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Cold Atmospheric Plasma (CAP) and CAP-Stimulated Cell Culture Media Suppress Ovarian Cancer Cell Growth – A Putative Treatment Option in Ovarian Cancer Therapy

DOMINIQUE KOENSGEN[1], ILMA BESIC[1], DENIS GÜMBEL[2], ANNE KAUL[1], MARTIN WEISS[3], KAROLINE DIESING[1], AXEL KRAMER[4], SANDER BEKESCHUS[5], ALEXANDER MUSTEA[1*] and MATTHIAS B. STOPE[6*]

Departments of [1]Gynecology and Obstetrics, [2]Trauma and Orthopaedic Surgery, [4]Hygiene and Environmental Medicine and 6Urology, University Medicine Greifswald, Greifswald, Germany; [3]Department of Obstetrics and Gynecology, University Hospital Tuebingen, Tuebingen, Germany; [5]ZIK Plasmatis, Leibniz-Institute for Plasma Science and Technology (INP Greifswald), Greifswald, Germany

Abstract. *Background/Aim: Ovarian cancer (OC) is a gynecologic tumor with poor prognosis. Despite radical cytoreductive surgery and platinum-based adjuvant systemic treatment, OC will relapse in the majority of the cases. Thus, cold atmospheric plasma (CAP), a highly reactive physical state bearing diverse biological activities being suited for anticancer therapy, may be a promising option in OC therapy. Materials and Methods: OC cell lines were exposed either directly to the CAP or to cell culture medium previously exposed to CAP. Cell proliferation and cell motility was measured. Results: The data demonstrated, that even a single application of a short-term CAP treatment led to an attenuation of OC cell growth and motility. Moreover, incubation with CAP-treated cell culture medium gave similar effects. Results were consistent in four OC cell lines. Conclusion: In summary, the CAP application in oncological surgery leads to strong anti-proliferative effects and opens up novel opportunities for the OC treatment.*

Although ovarian cancer (OC) ranks eighth among the most common female malignancies, it is the fifth cause of death from cancer in women (1). This discrepancy is largely attributed to the fact that the majority of patients present with advanced disease at the time of diagnosis. OC entities are composed of several sub-types with widely differing clinicopathological, genetic, and molecular features (2). The pronounced morphologic and molecular heterogeneity combined with non-specific symptoms in already advanced stages hinders striking OC therapy strategies in most of the cases.

OC therapy consists of an upfront surgery aiming at macroscopic complete resection and combined platinum-based chemotherapy with carboplatin and paclitaxel and, most recently, bevacizumab (3). Despite radical cytoreductive surgery and platinum-based adjuvant systemic treatment, OC will relapse in the majority of cases.

Based on studies on other cancer entities including prostate and breast cancer (4-6) as well as different OC cell lines *in vitro* and *in vivo* (7-11), the application of cold atmospheric plasma (CAP) may become a promising option for OC therapy, particularly for intraoperative application adjacent to critical sites. CAP is defined as a highly reactive partially ionized physical state containing diverse biologically reactive factors including reactive oxygen and nitrogen species (ROS, RNS). This broad spectrum of bioactive agents in combination with charged particles, electric field and shock waves is not achievable with any other therapeutic method. Biological efficacy of CAP is primarily characterized by cell type-specific cell growth modulation, promotion of wound healing and scar formation, activity against microorganisms and viruses, and the modulation of inflammation, leukocyte behaviour, and apoptosis (5, 6, 12-19). Notably, due to a weaker antioxidant capacity, CAP selectively attacks cancer cells and induces immunogenic cell death with potentially improve the imunnogenicity of cancer cells (20, 21).

In the presented study, we performed proof-of-concept experiments for the application of CAP in OC treatment *in vitro*.

*These Authors contributed equally to this work.

