Anticancer activity of plasma activated liquids for the treatment of epithelial ovarian cancer cells

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Abstract: The treatment of liquids by means of cold atmospheric pressure plasma (CAP) enables the production of plasma-activated liquids (PALs) containing reactive oxygen and nitrogen species having anticancer activity. In this work, treatments with undiluted and diluted PAL, hydrogen peroxide and nitrite solutions were performed on epithelial ovarian cancer cell lines. These experiments are aimed to evaluate antitumor effects and understand the mechanism of interaction between different concentrations of PAL's components.

Keywords: cold atmospheric pressure plasma, plasma activated liquids, ovarian carcinosis.

1. Introduction

Epithelial Ovarian Cancer (EOC) is the fifth leading cause of cancer-related death among women; this disease is characterised by the diffusion of nodules or plaques from the ovary to the peritoneal surfaces (carcinosis). Currently available therapeutic options include tumor debulking surgery and chemotherapy which cannot eradicate the disease and show low efficacy against resistant tumor subclones [1]. Thus, innovative solutions have to be found. Among these, delivering reactive oxygen and nitrogen species (RONS) to cancer tissues by washing the peritoneal cavities with PALs, might be a convenient strategy having significant analogies with the conventional intra-peritoneal chemotherapy procedures. Indeed, it was demonstrated *in vitro* and *in vivo* experiments that, depending on their RONS content, PALs might exert anticancer effects, even in a selective manner [2,3]. PALs are produced exposing a liquid substrate to CAP; when high voltage is applied, plasma filaments are generated in the gas phase, leading to the formation of a flow of free radicals, electrons, ions, reactive species and UV radiations that impinge on the liquid surface. Subsequently, gas phase species solvate into the liquid and give rise to additional reactions with the production of RONS in liquid, such as nitrite (NO_2^-), nitrate (NO_3^-), peroxynitrite ($OONO^-$), hydroxyl radical ($OH^\cdot$), hydrogen peroxide (H_2O_2), etc... [4]. RONS (in particular H_2O_2 and NO_2^-) have a significant role in cancer therapy due to their influence on cell death mechanisms. In proper concentrations, RONS can induce oxidative and nitrosative stresses leading cancer cells to apoptosis in a selective manner, avoiding damages to healthy tissues.

In this work, *in vitro* experiments aimed at evaluating the cytotoxic effect of PALs and the separate and combined effect of H_2O_2 and NO_2^- solutions on immortalized EOC cell lines are presented.

2. Material and methods

2.1 Plasma device

In this work, PAL is produced by exposing liquid to a micropulsed corona discharge. The plasma source (Fig. 1) consists of a multiwire corona driven by a high voltage generator (Alma*PULSE*, AlmaPlasma s.r.l.). The high voltage electrode consists of four steel wires individually fixed on aluminium supports through threaded screws; while the ground electrode consists of an aluminium sheet fixed on the bottom of the vessel containing the liquid substrate. A polymethylmethacrylate (PMMA) box was designed to encase the plasma source and guarantee a controlled atmosphere during treatment; moreover, the box was equipped with a fan.

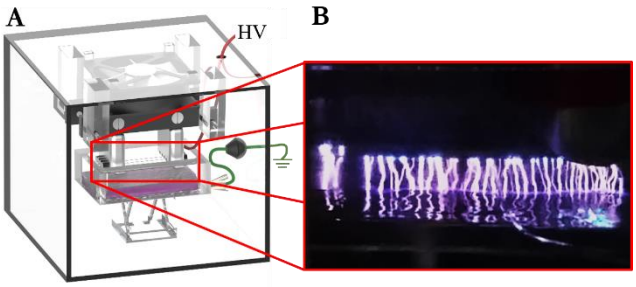

Fig. 1. Multiwire corona plasma source (A) encased in a PMMA box equipped with a fan. (B) Picture of plasma generated by a multiwire corona plasma source on liquid substrate.

2.2 PAL and chemical solutions production

20 ml of RPMI-1640 medium were exposed to the plasma for 10 minutes. The gap between high voltage electrodes and liquid surface was fixed to 5 mm, while peak voltage (PV) and pulse repetition frequency (PRF)

were set to 18 kV and 1 kHz, respectively. After plasma treatment, quantitative measurements of H_2O_2 and NO_2^- were evaluated following the same procedure described by Cerasale *et* al. [5]. Subsequently, three solutions of RPMI-1640 containing H_2O_2, NO_2^- and H_2O_2 + NO_2^- at the same concentrations of PAL were produced adding a proper amount of H_2O_2 (Sigma-Aldrich, 216763) and NO_2^- (Alfa Aesar, 43015) standard solutions to untreated RPMI-1640.

