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Cold Physical Plasma Selectively Elicits Apoptosis in Murine Pancreatic Cancer Cells *In Vitro* and *In Ovo*

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Abstract. *Background/Aim: Poor prognosis of pancreatic cancer has remained almost unchanged in recent years. Cold physical plasma was suggested as an innovative anticancer strategy, but its selective killing activity of malignant over non-malignant cells has only partially been explored. The present study aimed at exploring the effect of cold physical plasma on cellular viability. Materials and Methods: Induction of cell death and apoptosis by cold physical plasma was investigated in murine PDA6606 pancreatic cancer cells and primary murine fibroblasts in vitro (2D and 3D cultures) and in ovo. Results: Plasma increased apoptosis in PDA6606 to a significantly higher extent compared to fibroblasts. Antioxidants abrogated these effects, suggesting a prime role of reactive oxygen species in plasma-induced apoptosis. Plasma increased apoptosis of 3D PDA6606 multicellular spheres grown in vitro and in ovo, to significantly higher rates compared to that of fibroblasts, with minimum in ovo inflammation or necrosis observed by hematoxylin and eosin staining (H&E). Conclusion: These data support the future intra-operative application of cold physical plasma for the treatment of microscopic residual tumor tissue after surgical resection.*

Annually, pancreatic cancer causes over 330,000 deaths worldwide (1). Its incidence is rising in both genders, counteracting a global trend in cancer epidemiology (2). The absence of specific early symptoms leads to delayed diagnosis. At late stages, only 20-30% of the patients present with resectable tumors (3). Both aggressive local growth and early metastatic dissemination often impede curative therapy and result in a high rate of surgical R1-resections. Micrometastasis in these margins are the major cause for tumor relapse in pancreatic cancer patients (4). This results in median survival of only 18 months (5), calling for new therapeutic strategies in the treatment of pancreatic cancer.

Cold physical plasma is a partially ionized gas expelling a variety of reactive oxygen species (ROS) (6-8). This cocktail of plasma-derived ROS was shown to display considerable toxicity towards tumors in different kinds of cancers *in vitro*, including for example melanoma (9-11), glioblastoma (12-14), leukemia (15-17), ovarian cancer (18-20), prostate cancer (21-23), and colon cancer (24-26). Several groups have determined profound anticancer activity of cold physical plasmas in tumor animal models (27-29). Successful application of the atmospheric pressure argon plasma jet kINPen MED in cancer patients has been reported (30-32). Importantly, this plasma device was tested negative for genotoxic effects using appropriate model systems (33-35). Yet, it is unclear to what extent the kINPen MED plasma jet provides selective toxicity in malignant versus non-malignant cells.

To this end, the toxicity of cold physical in murine PDA6606 pancreatic cancer cells was compared to that in non-malignant, primary murine fibroblasts. Using cells grown in 2D and 3D *in vitro*, and in 3D on fertilized eggs (*in ovo*) according to the TUM-CAM assay (36), a selective and thus tumor-toxic effect of cold physical plasmas towards pancreatic cancer cells was clearly identified in these model systems. Our results suggest a potential implementation of cold physical plasma, maybe, as adjuvant treatment in the therapy of pancreatic cancer.

Materials and Methods

Cell line and culture. 6606PDA cells (a kind gift from David Tuveson, John Hopkins University, Philadelphia, USA) have been isolated from a pancreatic adenocarcinoma of a transgenic C57BL/6 $Kras^{D12G}$ knock-in mouse (37). Primary, non-immortalized murine

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fibroblasts were isolated from C57BL/6 embryos, as previoulsy described (38). Both cell entities were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Cells were subcultured twice a week for up to 10 passages. Cell culture reagents were obtained from Gibco (Invitrogen; Carlsbad, CA, USA). Cell cultures were kept pathogen-free in a humidified incubator at 37 ˚C in an atmosphere of 5 % CO_2, and were regularly tested negative for mycoplasma contamination.

