of the same malignancy and are indicative of a general different CAP sensitivity of cell populations from different origins (32).

Regarding the clinical use of CAP, there are innovative experimental approaches for combination of CAP with other therapeutic procedures. A combination of CAP with a static magnetic field shows a significantly stronger antiproliferative effect on MDA-MB-231 cells than CAP treatment alone (33). Similarly, CAP treatment of MC cells can be performed in combination with nanoparticles, which also inhibits cell growth more than the mono treatment (34, 35).

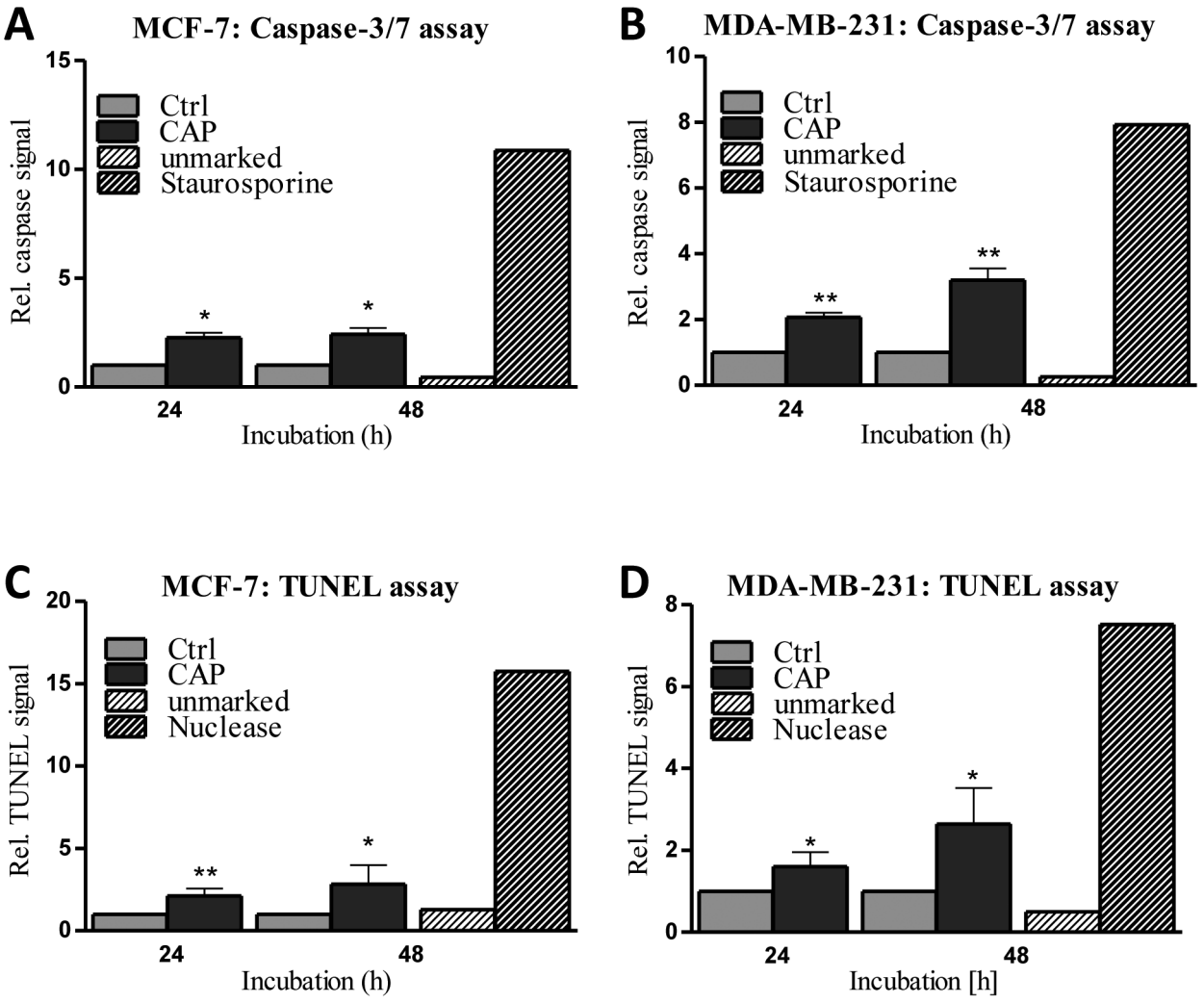

Figure 4. *Apoptosis detection by caspase-3/7 assay (A and B) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (C and D) MCF-7 (A and C) and MDA-MB-231 cells (B and D) were seeded and incubated for 24 h and 48 h. Analysis of the assays was performed by using the CellEvent Caspase 3/7 Green Detection Reagent (Thermo Fisher Scientific; A and B) and the colorimetric apoptosis detection kit TiterTACS (Trevigen; C and D) according to manufacturer's instructions. Unmarked approaches served as negative controls, incubations with staurosporine (A and B) and DNA nuclease (C and D) served as positive controls. The signal intensities were related to the cell number and are depicted as mean values ±SD. p≤0.05 (*), p≤0.01 (**) and p≤0.001 (***) were defined as statistically significant.*