2.3 Cell culture

Human EOC cell lines SKOV-3 and OV-90 were purchased from ATCC®. All cell lines were grown in RPMI-1640 medium with 10% heat inactivated fetal bovine serum (FBS), 1% antibiotic solution (penicillin/streptomycin) and 1% L-glutamine solution (all purchased from Euroclone, Milan, ITALY). Cells were cultured at 37°C and 5% CO_2 for 24, 48 and 72 hours.

2.4 Cell treatment and viability assay

Immediately after plasma treatment, 100 µl of undiluted and diluted (from 1:2 to 1:32) PAL were transferred to 96 well plates. 2 hours after treatments (T = 0 h), cells were washed in phosphate buffered solution (PBS) and cultured in RPMI-1640 medium at 37°C and 5% CO_2. Their viability was measured with Sulforhodamine B (SRB) assay 24, 48 and 72 hours after treatments. Treated cells were fixed with 50% cold trichloroacetic acid (TCA) for 1 h, washed 5 times with distilled water to eliminate TCA, and stained with 0.4% SRB for 30 minutes. Protein-bound dye was dissolved in 10 mM pH 10.5 Tris base solution after 4 washes with 1% acetic acid to remove unbound dye. Optical density values were determined at 570 nm using a 96-well Multilabel Plate Reader VICTOR^3 (1420 Multilabel Counter-PerkinElmer, Turku, Finland). Percentage of growth was calculated considering untreated cells (CTR) at T = 0 h as 100%.

3. Results and discussion

3.1 Chemical analysis

In Fig. 2, the concentrations of H_2O_2 and NO_2^- in 20 ml of RPMI-1640 medium are reported. All the measurements were carried out immediately after the end of the treatment.

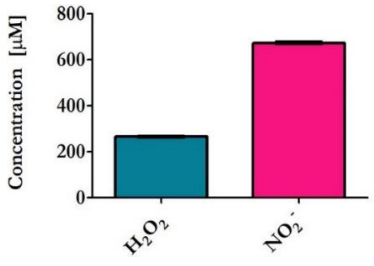

Fig. 2. H_2O_2 and NO_2^- concentrations of undiluted PAL after 10 min of plasma treatment at PV 18 kV and PRF 1 kHz. The measurements were performed using Amplex® Red Hydrogen Peroxide Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and Nitrate/Nitrite colorimetric assay (ROCHE, Basel, Switzerland).

3.2 PALs treatments induce cytotoxic/cytostatic effect on cancer cell lines

For the evaluation of cytotoxic effect on SKOV-3 and OV-90 cell lines with different values of RONS concentration, treatments with undiluted and diluted PAL, H_2O_2, NO_2^- and H_2O_2 + NO_2^- solutions were performed. For both cancer cell lines, control groups treated with RPMI-1640 without FBS (CTR no FBS) were analysed to ensure that the absence of essential nutrients does not significantly affect cells growth. As can be seen in Fig. 3, undiluted PAL and its dilutions induce a cytotoxic effect (50-70%) on both cancer cell lines at T = 0 h. Diluted PAL treatments induced different effects to different types of cancer cells; indeed, the effect recorded on OV-90 is dose-dependent in contrast with the one induced on SKOV-3, which is not dose-dependent. The analysis at different times after treatment show a cytostatic effect on SKOV-3 viability by undiluted PAL up to 1:8 dilution. This effect was also observed on OV-90 treated with undiluted PAL and its dilutions up to 1:4, until 72 h after treatments. The dilutions 1:16 and 1:32 have no effect in both cancer cell lines.

