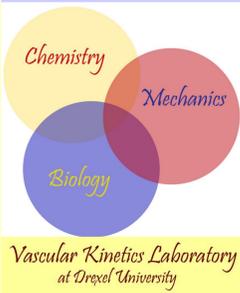


Low Dose Non-Thermal Plasma Enhances Endothelial Cell Proliferation Through Fibroblast Growth Factor 2 Release

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INTRODUCTION

Cold atmospheric pressure plasma is currently being investigated for a wide range of clinical applications, including skin sterilization, blood coagulation [1, 2], malignant cell apoptosis [1], and wound healing [1]. However, the effect of non-thermal plasma on the vasculature is unclear. Blood vessels are affected during plasma treatment of all tissues, and vessels themselves may be an important potential target for clinical plasma therapy. We investigated the effect of cold plasma treatment on endothelial cells, which line the inner surface of blood vessels. Endothelial cells play a guiding role in angiogenesis, the growth of new blood vessels from existing vessels. In various disease conditions, healing may result from promoting or blocking angiogenesis. We present enhanced proliferation in low dose plasma treated endothelial cells *in vitro*, as well as mechanisms for the observed effect.

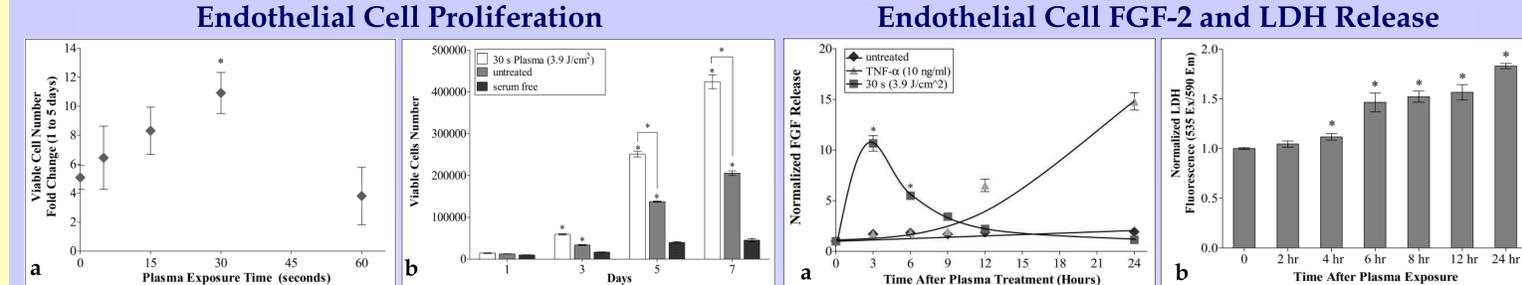
HYPOTHESIS

We hypothesize that non-thermal plasma treatment can be varied to grow or regress blood vessels

METHODS

- PAEC on glass cover slips were exposed to low power plasma for 0 to 60 seconds. Following plasma treatment, cover slips were immediately placed in a new 12-well plate, and samples were incubated in 1.5 ml of fresh medium
- Viable endothelial cell number was determined by counting trypsin-detached cells in a Coulter counter. Cell proliferation was measured through cell counts either on directly treated cells or through a conditioned media assay.
- FGF2 release was measured by enzyme linked immunosorbent assay (ELISA). FGF2 effects were blocked using an FGF2 neutralizing antibody (10 µg/ml),
- Data are mean ± SD. Statistical significance (p<0.05, #) was evaluated using Student's t test (2 groups) and ANOVA (>2 groups).

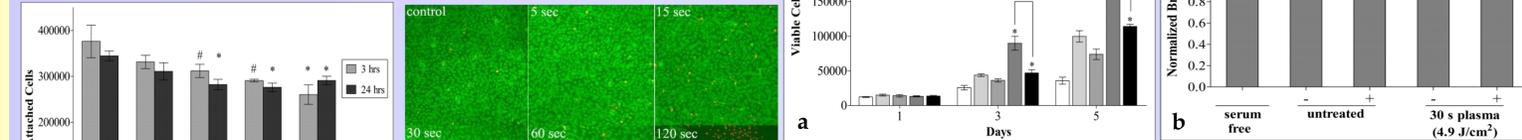
RESULTS



Non-thermal plasma induces endothelial cell proliferation. (a) Endothelial cell fold growth is enhanced in non-thermal plasma treated cells 5 days after treatment. Plasma treated cells were counted using a Coulter counter 1 and 5 days after treatment. * p < 0.01 as compared to control. (b) Endothelial cell proliferation is enhanced in cells incubated in non-thermal plasma treated conditioned medium. Conditioned medium was collected from confluent endothelial cells 3 hours after treatment and added to subconfluent untreated cells. Cell number was counted using a Coulter counter. * p < 0.05 as compared to day 1; # p < 0.05 comparing untreated cells with 30 seconds plasma treatment on days 5 and 7