Cold physical plasma. Cold physical plasma (Figure 1) was generated by the atmospheric pressure argon plasma jet kINPen MED (neoplas tools; Greifswald, Germany). It has received accreditation as medical device class IIa (39). Embedded in the hand-held unit and shielded by a dielectric capillary, the argon gas (purity greater 99.999%) was excited by a rod-like electrode using three standard liters of argon gas flow. Application was carried out from a distance of 5 mm of the plasma tip from the tissue surface. As sham treatment, argon gas flow alone (without electrical excitation or plasma generation) was used in some experiments.

Metabolic activity in vitro. For the assessment of metabolic activity *in vitro*, 2×10^3 cells were seeded into each well of a 96-well plate. After overnight attachment, supernatants were discarded and fresh cell culture medium was added. Subsequently, the argon plasma jet was directly hovered over the center of each well for the indicated time length. In some experiments, the culture medium was supplemented with the antioxidant N-acetylcysteine (NAC; Sigma; St. Louis, MO, USA). Following treatment, cells were returned to the incubator. After 24 h cells were washed, incubated with resazurin (Alfa Aesar; Ward Hill, MA, USA) for 3 h, and its transformation to resorufin was determined by fluorescence measurement in a microplate reader (Tecan; Männedorf, Switzerland) at λ_{ex}=535 nm and λ_{em}=590 nm.

Detection of cell death and apoptosis of 3D multicellular spheres in vitro. To grow three-dimensional multicellular spheres, 5×10^5 PDA6606 cells or fibroblasts were added into cell culture inserts (5 mm plastic templates, self-made) in 12-well plates. After overnight incubation, inserts were removed and supernatant was exchanged with 500 μl of fresh medium leaving only a thin liquid film on the cells. Then, plasma treatment was carried out for 20 sec. The control group was exposed for 20 sec to argon gas only. After treatment, cells received 2 ml of fresh medium, and the dish was returned to the cell culture incubator. Cell death and apoptosis analyses were performed using allophycocyanin-labeled annexin V and propidium iodide (both BD Pharmingen; Heidelberg, Germany) at 1, 24, and 48 h following plasma treatment. Briefly, cells were detached with Trypsin/EDTA, washed, and incubated in annexin V binding buffer (BioLegend; London, UK) containing annexin V and propidium iodide. Cells were washed, fluorescent signals were acquired by flow cytometry (LSR II; Becton Dickinson, Heidelberg), and data were analyzed using FlowJo software (TreeStar; Ashland, OR, USA).

In ovo tumor-chorio-allantoic membrane (TUM CAM) fertilized chicken embryo model. Pathogen-free fertilized eggs were obtained from VALO Biomedia (Osterholz-Scharmbeck, Germany). After delivery, eggs were incubated for 6 days at 37˚C and 65% relative humidity in a motorized incubator (Thermo-De-Luxe 250; J. Hemel Brutgeräte; Verl, Germany). On day 6, a small hole (<1 mm) was

Figure 1. *The atmospheric pressure argon plasma jet kINPen MED. The jet excites a flux of argon gas at the tip of a rod-like electrode in the inner part of the head (smaller part on the left of the pen-like housing at the bottom of the image). The excited argon molecules are driven to the ambient air, reacting with room air oxygen and nitrogen, to generate a mixture of reactive oxygen and nitrogen species (violet plasma effluent at the lower left of the image).*

punched into the pointed end of the eggs using a cannula (20G) to generate an air cell. The hole was then closed with a self-adhesive tape, and eggs were incubated for two additional days. On day 8, eggs were carefully opened at the pointed end and the surface of the chorio-allantoic membrane (CAM) was slightly roughened using a 5×5 mm filter paper (TISSUE-TEK II; Vogel, Germany) saturated with diethyl ether. Onto this area, a sterile silicone ring was placed (5 mm inner and 6 mm outer diameter). Thereafter, 2×10^6 PDA6606 cells or fibroblasts were resuspended in 10 μl BD Matrigel (BD Biosciences, USA) and 5 μl DMEM. This suspension was carefully filled into the silicone ring. Eggs were covered with Tegaderm (3M Healthcare; Neuss, Germany) to avoid evaporation and were returned to the incubator. On day 12, multicellular spheres were exposed to plasma *in ovo*, while controls received argon gas only. Eggs were placed back into the incubator for another 48 h (Figure 2).