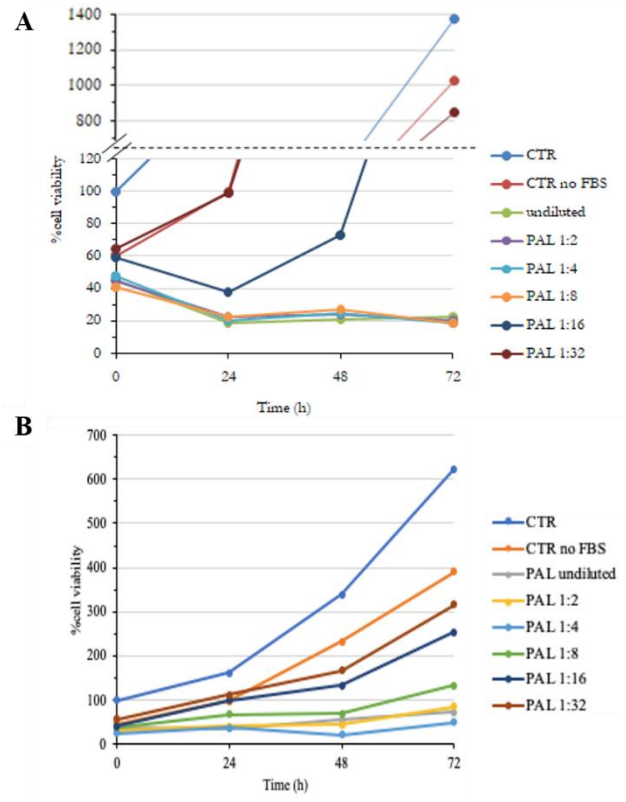

Fig. 3. Cell viability at 2 h (T = 0 h), 24, 48 and 72 h after PAL treatments. In the graphs shows (A) SKOV-3 and (B) OV-90 cancer cell lines (*n*=1).

3.3 PAL reactive species having anticancer effect

To evaluate if the PAL's effect on cell viability could be due by a synergistic effect between produced reactive species or induced by single ones, the effect of H_2O_2, NO_2^-

and H_2O_2 + NO_2^- solutions on cancer cells viability was evaluated.

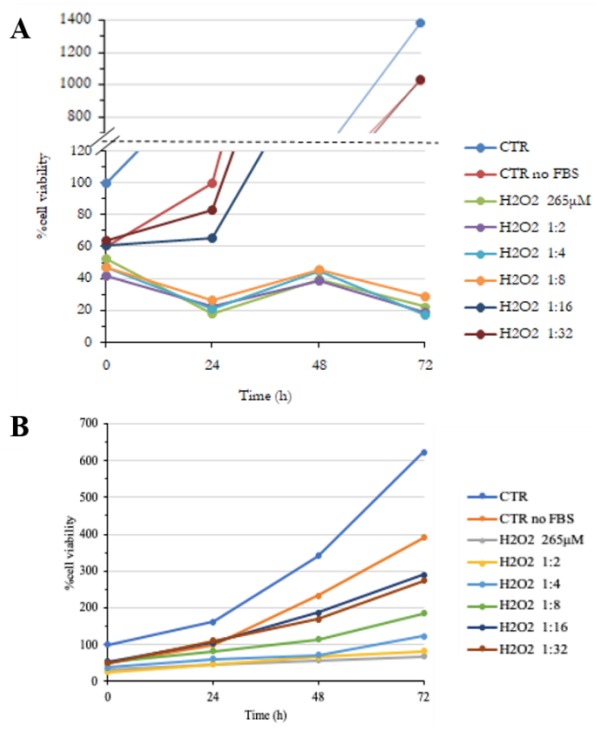

Fig. 4. Cell viability at 2 h (T = 0 h), 24, 48 and 72 h after the treatment of H_2O_2 solutions. In the graphs shows (A) SKOV-3 and (B) OV-90 cancer cell lines (n=1).

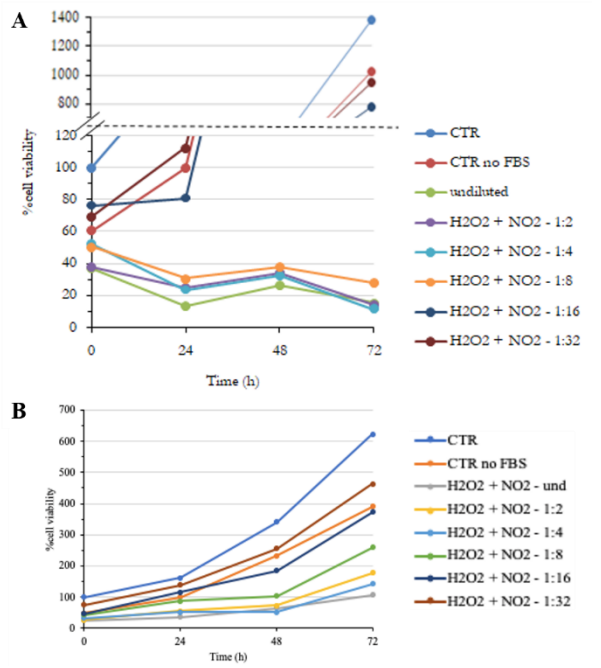

Fig. 5. Cell viability at 2 h (T = 0 h), 24, 48 and 72 h after the treatment of H_2O_2 + NO_2^- solutions. In the graphs shows (A) SKOV-3 and (B) OV-90 cancer cell lines (n=1).