Furthermore, the combination of CAP treatment with an immediate local (hyperthermic) intraperitoneal chemotherapy is also promising. Due to the CAP-mediated permeabilization of the cytoplasmic membrane, low-molecular substances can enter the tumor cells more easily (36). This leads to a resensitization of the tumor cells to chemotherapeutic agents and additionally increases the penetration depth of the drugs during hyperthermic intraperitoneal chemotherapy. For example, tamoxifen-resistant MCF-7 cells became re-susceptible to tamoxifen therapy after CAP treatment (37). This application horizon of CAP is potentially attractive for all tumor operations in the abdomen.

Conclusion

Treatment of MC cells with CAP leads to the inhibition of cell growth and motility and induces apoptotic mechanisms in cancer cells. Due to these antioncogenic properties, CAP treatment is therefore an innovative and highly promising physical approach to expand and complement the treatment options for MC. In addition to the local and side-effect free anticancer therapy with CAP, a particularly promising option is to combine CAP treatment with classical MC therapies, thus significantly increasing the efficacy of both therapeutic components. Further development of the available CAP devices

for endoscopic and laparoscopic techniques will further expand and facilitate the future applications in plasma oncology.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

Conceptualization, M.B.S., A.M.; methodology, R.B., C.S., A.N.; formal analysis, M.B.S., R.B., C.S., L.H., A.N.; investigation, M.B.S., R.B., C.S.; writing – original draft preparation, M.B.S., L.H., E.E.; writing – review and editing, M.B.S., E.E., A.M.; visualization, R.B., C.S., A.N.; supervision M.B.S., A.M.

References

1 Kramer A, Meinke M, Patzelt A, Stope MB, Lademann J and Jacobs K: Perspektiven physikalischer Verfahren der Antiseptik und Desinfektion. Hyg Medizin 45: 39-45, 2020.

2 van Gaens W and Bogaerts A: Kinetic modelling for an atmospheric pressure argon plasma jet in humid air. J Phys D Appl Phys *46*: 79502, 2013. DOI: 10.1088/0022-3727/46/27/275201

3 Schmidt-Bleker A, Winter J, Bösel A, Reuter S and Weltmann KD: On the plasma chemistry of a cold atmosphericargon plasma jet with shielding gas device. Plasma Sources Sci Tech *25*: 15005, 2016. DOI: 10.1088/0963-0252/25/1/015005

4 Mitra S, Nguyen LN, Akter M, Park G, Choi EH and Kaushik NK: Impact of ROS generated by chemical, physical, and plasma techniques on cancer attenuation. Cancers *11*: 1030, 2019. PMID: 31336648. DOI: 10.3390/cancers11071030

5 Koensgen D, Besic I, Gümbel D, Kaul A, Weiss M, Diesing K, Kramer A, Bekeschus S, Mustea A and Stope MB: Cold atmospheric plasma (CAP) and CAP-stimulated cell culture media suppress ovarian cancer cell growth - a putative treatment option in ovarian cancer therapy. Anticancer Res *37*: 6739-6744, 2017. PMID: 29187451. DOI: 10.21873/anticanres.12133

6 Kim JY, Ballato J, Foy P, Hawkins T, Wei Y, Li J and Kim S-O: Apoptosis of lung carcinoma cells induced by a flexible optical fiber-based cold microplasma. Biosens Bioelectron *28*: 333-338, 2011. PMID: 21820891. DOI: 10.1016/j.bios.2011.07.039

7 van Loenhout J, Flieswasser T, Freire Boullosa L, Waele J de, van Audenaerde J, Marcq E, Jacobs J, Lin A, Lion E, Dewitte H, Peeters M, Dewilde S, Lardon F, Bogaerts A, Deben C and Smits E: Cold atmospheric plasma-treated PBS eliminates immunosuppressive pancreatic stellate cells and induces immunogenic cell death of pancreatic cancer cells. Cancers *2019*: 11, 2019. PMID: 31635070. DOI: 10.3390/cancers11101597

8 Weiss M, Gümbel D, Hanschmann E-M, Mandelkow R, Gelbrich N, Zimmermann U, Walther R, Ekkernkamp A, Sckell A, Kramer A, Burchardt M, Lillig CH and Stope MB: Cold atmospheric plasma treatment induces anti-proliferative effects in prostate cancer cells by redox and apoptotic signaling pathways. PLoS One *10*: e0130350, 2015. PMID: 26132846. DOI: 10.1371/journal.pone.0130350