Immunofluorescence and histology. On day 14, multicellular spheres were excised, stored overnight in formalin, and embedded in paraffin. Thin sections (2 μm) were cut vertically and mounted on glass slides. Following deparaffinization with xylene and ethanol (100%), sections were stained with H&E according to standard protocols. To quantify apoptotic cells, TUNEL assay (CALBIOCHEM, Merck, Darmstadt, Germany) was performed according to manufacturer's specifications. Using fluorescence microscopy (Keyence; Neu-Isenburg, Germany) the ratio of TUNEL-positive (DAPI-positive) cells was determined.

Statistical analysis. Graphics and statistical analyses were performed using prism 7.04 (GraphPad Software, USA). *In vitro* experiments were repeated at least three independent times. Statistical comparison was performed using multiple *t*-tests with Holm-Sidak post-testing correcting for multiple comparisons.

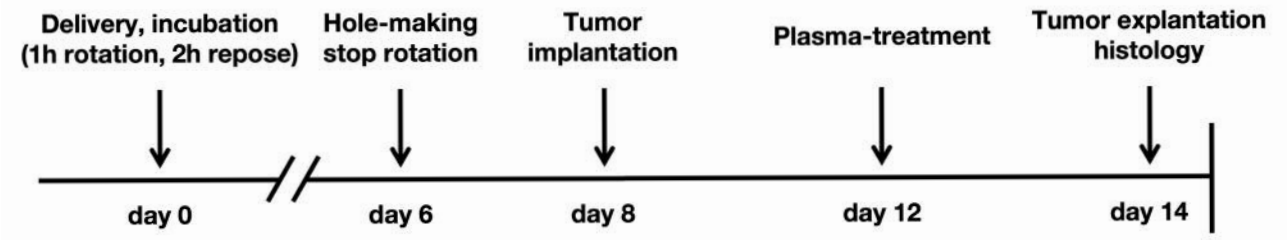

Figure 2. *Experimental design of TUM-CAM in ovo assay. After delivery, eggs were stored in the incubator with intermittent rotation. On day 6, small holes were punched in the eggshell for creating an air pocket. On day 8, PDA6606 murine pancreatic cancer cells or murine primary fibroblasts were implanted, and chicken embryos were again incubated. On day 12, cold physical plasma treatment was performed. On day 14, PDA6606 or fibroblasts spheres were removed and analyzed via immunofluorescence for apoptosis and histologically.*

Significance levels were indicated as follows: *α=0.05, **α=0.01, and ***α=0.001. Data are shown as individual data points and mean, or mean±standard error.

Results

Cold physical plasma selectively inactivated murine pancreatic cancer cells over murine fibroblasts via release of reactive oxygen species in vitro. Cold physical plasma is a partially ionized gas expelling various reactive oxygen species (ROS) that have been shown to be toxic to tumor cells. To address the selectivity of this novel treatment regimen, the toxicity of cold plasma was examined using murine PDA6606 pancreatic cancer cells and murine primary fibroblasts. To this end, the metabolic activity of cells was assessed 24 h after plasma treatment of 2D monolayer cell cultures *in vitro*. Compared to mock-treated argon gas controls, plasma treatment significantly reduced metabolic activity of pancreatic cancer cells but not that of fibroblasts (Figure 3A). Supplementation with the ROS-scavenging antioxidant N-acetylcysteine (NAC) reversed plasma-mediated toxicity in a concentration-dependent manner. At 2 and 4 mmol/l of NAC, exposure to plasma significantly decreased the metabolic activity of pancreatic cancer cells compared to that of murine fibroblasts (Figure 3A). At higher NAC concentrations, there was still a differential response of these cell types. Interestingly, the lowest NAC concentrations nearly abolished plasma effects in fibroblasts, whereas pancreatic cancer cells were only partially protected. This difference might offer a therapeutic window for minimizing potential harmful effects of plasma treatment in patients by adding small amount of scavenger. Altogether, these results indicate that plasma treatment is more toxic to pancreatic cancer cells than fibroblasts in the presence of NAC.