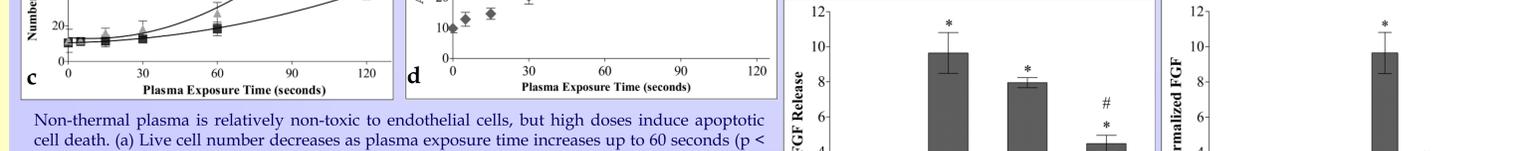
(a) FGF2 release increases up to 3 hours post plasma treatment and then decreases up to 24 hours post plasma treatment. FGF2 was measured in conditioned media samples by ELISA. * p < 0.01 as compared to control (0 h). (b) LDH release increases up to 24 hours post plasma exposure. * p < 0.01 as compared to untreated cells.

Toxicity of Non-Thermal Plasma Treatment

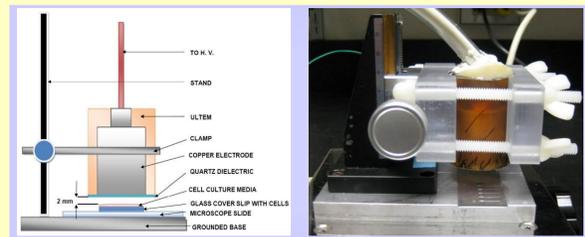


(a) FGF2 blockade in the conditioned medium reduces the endothelial cell proliferation rate. Conditioned medium from plasma treated cells was incubated with 1 mg/ml FGF2 neutralizing antibody prior to application to untreated cells. Cell number was measured using a Coulter counter. * p < 0.01 as compared to day 1; # p < 0.05 comparing plasma treated cells with FGF-neutralizing antibody to plasma treated cells without FGF-neutralizing antibody on days 3 and 5. (b) FGF-2 blockade in the conditioned medium also reduces incorporation of BrDU by endothelial cells. Conditioned medium from plasma treated cells was incubated with 1mg/ml FGF-2 neutralizing antibody prior to application to untreated cells. BrDU incorporation was measured using BrDU assay according to the manufacturer's instructions 18h after addition of conditioned medium. * p < 0.01 as compared to untreated cells without FGF-neutralizing antibody. # p < 0.05 as compared to 30 s treatment without FGF-neutralizing antibody.

Mechanisms of FGF-2 Release Post Plasma Treatment



Endothelial cell FGF2 release is linked to neutral ROS (a) Direct plasma treatment with or without a mesh (indirect) induces greater cellular FGF2 release compared to separated treatment. * p < 0.01 as compared to control; # p < 0.05 as compared to direct treatment (b) Intracellular (4 mM NAC - N-Acetyl Cysteine) and extracellular (50 mM SP - Sodium Pyruvate) ROS scavengers block FGF2 release from endothelial cells post plasma treatment. * p < 0.01 as compared to control (0). # p < 0.05 as compared to direct treatment (D). NSP: 4mM N-Acetyl Cysteine and 10 mM Sodium Pyruvate together.



NON-THERMAL PLASMA SETUP & PARAMETERS

Parameter	Value
Excitation	Microsecond Pulsed
Voltage	20 kV p-p
Rise Time	5 V/ns
Pulse Width (FWHM)	1.65 µs
Frequency	0.5 - 1.5 kHz
Power Density	0.1 - 1 W/cm ²
Rotational Temperature	300 - 350 K
Vibrational Temperature	3000 - 4000 K

CONCLUSIONS

- Non-thermal plasma is relatively non-toxic to endothelial cells at short exposure times while longer exposure times are cytotoxic.
- Endothelial cell proliferation is enhanced following plasma treatment, which is likely related to FGF2 release caused by plasma-produced ROS.
- By tuning plasma properties, angiogenesis could be controlled.
- Low power non-thermal plasma treatment shows promise for novel therapies focused on promotion or inhibition of endothelial cell mediated angiogenesis.

FUTURE WORK

- We believe that non-thermal plasma could be used *in vitro* and *in vivo* to stimulate angiogenesis.
- Potential applications of plasma treatment include vascularizing tissue engineering structures, enhancing incorporation of transplanted tissues, and accelerating wound healing
- In the future, this research will examine endothelial cell migration in two and three dimensions, as well as angiogenic tube formation.
- Finally we will determine non-thermal plasma efficacy in blood vessel formation in tissue engineering, transplantation, and wound healing.

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