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Supplementary information

Evaluation of the effects of cold atmospheric plasma and plasma-treated liquids in cancer cell cultures

In the format provided by the authors and unedited

Supplementary Information: **Selected review on the influence of plasma jets and liquid sample parameters on the cytotoxic outcomes of the treatment.**

Supplementary Table 1. Influence of modifying different cold atmospheric plasma jet parameters (type of gas, gas flow) or of the liquid treated (volume, type: cell culture media, saline solutions, etc.) on the biological response (cell viability) of a variety of cell lines.

PLASMA SETTINGS						SAMPLES		BIOLOGICAL EFFECTS		Ref
Flow rate (L/min)	**Gas**	**U** (kV)	**f** (kHz)	**d** (mm)	**t** (s)	**V** (µL)	**Liquid**	**Cell lines**	**Cell viability** (%)	
2	Ar	7	-	13	180	3000	DMEM & DMEM+P/S+FBS	U251SP (Human glioblastoma cell line)	C_v^{24h} ~10-12%	[1]
								MCF10A (Human mammary epithelial cell line)	C_v^{24h} ~95-98%	
3		2-6	1000	10	300	2000	PBS	U87, U251 and LN229 (Glioblastoma human GBM cell lines)	C_v^{24h} ~2-5%	[2]
					540					
0.05	He	8	20	10	120	500	PBS (Ca^{2+}/Mg^{2+}) +DMEM	NHSF (Normal human skin fibroblasts)	C_v^{24h} ~4%	[3]
								$MRC5V_i$ cell line (derived from normal human lung fibroblasts MRC5)	C_v^{24h} ~10%	
								HCT116 (Human colon cancer cells)	C_v^{24h} ~45%	
								Lul205 (Melanoma cell line)	C_v^{24h} ~30%	
					480			NHSF (Normal human skin fibroblasts)	C_v^{24h} ~ 1%	
								$MRC5V_i$ cell line (derived from normal human lung fibroblasts MRC5)		
								HCT116 (Human colon cancer cells)		
								Lul205 (Melanoma cell line)		
0.1		8	15	5	120	100	DMEM+FBS	Glioblastoma (U87MG)	C_v^{24h} ~60.8% C_v^{48h}~45-49%	[4]
4.7		3.16	-	30	240	1000		MDA-MB-231 (Human breast cancer cells)	C_v^{72h} ~40%	[5]

							DMEM, modified-DMEM	U87MG (Human glioblastoma cells)	C_v^{72h} ~15%	
								PA-TU-8988T (Pancreatic cancer cells)	C_v^{72h} ~20%	
5		5-9	5	25	120	500	MEM +10% FBS	ScaBER (from human bladder cancer)	C_v^{12h} ~50% C_v^{48h} ~50%	[6]
					240				C_v^{12h} ~10% C_v^{48h} ≤5%	
8	He + O_2	28	2	-	20-300	300	Phenol-free RPMI 1640	Bel7402 (Human hepatocellular carcinoma)	C_v^{24h} ≤10%	[7]
					20			5-FU-resistant Bel7402/5FU cells	C_v^{24h} ~ 92%	
					40				C_v^{24h} ~ 80%	
					60				C_v^{24h} ~ 60%	
					120				C_v^{24h} ~ 40%	
					300				C_v^{24h} ~ 10%	

References

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4. Chen Z. *et al.* A novel micro cold atmospheric plasma device for glioblastoma both in vitro and in vivo. *Cancers* **9**, 61, doi:10.3390/cancers9060061 (2017).

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7. Yang H. *et al.* Effects of atmospheric pressure cold plasma on human hepatocarcinoma cell and its 5-fluorouracil resistant cell line. *Phys. Plasmas* **22**, 122006 doi:10.1063/1.4933405 (2015).

Supplementary Method 1

RNA extraction and RT-PCR analysis of CAP or PCM-treated U-2 OS cells.

Kits

RNA RNeasy Mini Kit (Qiagen, # 74104).

Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, # K1671).

SYBR™ Green PCR Master Mix (Applied Biosystems™).

Equipment

2720 Thermal Cycler (Applied Biosystems™).

7500 Fast Real-Time PCR System (Applied Biosystems™).

Spectrophotometer (Denovix DS-11, Denovix).