Cold physical plasma selectively induced apoptosis and terminal cell death in murine pancreatic cancer cells over murine fibroblasts in vitro. To investigate cell death in a more relevant three-dimensional culture system, tumor or fibroblasts were grown in tissue-like spheres *in vitro*, before being subjected to plasma treatment. Spheres were dissociated and the total percentage of dead and apoptotic cells was evaluated by flow cytometry. There was a significant increase of apoptotic cells in plasma-treated PDA6606 pancreatic cancer cells over primary murine fibroblasts assayed 24 h and 48 h after plasma treatment (Figure 3B). Notably, apoptotic rates in fibroblasts decreased between 24 h and 48 h whereas they were increasing in pancreatic cancer cells. This suggests stronger counter-regulation mechanisms in fibroblasts but not in tumor cells. The significantly elevated percentage of apoptotic fibroblasts over cancer cells at 1 h may be explained by a higher percentage of dead fibroblasts in the controls. Since fibroblasts were isolated from primary murine tissue, they are more prone to cell culture-induced toxicity. By contrast, quantifying the percentage of terminally dead (propidium iodide$^+$) cells at 1 h, PDA6606 pancreatic cancer cells were found to be significantly more vulnerable (Figure 3C) compared to murine fibroblasts. This finding became more pronounced with increasing incubation times of 24 h or 48 h post plasma treatment.

Cold physical plasma elicited apoptosis preferentially in spheres grown from murine pancreatic cancer cells over that of primary fibroblasts. Plasma treatment selectively induced apoptosis of murine pancreatic cells over fibroblasts in a ROS-dependent manner in 2D monolayers and 3D sphere cultures *in vitro*. However, even 3D *in vitro* tissue cultures lack parts of the tumor microenvironment in living hosts. To address this complexity while minimizing the need for animal experiments (*in vivo*), the TUM-CAM assay has been developed which utilizes fertilized chicken embryos harboring a pre-mature central nervous system up to day 15 (36). Accordingly, we implanted either murine PDA6606 pancreatic cancer cells or murine primary fibroblasts onto

chorio-allantoic membranes of eggs (*in ovo*) and assayed the tissue response following exposure to cold physical plasma. Counterstaining of nuclei with DAPI and DNA fragments of apoptotic cells with the TUNEL assay revealed overall similar spontaneous apoptosis for either of the cell types in the argon gas mock treatment group (Figure 4). By contrast, there was a notable increase in apoptosis in PDA6606 murine pancreatic cancer cells over primary murine fibroblasts following plasma treatment for 10 sec or 30 sec. Quantifying the percentage of apoptotic cells in both cell types, fibroblasts were significantly less affected by plasma treatment compared to cancer cells. Consistent with the *in vitro* observations, the *in ovo* results suggested fibroblasts to be significantly more resistant to cold physical plasma-induced apoptosis than pancreatic tumor cells. This was confirmed by H&E staining of sections of *in ovo* spheres exposed either to mock treatment with argon gas or cold physical plasma. Fibroblast spheres did not show any morphological changes following 30 sec of plasma treatment (Figure 5A and 5B). However, plasma treatment of PDA6606 cells *in ovo* elicited morphological signs of apoptotic cell death such as cellular shrinking, condensed and bright eosinophilic cytoplasm, and pycnotic dark small nuclei due to chromatin condensation (Figure 5C and 5D). Nevertheless, the rest of cells retained their malignant phenotype with some showing mitotic activity. In general, no inflammatory reaction or large necrotic areas were noted following plasma treatment.