Correspondence to: Matthias Stope, Department of Urology, University Medicine Greifswald, Ferdinand-Sauerbruch-Strasse, 17475 Greifswald, Germany. Tel: +49 38348680436, Fax: +49 38348680435, e-mail: matthias.stope@uni-greifswald.de

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Materials and Methods

Cell culture and CAP treatment. The OC cell lines OVCAR-3 (Cell Lines Service, Eppelheim, Germany), SKOV-3 (Cell Lines Service, Eppelheim, Germany), TOV-21G (American Type Culture Collection, Manassas, VA, USA), and TOV-112D (American Type Culture Collection, Manassas, VA, USA) were propagated in RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 10% fetal bovine serum (Biochrom, Berlin, Germany), 0.125% gentamicin (Ratiopharm, Ulm, Germany), and 0.1% insulin (Novo Nordisk, Mainz, Germany) (OVCAR-3), DMEM/F12 (Life Technologies, Darmstadt, Germany) containing 5% fetal bovine serum (Biochrom, Berlin, Germany), and 0.125% gentamicin (Ratiopharm, Ulm, Germany) (SKOV-3), and MCDB105 (tebu-Bio, Offenbach, Germany)/medium 199 (Biochrom, Berlin, Germany) mixture containing 15% fetal bovine serum (Biochrom, Berlin, Germany), and 0.125% gentamicin (Ratiopharm, Ulm, Germany) (TOV-21G, TOV-112G), respectively. Cells were passaged twice a week in a humidified atmosphere at 37˚C and 5% CO_2.

For CAP treatment, 3.0×10^4 cells in 500 μl cell culture medium were treated with CAP for indicated times with the atmospheric pressure argon plasma jet kINPen MED (neoplas tools, Greifswald, Germany) at three standard liters per minute, 65 V supply voltage and 1.1 MHz frequency. After CAP exposure, cells were incubated for 120 h. Control cells were treated with argon gas. Alternatively, cell culture medium was exposed to CAP and subsequently diluted with cell suspension 1:2.

Proliferation assay. Cellular growth was assessed by use of a CASY Cell Counter and Analyzer Model TT (Roche Applied Science, Mannheim, Germany). Cells were diluted in CASYton solution (1:100, Roche Applied Science) and 400 μl of cell suspension were measured in triplicates using a capillary of 150 μm in diameter and gate settings of 9.00 μm/15.75 μm (OVCAR-3), 7.00 μm/15.15 μm (SK-OV-3), 5.25 μm/10.15 μm (TOV-21G), and 6.15 μm/11.00 μm (TOV-112D).

Cell motility assay. A total of 1.0×10^5 cells per well were CAP treated for 15 s (OVCAR-3, SKOV-3, TOV-21G), and 30 s (TOV-112G), seeded in a 24-well cell culture plate and incubated at 37˚C and 5% CO_2 for 24 h. Subsequently, cell layers were scratched with a 200-μl pipette tip and imaged for 48 h in a life cell imaging setup at 37˚C and 5% CO_2. Light microscopical analysis in an Axio Observer Z1 microscope (Carl Zeiss, Oberkochen, Germany) was performed every 6 h and cell translocation into the cell-free scratch was analyzed using ZEN pro 2012 software (Carl Zeiss, Oberkochen, Germany). Cell motility was expressed as a relative decrease of the cell-free space (cell-free growth area) of the scratch compared to t=0.

Statistics. Data are given as the mean±standard deviation (SD). Statistical comparison was performed using the unpaired Student's *t*-test. $p\leq0.05$, $p\leq0.01$, and $p\leq0.001$ were considered as significant.

Results

CAP exposure leads to anti-proliferative effects on OC cells. The anti-proliferative efficacy of CAP was dependent on treatment duration as well as on the used OC cell line. Generally, growth inhibition after CAP treatment increased with the length of the treatment (Figure 1). TOV-21G cells, however, appeared more sensitive to CAP because their proliferation was significantly inhibited after 10 s and 15 s, respectively (Figure 1G-I). In contrast to TOV-21G cells, TOV-112G cells demonstrated reduced sensitivity to CAP, resulting in prolonged treatment exposure of 10 s, 20 s, and 30 s (Figure 1J-L) for similar effects compared to the other OC cell lines.