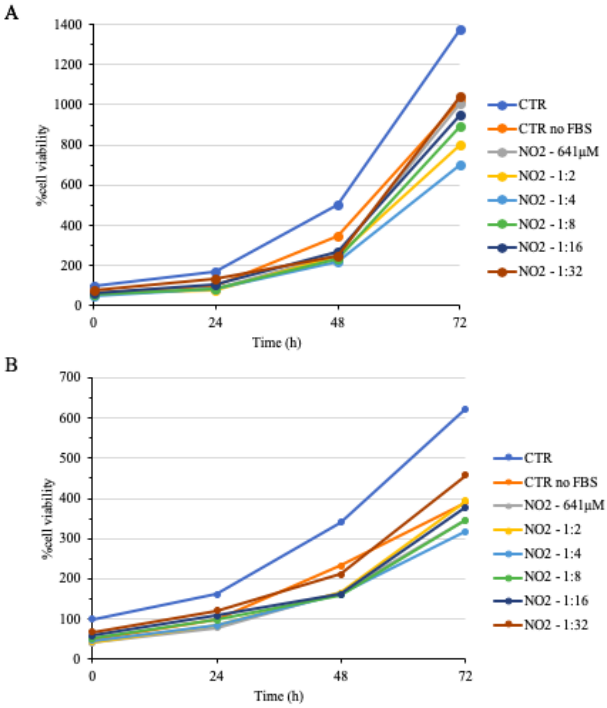

Fig. 6. Cell viability at 2 h (T = 0 h), 24, 48 and 72 h after the treatment of NO_2^- solutions. In the graphs shows (A) SKOV-3 and (B) OV-90 cancer cell lines (n=1).

In all the cases investigated, a cytotoxic effect was observed at T = 0 h in both cancer cell lines. 24 h after H_2O_2 (Fig. 4) and H_2O_2+NO_2^- (Fig. 5) solution treatments a cytostatic effect was recorded for the undiluted up to dilutions 1:4 on OV-90 and 48 h after treatments the same effect was observed up to 1:8 dilution on SKOV-3. In OV-90 cancer cell lines the effect is dose-dependent in contrast to what has been observed on SKOV-3. The NO_2^- solution treatments (Fig. 6) and the high dilutions (1:16 and 1:32), in all the examined cases, have no effect on cancer cell lines.

4.Conclusions

Oxidative stress has a major role in many biological processes, such as proliferation and differentiation, while, over a certain intracellular level, ROS are responsible for cytotoxic and cytostatic effects [6]. CAP treatment of liquid induced the generation of RONS, such as H_2O_2 and NO_2^-, which are responsible for the anticancer effect [7]. In this study, we demonstrated that PAL antitumor effect is mainly due to a synergic effect between H_2O_2 and NO_2^-. However, the CAP-treated medium (PAL) and H_2O_2 + NO_2^- solution treatments induced similar anticancer effects even if the first one had a greater impact on cells viability than the second one. Thus, suggest that components other than H_2O_2 and NO_2^- in plasma-activated liquid are also responsible for anticancer effect.

Future studies will focus on selectivity of PALs effect on the viability of immortalized EOC cells and peritumoral fibroblasts, derived from a biobank of primary cultures of

patients *in vitro*. The final aim is the development of a novel intraperitoneal therapy for cancer treatment, even in synergy with chemotherapy, using liquids suitable for the clinical application (e.g. Ringer Lactate) treated with plasma.

5.Acknowledgments

This work was supported by AlmaIDEA Senior Grant by Alma Mater Studiorum-Università di Bologna: "Chemo-physical and biological mechanisms behind the anticancer activity of plasma activated liquids for the treatment of peritoneal carcinosis from primitive epithelial ovarian/fallopian tube tumor".

6. References

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