Discussion

Notwithstanding general translational and clinical progress in cancer treatment, prognosis for patients suffering from pancreatic cancer remains very poor. In search of possible add-on treatment procedures, the toxicity and selectivity of cold physical plasma in murine pancreatic cancer cells was investigated and compared to non-malignant, primary fibroblasts.

Cold physical plasma treatment reduced metabolic activity and induced apoptosis, as well as early terminal cell death in pancreatic cancer cells. This is in line with other findings reporting on pro-apoptotic effects of plasma treatment in human or mouse cancer cell lines (40-45). We have also observed apoptosis in COLO-357 tumor spheres grown *in ovo* (TUM-CAM) following exposure to an argon plasma jet earlier (36). It is subject of ongoing research which main active components in plasmas mediate such biological response. In principle, cold physical plasmas are multi-component systems releasing mild heat, electrons and ions, electric fields, UV-radiation, and reactive oxygen and nitrogen species (ROS/RNS) (46). In 2D cell culture systems, adherent cells are covered by cell culture medium to protect them from drying out. Plasma components need to

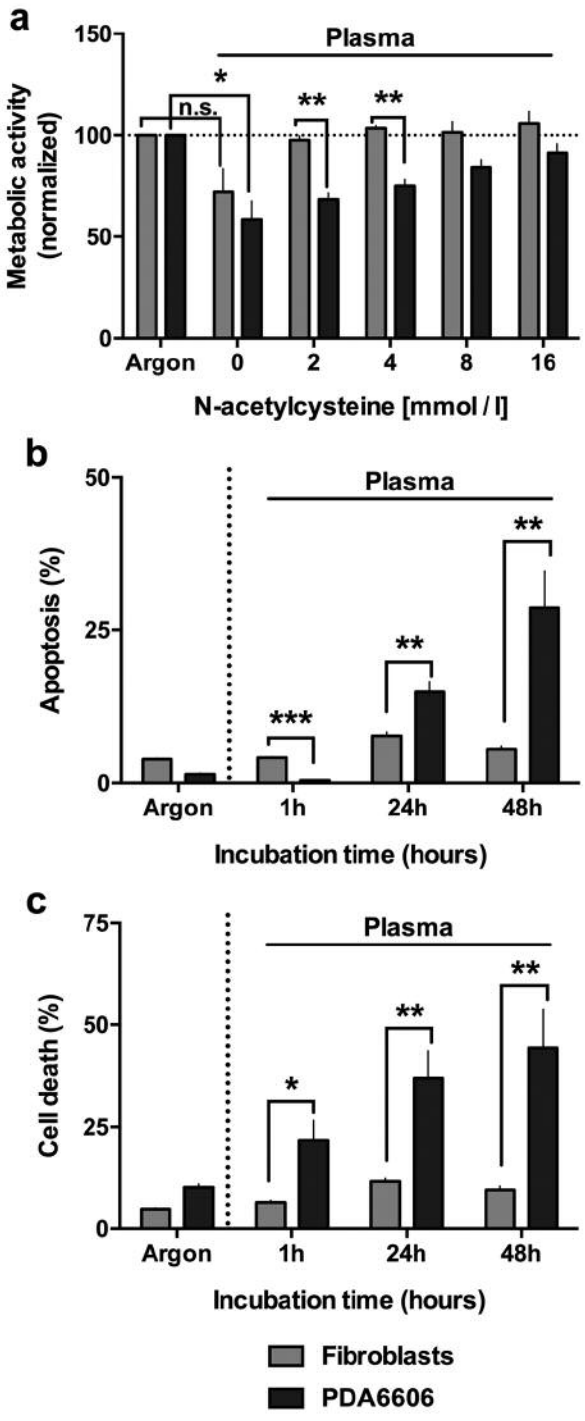

Figure 3. *Cold physical plasma reduced metabolic activity and induced apoptosis in pancreatic cancer cells grown in vitro as 2D monolayers or 3D multicellular spheres. (A) Metabolic activity of PDA6606 murine pancreatic cancer cells and fibroblasts 24 h post exposure to cold physical plasma (60 sec) in the presence of ROS-scavenger N-acetylcysteine (NAC); (B, C) Percentage of annexin V+ apoptotic (B) and propidium iodide+ terminally dead cells (C) in argon gas control and plasma-treated (20 sec) PDA6606 murine pancreatic cancer cells and murine primary fibroblasts grown in 3D multicellular spheres in vitro. Data show mean + standard error of 4-5 independent experiments.*