The anti-proliferative efficacy of CAP treatment depends on the cell culture medium composition. To investigate CAP's indirect impact on OC cells *via* CAP activated biofluids, cell culture medium was treated as mentioned above followed by an incubation of untreated OC cells with treated medium for 120 h. Neither medium A (DMEM F12; Figures 2 A to C) nor medium B (MCDB105/Medium 199; Figures 2 D to F) interfered with OC cells after CAP activation (Figure 2). The volume of treated medium was not important in mediating CAP-dependent effects. Performing these experiments using 1000 μl of CAP treated medium A and medium B, respectively, gave no growth deceleration (data not shown). Only medium C (RPMI 1640; Figure 2 G to I) conveyed CAP effects on OC cells and inhibited untreated OVCAR-3 cells after CAP treatment of 200 μl of medium. Again, application of 1000 μl of CAP treated medium C demonstrated very similar effects on OC cell growth (Figure 2J-L).

CAP exposure inhibits the cellular motility of OC cells. Beside cellular growth, the complex network of cellular motility, invasion, and metastasis is one of the primary hallmarks of cancer. Performing scratch assays with CAP-treated OC cells, anti-metastatic efficacy of CAP has been demonstrated. Specifically, SKOV-3 (Figure 3B) and TOV-21G (Figure 3C) cells showed an attenuated but non-significant influx into scratched areas. In contrast, CAP treatment of OVCAR-3 (Figure 3A) and TOV-112G (Figure 3D) cells significantly inhibited the motility of both cell lines over a period of 48 h.

Discussion

First, in 2012, Iseki *et al*. have suggested anti-proliferative CAP effects on OC cell lines SKOV-3 and HRA (22). The authors demonstrated a reduced cell metabolic activity 72 h after CAP exposure in both cell lines, pointing to a declined cellular viability. Omran *et al*. confirmed with a transporting and flexible plasma jet the eradication of OC cells *in vitro* (11) and Utsimi *et al*. demonstrated anticancer effects of CAP-activated medium on chemoresistant OC cells *in vitro* and *in vivo* (8, 9). Notably, the more aggressive population of OC cells derived from patients ascites was more sensitive to CAP treatment than the less aggressive type (10).