Software

https://primer3.ut.ee/

Microsoft Office Excel 2016 (Microsoft).

GraphPAD 8.

Procedure:

Prior to proceeding, check cells under the microscope to confirm that cells are detached from the surface.

Perform Steps 66-73 for each experimental condition, using PCM or CAP. Store cell pellet at -80 ° C, until use. Or alternatively, use fresh pellet to proceed with RNA Extraction

RNA Extraction:

The extraction of total RNA is carried out by column purification using the RNA RNeasy Mini Kit (Qiagen, # 74104).

Total RNA is extracted following the manufacturer's instructions from frozen or fresh pellets. RNA is resuspended in RNAase-free water (provided by the kit) and its concentration and purity quantified by measuring absorbance at 260 nm and 280 nm in a spectrophotometer (Denovix DS-11, Denovix). The RNA concentration is obtained at the absorbance of 260 nm, and the ratio between A260 / A280 shows the purity of the sample. We only use samples with a purity greater than A260 / 280 ≥2. Finally, store RNA samples at -80 ºC until use.

cDNA synthesis[1,2]

For the reverse transcription reactions, we use Maxima First Strand cDNA Synthesis kit for RT-qPCR (K1671, ThermoFisher, USA) with 1 µg of RNA following the manufacturer's instructions. To perform cDNA synthesis, we use a 2720 Thermal Cycler (Applied Biosystems™). After 25 minutes of reaction, the cDNA is diluted 8 times in sterile water before its subsequent use in PCR reactions to avoid cross-reactivity.

qRT-PCR[1,2]

The PCR reaction is carried out with 2.5 µL of 1/8 diluted cDNA, adding: 3.75 µL primers (Forward and Reverse) at 4.5 pmol concentration, 7.5 µL of sterile water and finally 18.75 µL of power SYBR green PCR master mix (Applied Biosystems) containing a fluorophore that binds to double-stranded DNA. All samples are analyzed in duplicate PCR reactions. Expression of the β-actin is used as house-keeping.

The Primers can be designed using the Primer3 tool (https://primer3.ut.ee/). Taking the cDNA sequence of the gene of interest, and selecting oligonucleotides with optimal designs: 90-100 base pairs, 60 ºC melting and 20 nucleotides to avoid generating primer-dimers.

Table. List of primers used in this method.

Name	Forward (5'→3')	Reverse (5'→3')
ATF3	TGCCTCGGAAGTGAGTGCTT	GCAAAATCCTCAAACACCAGTG
ATF6	GGAAAGTGGCAAAGAGCTTG	GTGGTGGGAACTTTGGCTG
BETA ACTIN	GTCCTCTCCCAAGTCCACAC	GGGAGACCAAAAGCCTTCAT
GADD153	TCTCCTCAGGTTCCAGCTCT	TCTAAGGCAGCACTGAGCGTATCA
TRB3	TGGTACCCAGCTCCTCTACG	GACAAAGCGACACAGCTTGA
ATF3	TGCCTCGGAAGTGAGTGCTT	GCAAAATCCTCAAACACCAGT G
HSPA5	CTATTGGGGTGTTTCGCGAG	GAGAGCTTCATCTTGCCAGC

We use a 7500 Fast Real-Time PCR System (Applied Biosystems™) device to run RT-PCR reactions, with standard conditions: a first denaturation step of 10 minutes at 95 ºC, followed by 40 cycles with 15 s at 95 ºC and 1 min at 60 ºC and curve step of dissociation with 1 minute at 95 ºC, 30 s at 55 ºC and 30s at 95 ºC.

Data Analysis

The data is analyzed with the 7500 Fast Real-Time PCR System (Applied Biosystems™). The software processes the level of fluorescence emitted by the samples to generate a threshold cycle for each sample (CT), this being the PCR cycle in which an increase in fluorescence is detected above the baseline signal. The quantification of the relative expression level is carried out in four steps following the method described in [3].

i. Quantification of ΔCt1 (the difference between the Ct values in treated sample) = Ct gene - Ct beta actin gene.
ii. Quantification of ΔCt2 (the difference between the Ct values in control sample) = Ct gene - Ct beta actin gene.
iii. Quantification of the difference in the ΔCt values between the experimental and control samples ΔΔCt = (ΔCt1 - ΔCt2).
iv. Gene expression level: The fold-change in expression of the gene of interest between the two samples is then equal to $2^{-\Delta\Delta Ct}$.