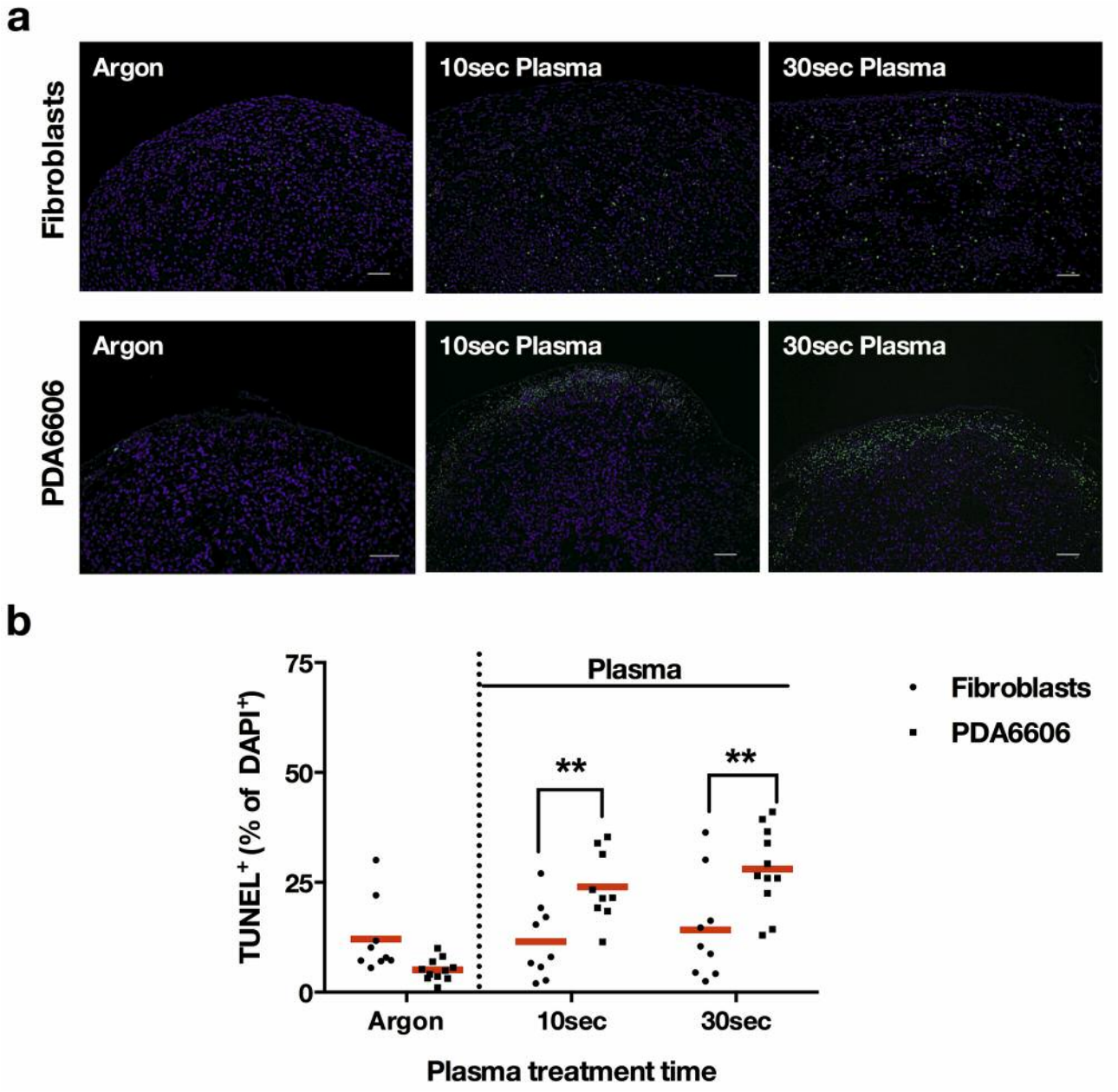

Figure 4. *Exposure to cold physical plasma elicited apoptosis preferentially in spheres grown in ovo from murine pancreatic cancer cells over that from primary fibroblasts. (A) Representative images of sphere sections stained for nuclei (DAPI, blue) and apoptotic cells (TUNEL, green); (B) ratio of TUNEL+ over DAPI+ cells among all treatment groups. Data are from 9-11 tissue sections per group; scale bars represent 50 μm, **p<0.01.*

travel through this liquid in order to be active. Damaging, long-lived UV radiation (UV-C) only penetrates in the order of 100 nm (47), eliminating this possible effector. Temperature measurements of plasma-treated liquids have ruled out this mode of action. Electrons and ions quickly deteriorate at the plasma gas-liquid interphase (48). Electric fields are negligible with the atmospheric pressure argon plasma jet kINPen MED used in this study (49). This leaves ROS/RNS as main driver of plasma effects in *in vitro* cultures. Many kinds of reactive species were so far identified in plasma-treated liquids (50) including superoxide, singlet delta oxygen, peroxynitrite, and hydrogen peroxide (51-54). Disassembling the plasma-derived redox chemistry was out of the scope of the current study, but