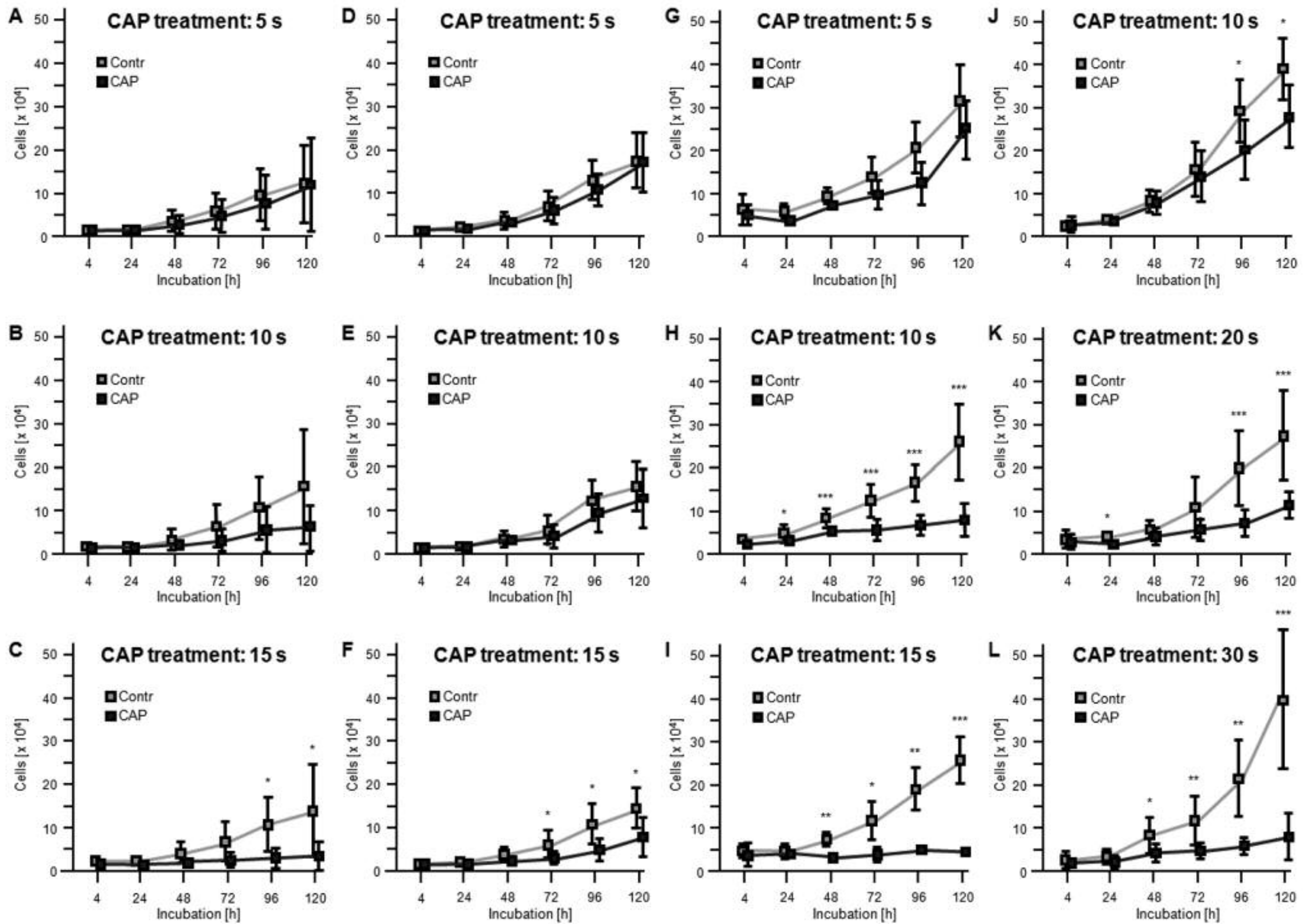

Figure 1. *Anti-proliferation after CAP treatment of OC cells. OC cells OVCAR-3 (A-C), SKOV-3 (D-F), TOV-21G (G-I), and TOV-112G (J-L) were exposed to CAP (CAP) for 5s, 10 s, and 15 s (OVCAR-3, SKOV-3, TOV-21G) and 10 s, 20 s, and 30 s (TOV-112G), respectively, and cell counts were performed at the indicated time points. Control cells (Contr) were treated similarly with argon carrier gas. Data are given as the mean±SD with p≤0.05 (*), p≤0.01 (**), and p≤0.001 (***) as determined by the Student's t-test.*

In our study, we performed CAP exposure experiments utilizing the OC cell culture model consisting of SKOV-3, OVCAR-3, TOV-21G, and TOV-112G cells, which represents a well-characterized established *in vitro* model particularly in the field of OC treatment resistance and cancer progression research (23-26). Our data demonstrated remarkable anticancer effects over a period of 120 h by cell counting, indicating direct cell number alterations. As expected, the anti-proliferative efficacy of CAP was dependent on the individual OC cell type and the exposure time, reflecting OC heterogeneity and dose-response effects. A decrease in scratch re-population was observed for all four OC cell lines. Moreover, CAP effects could be indirectly transferred to cells by treatment of the cell culture medium. This effect did not interfere with the liquid volume which has been treated with CAP, but with the chemical composition of the medium. Even though the protein composition in cell culture medium remains unchanged after CAP exposure (27), however, several biologically effective species are generated, *e.g.* superoxide (O_2^-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and ozone (O_3) (28). Consequently, physical parameters of the medium which determine cellular viability, *e.g.* pH, electrical conductivity, and UV transmittivity, may be affected by CAP treatment according to the composition of the cell culture medium. As a result, reactive species together with critical changes in liquid physics might enable to mediate CAP's efficiency even indirectly (27, 29, 30). Due to the high content of amino acids, vitamins, and other organic compounds and due to the higher buffer capacity of DMEM compared to RPMI (31), DMEM medium appears more appropriate to neutralize CAP effects in liquids. In the clinical context, coagulating