9 Jacoby JM, Strakeljahn S, Nitsch A, Bekeschus S, Hinz P, Mustea A, Ekkernkamp A, Tzvetkov MV, Haralambiev L and Stope MB: An innovative therapeutic option for the treatment of skeletal sarcomas: Elimination of osteo- and Ewing's sarcoma cells using physical gas plasma. Int J Mol Sci *21*: 4460, 2020. PMID: 32585948. DOI: 10.3390/ijms21124460

10 Kramer A, Bekeschus S, Matthes R, Bender C, Stope MB, Napp M, Lademann O, Lademann J, Weltmann KD and Schauer F: Cold physical plasmas in the field of hygiene – relevance, significance, and future applications. Plasma Process Polym *12*: 1410-1422, 2015. DOI: 10.1002/ppap.201500170

11 Weiss M, Daeschlein G, Kramer A, Burchardt M, Brucker S, Wallwiener D and Stope MB: Virucide properties of cold atmospheric plasma for future clinical applications. J Med Virology *89*: 952-959, 2017. PMID: 27696466. DOI: 10.1002/jmv.24701

12 Daeschlein G, Hillmann A, Gümbel D, Sicher C, Podewils S, Stope MB and Jünger M: Enhanced anticancer efficacy by drug chemotherapy and cold atmospheric plasma against melanoma and glioblastoma cell lines *in vitro*. IEEE Transactions Radiation Plasma Med Sci *2*: 153-159, 2018. DOI: 10.1109/TRPMS.2018.2789659

13 Siegel RL, Miller KD and Jemal A: Cancer Statistics, 2017. CA Cancer J Clin *67*: 7-30, 2017. PMID: 28055103. DOI: 10.3322/caac.21387

14 Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A: Global cancer statistics, 2012. CA Cancer J Clin *65*: 87-108, 2015. PMID: 25651787. DOI: 10.3322/caac.21262

15 Balzer J, Demir E, Kogelheide F, Fuchs PC, Stapelmann K and Opländer C: Cold atmospheric plasma (CAP) differently affects migration and differentiation of keratinocytes via hydrogen peroxide and nitric oxide-related products. Clin Plasma Med *13*: 1-8, 2019. DOI: 10.1016/j.cpme.2018.11.001

16 Adachi T, Tanaka H, Nonomura S, Hara H, Kondo SI and Hori M: Plasma-activated medium induces A549 cell injury via a spiral apoptotic cascade involving the mitochondrial-nuclear network. Free Radic Biol Med *79*: 28-44, 2015. PMID: 25433364. DOI: 10.1016/j.freeradbiomed.2014.11.014

17 Yan D, Talbot A, Nourmohammadi N, Cheng X, Canady J, Sherman J and Keidar M: Principles of using cold atmospheric plasma stimulated media for cancer treatment. Sci Rep *5*: 18339, 2015. PMID: 26677750. DOI: 10.1038/srep18339

18 Lee S, Lee H, Bae H, Choi EH and Kim SJ: Epigenetic silencing of miR-19a-3p by cold atmospheric plasma contributes to proliferation inhibition of the MCF-7 breast cancer cell. Sci Rep *6*: 30005, 2016. PMID: 27445062. DOI: 10.1038/srep30005

19 Park S-B, Kim B, Bae H, Lee H, Lee S, Choi EH and Kim SJ: Differential epigenetic effects of atmospheric cold plasma on MCF-7 and MDA-MB-231 breast cancer cells. PLoS One *10*: e0129931, 2015. PMID: 26042423. DOI: 10.1371/journal.pone.0129931

20 Xiang L, Xu X, Zhang S, Cai D and Dai X: Cold atmospheric plasma conveys selectivity on triple negative breast cancer cells both *in vitro* and *in vivo*. Free Radic Biol Med *124*: 205-213, 2018. PMID: 29870749. DOI: 10.1016/j.freeradbiomed.2018.06.001

21 Hostanska K, Nisslein T, Freudenstein J, Reichling J and Saller R: Inhibitory effect of an isopropanolic extract of black cohosh on the invasiveness of MDA-MB 231 human breast cancer cells. In Vivo *21*: 349-355, 2007. PMID: 17436587

22 Chen Z, Zhang S, Levchenko I, Beilis II and Keidar M: In vitro demonstration of cancer inhibiting properties from stratified self-organized plasma-liquid interface. Sci Rep *7*: 12163, 2017. PMID: 28939877. DOI: 10.1038/s41598-017-12454-9

23 Nguyen NH, Park HJ, Yang SS, Choi KS and Lee J-S: Anti-cancer efficacy of nonthermal plasma dissolved in a liquid, liquid plasma in heterogeneous cancer cells. Sci Rep *6*: 29020, 2016. PMID: 27364630. DOI: 10.1038/srep29020