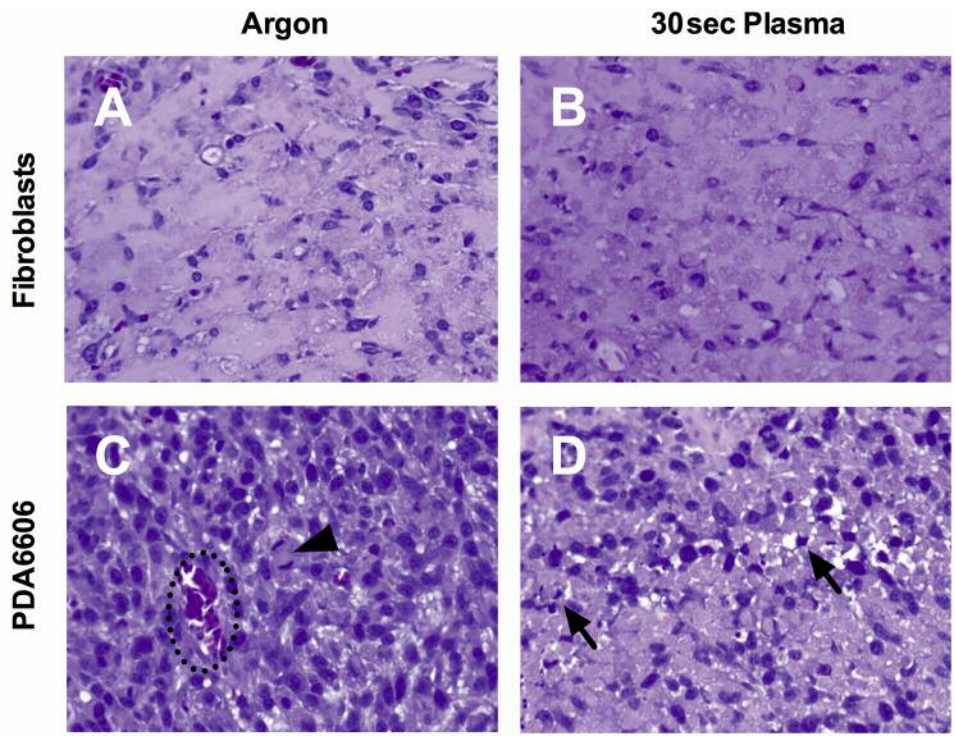

Figure 5. *H&E staining of solid spheres from pancreatic cancer cells and non-cancer spheres of murine fibroblasts. (A, B) showing spindle-shaped nuclei and bigger intercellular space in comparison to cancer cells (C, D), murine fibroblast spheres grown on chicken embryos in ovo displayed no differences when comparing argon gas, mock treatment, (A) to 30 sec of cold physical plasma treatment (B); (C) mock-treated, argon gas treated, cancer cells showed big cell nuclei, tightly stuck together causing small intercellular spaces, mitotic figures (arrowhead) and a strong vascularization (interrupted line), hallmarks of malignant tissue; (D) after 30 s exposure to cold physical plasma, pancreatic cancer tumors showed signs of cell death, as seen with generation of apoptotic bodies, pycnotic, dark small nuclei, and a drained cell organization with eosinophilic cytoplasm rests (arrows). Representative images are shown, original magnification 400×.*

results using the clinically established ROS-scavenger NAC (55) suggested a main role of radical chemistry in cytotoxic effects. In principal, this accounts also for 3D cell spheres grown *in vitro* and *in ovo* that were not directly protected from a large liquid film and thus directly accessible to plasma-derived short-lived radical species.

Apart from plasma and liquid redox chemistry, our results clearly indicate a selective toxicity of plasma-derived reactive species towards malignant pancreatic tumor cells over non-malignant primary fibroblasts. This corroborates previous findings that demonstrated selective toxicity in tumor cells using cold physical plasma sources (56-61). There are different theories of how this selectivity is achieved with none of them being confirmed so far. One is the assumption that cancer cells have more aquaporins in their cell membrane (62). These water channels not only carry water in and out of cells but also facilitate the uptake of long-lived plasma-derived