In all cases, we recommend to perform the melting curve to verify a single dissociation peak for each gene.

Microsoft Office Excel 2016 (Microsoft) is used to perform data analysis and calculate fold change, comparing the gene expression level of treated cells vs untreated cells (control)[1] and GraphPAD 8 software to represent data.

References:

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Supplementary Method 2

Human Phospho-Kinase Array

Reagents

Proteome Profiler Human Phospho-Kinase Array Kit (RD Systems, ARY003B).

Equipment

Odyssey® Fc Imaging System (LI-COR Biosciences).

Software

Image Studio Lite Software (LI-COR Biosciences).

ImageJ Software. (Free licence).

Microsoft Office Excel 2016 (Microsoft).

REAGENT PREPARATION

Bring all reagents to room temperature before use. Proteome Profiler Human Phospho-Kinase Array Kit (RD Systems, ARY003B) contains Part A and Part B that should be used together for optimal analysis efficiency. Handle the membranes only with gloved hands.

I. Reconstitute Detection Antibody Cocktail A in 100 µL of deionized or distilled water.
II. Reconstitute Detection Antibody Cocktail B in 100 µL of deionized or distilled water.
III. Add 2 mL of 5x Array Buffer 2 Concentrate to 8 mL of Array Buffer 3. Prepare fresh for each use.
IV. Add 40 mL of 25x Wash Buffer Concentrate to 960 mL of deionized or distilled water to prepare 1000 mL of 1x Wash Buffer.
V. Chemi Reagent 1 and 2 should be mixed in equal volumes within 15 minutes of use. Protect from light. 1 mL of the resultant mix is required for each set of membranes (A and B).

Procedure [1]

Scraping of PCM-Treated Cells

The phosphorylation profile was analyzed in the PCM treated cells using the Proteome Profiler Human Phospho-Kinase Array (R&D Systems, cat.no ARY003B, Minneapolis, MN, USA). Cells (750×10^3) were plated in a 100-mm dish, on the following day, the culture medium was replaced with 8000 µL of PCM using 60 s in U-2 OS cells. 6 h post-treatment cells were collected by scraping, according to Box 1.

Rinse cells with DPBS 1x by scraping, making sure to remove any remaining DPBS before adding 1 mL of lysis buffer. Pipette up and down to resuspend lysates gently at 2-8 °C for 30 min. Store the samples overnight at – 80 °C to improve lysis. Microcentrifuge at 14.000 x g for 5 min, and transfer the supernatant into a clean test tube. Lysates should be used immediately or aliquoted and stored at ≤ -80 °C. Avoid repeated freeze-thaw cycles. Thawed lysates should be kept on ice prior to use.

Array Procedure

Bring all reagents to room temperature before use. Keep samples on ice. For the parallel determination of the relative levels of protein phosphorylation follow the manufacturers' protocol provided by the kit (RD Systems, Catalog Number ARY003B).

DATA ANALYSIS

The bound material was detected by adding the biotinylated antibodies followed by streptavidin conjugated with HRP. Chemiluminescence was detected using Odyssey Fc imaging system and the software Image Studio from LI-COR (Lincoln, NE, USA). The pixel density of the background was subtracted from the signal of each spot, and the average of duplicate spots was determined using the ImageJ software.

I. Create a template to analyze pixel density in each spot of the array.
II. Export signal values to Microsoft Excel.
III. Determine the average signal (pixel density) of the pair of duplicate spots representing each phosphorylated kinase.
IV. Subtract an averaged background signal from each spot. Use a signal from a clear area of the array or negative control spots as a background value.
V. Compare corresponding signals on different arrays to determine the relative change in phosphorylated kinase proteins between samples.

Reference

1 Tornin, J. *et al.* Pyruvate Plays a Main Role in the Antitumoral Selectivity of Cold Atmospheric Plasma in Osteosarcoma. *Sci Rep* **9**, 10681, doi:10.1038/s41598-019-47128-1 (2019).