Figure 2. *Indirect CAP effects of CAP-treated cell culture media on untreated OC cells. Untreated OC cells SKOV-3 (A-C), TOV-21G (D-F), and OVCAR-3 (G-L) were incubated with medium A (DMEM/F12; A-C), medium B (MCDB105/Medium 199 mixture; D-F), and medium C (RPMI 1640; G-L), respectively, which were exposed to CAP for 5 s, 10 s, and 15 s, respectively, and cell counts were performed over a period of 120 h at the indicated time points. In control samples (Contr), media were treated similarly with argon carrier gas. Incubation experiments were performed using 200 µl (A-I) and 1000 µl (J-L) of CAP treated media. Data are given as the mean±SD with p≤0.05 (*), and p≤0.01 (**) as determined by Student's t-test.*

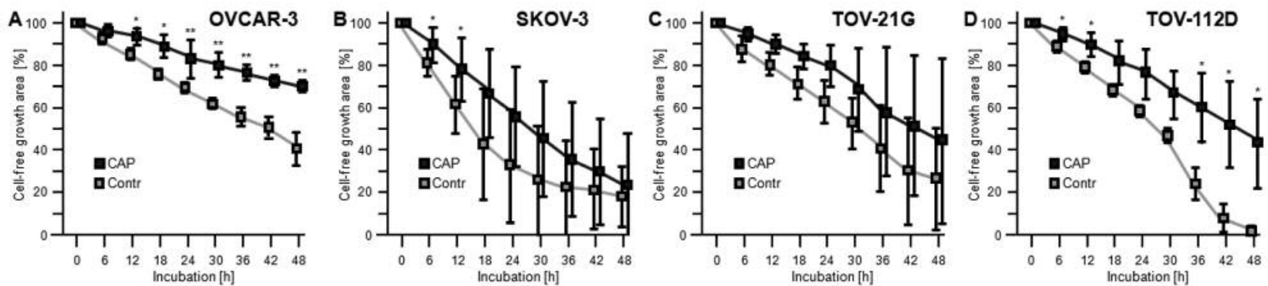

Figure 3. *Cell motility inhibitory effects of CAP on OC cells. OVCAR-3 (A), SKOV-3 (B), TOV-21G (C), and TOV-112G (D) cells were exposed to CAP for 15 s (OVCAR-3, SKOV-3, TOV-21G), and 30 s (TOV-112G), respectively, and propagated in 24-well cell culture plates for 24 h. Subsequently, monolayers were scratched and imaged for up to 48 h in a life cell imaging setup. Light microscopical analysis was performed every 6 h and cell translocation into the cell-free scratch was expressed as relativ decrease of the cell-free space of the scratch compared to t=0. Control cells (Contr) were treated similarly with argon carrier gas. Data are given as the mean±SD with p≤0.05 (*), and p≤0.01 (**) as determined by the Student's t-test.*

effects of CAP will also play an important role. CAP treatment efficiently achieves blood coagulation without thermal effects (32) and, thus, intraoperative CAP applications would primarily be restricted to the site of surgery without subsequent systemic effects. Moreover, previous studies demonstrated the kINPen Med CAP being genotoxically safe (33, 34).

First hints were generated to the mode of action of CAP suppressing ovarian and breast cancer cell growth. Macranthoside B, a saponin compound with anticancer efficacy, blocks proliferation and induces apoptosis of OC cells *via* reactive oxygen species accumulation (35). These findings, when considered together with the study of Yan *et al*. demonstrating that CAP treatment generated reactive oxygen species at the micromolar level (36), may provide a new prospective to understand the interaction between CAP and cancer cells.

In summary, the CAP application in oncological surgery opens up novel opportunities for OC therapy. The study presented here demonstrated that even a single application of a short-term CAP treatment leads to attenuation of cell growth as well as to inhibition of the metastatic capability of OC cells. The study, however, is limited by its restriction to an *in vitro* cell culture model. Consequently, semi-*in vivo* tests, *e.g*. the hen's egg test-chorio allantoic membrane (HET-CAM) and animal models, are needed to confirm these results.

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