24 Ninomiya K, Ishijima T, Imamura M, Yamahara T, Enomoto H, Takahashi K, Tanaka Y, Uesugi Y and Shimizu N: Evaluation of extra- and intracellular OH radical generation, cancer cell injury, and apoptosis induced by a non-thermal atmospheric-pressure plasma jet. J Phys D Appl Phys *46*: 425401, 2013. DOI: 10.1088/0022-3727/46/42/425401

25 Calugaru A, Cremer L, Herold A, Lupu A, Szegli G, Lungu C, Lungu A and Georgescu N: The effect of the plasma needle on tumoral cell lines apoptosis. Roum Arch Microbiol Immunol *64*: 57-64, 2005. PMID: 17405316.

26 Kumar N, Attri P, Choi EH and Uhm HS: Influence of water vapour with non-thermal plasma jet on the apoptosis of SK-BR-3 breast cancer cells. RSC Adv *5*: 14670-14677, 2015. DOI: 10.1039/C4RA15879B

27 Kim SJ, Chung TH, Bae SH and Leem SH: Induction of apoptosis in human breast cancer cells by a pulsed atmospheric pressure plasma jet. Appl Phys Lett *97*: 23702, 2010. DOI: 10.1063/1.3462293

28 Kalghatgi S, Kelly CM, Cerchar E, Torabi B, Alekseev O, Fridman A, Friedman G and Azizkhan-Clifford J: Effects of non-thermal plasma on mammalian cells. PLoS One *6*: e16270, 2011. PMID: 21283714. DOI: 10.1371/journal.pone.0016270

29 Kalghatgi S, Fridman A, Azizkhan-Clifford J and Friedman G: DNA damage in mammalian cells by non-thermal atmospheric pressure microsecond pulsed dielectric barrier discharge plasma is not mediated by ozone. Plasma Process Polym *9*: 726-732, 2012. DOI: 10.1002/ppap.201100156

30 Liu Y, Tan S, Zhang H, Kong X, Ding L, Shen J, Lan Y, Cheng C, Zhu T and Xia W: Selective effects of non-thermal atmospheric plasma on triple-negative breast normal and carcinoma cells through different cell signaling pathways. Sci Rep *7*: 7980, 2017. PMID: 28801613. DOI: 10.1038/s41598-017-08792-3

31 Wang M, Holmes B, Cheng X, Zhu W, Keidar M and Zhang LG: Cold atmospheric plasma for selectively ablating metastatic breast cancer cells. PLoS One *8*: e73741, 2013. PMID: 24040051. DOI: 10.1371/journal.pone.0073741

32 Haralambiev L, Bandyophadyay A, Suchy B, Weiss M, Kramer A, Bekeschus S, Ekkernkamp A, Mustea A, Kaderali L and Stope MB: Determination of immediate *vs*. kinetic growth retardation in physically plasma-treated cells by experimental and modelling data. Anticancer Res *40*: 3743-3749, 2020. PMID: 32620613. DOI: 10.21873/anticanres.14363

33 Cheng X, Rajjoub K, Shashurin A, Yan D, Sherman JH, Bian K, Murad F and Keidar M: Enhancing cold atmospheric plasma treatment of cancer cells by static magnetic field. Bioelectromagnetics *38*: 53-62, 2017. PMID: 27748977. DOI: 10.1002/bem.22014

34 Jalili A, Irani S and Mirfakhraie R: Combination of cold atmospheric plasma and iron nanoparticles in breast cancer: gene expression and apoptosis study. OncoTargets Ther *9*: 5911-5917, 2016. PMID: 27729800. DOI: 10.2147/OTT.S95644

35 Zhu W, Lee S-J, Castro NJ, Yan D, Keidar M and Zhang LG: Synergistic effect of cold atmospheric plasma and drug loaded core-shell nanoparticles on inhibiting breast cancer cell growth. Sci Rep *6*: 21974, 2016. PMID: 26917087. DOI: 10.1038/srep21974

36 Haralambiev L, Nitsch A, Einenkel R, Muzzio DO, Gelbrich N, Burchardt M, Zygmunt M, Ekkernkamp A, Stope MB and Gümbel D: The effect of cold atmospheric plasma on the membrane permeability of human osteosarcoma cells. Anticancer Res *40*: 841-846, 2020. PMID: 32014927. DOI: 10.21873/anticanres.14016

37 Lee S, Lee H, Jeong D, Ham J, Park S, Choi EH and Kim SJ: Cold atmospheric plasma restores tamoxifen sensitivity in resistant MCF-7 breast cancer cell. Free Radic Biol Med *110*: 280-290, 2017. PMID: 28666851. DOI: 10.1016/j.freeradbiomed.2017.06.017

Received September 15, 2020
Revised September 23, 2020
Accepted September 24, 2020