oxidants such as hydrogen peroxide (63). With dry argon as feed gas used in this study, our plasma jet produces only little hydrogen peroxide in the gas phase (64), suggesting that hydrogen peroxide is unlikely to have a significant contribution in our three-dimensional model cell spheres. Another theory claims a significance of membrane-bound catalase for plasma-derived reactive-species dependent tumor cell kill (65). Specifically, this enzyme is claimed to protect tumor cells from autocrine hydrogen peroxide and peroxynitrite-mediated tumor cell apoptosis. Yet, the *in vivo* relevance of this mechanism remains to be established. A third hypothesis attributes a role to membrane-based cholesterol in dictating the selectivity of plasma-mediated toxicity in cancer versus non-cancerous cells (66). However, experimental evidence is missing to support this theory. A fourth theory is that of "oxidative balance" (67-69). It proposes that metabolic reprogramming in cancer cells (*e.g.* from oxidative phosphorylation in mitochondria to glycolysis) leads to higher endogenous ROS levels compared to that of non-malignant cells (70). This would make cancer cells more prone to ROS-induced cell death as with cold physical plasma treatment (71). However, our own data suggest that mitochondria remain an important

target in cancer cells, also using cold physical plasmas (72). This is based on the general finding that some types of tumor cells employ both oxidative phosphorylation and glycolysis to meet their energy needs (73). Moreover, ROS not only damage cellular biomolecules but also serve as reactive agents in thiol-arrays responsible for redox-mediated signaling function (74). Yet, it remains to be explored which redox proteins are key switches in translating plasma-derived ROS into apoptotic responses in malignant over non-malignant cells.

Conflicts of Interest

The Authors declare that they have no competing interests regarding this work.

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