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Keywords (separated by '-')	Angiogenesis - Plasma medicine - Reactive oxygen species - Apoptosis - Wound healing	
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Endothelial Cell Proliferation is Enhanced by Low Dose Non-Thermal Plasma Through Fibroblast Growth Factor-2 Release

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Abstract—Non-thermal dielectric barrier discharge plasma is being developed for a wide range of medical applications, including wound healing, blood coagulation, and malignant cell apoptosis. However, the effect of non-thermal plasma on the vasculature is unclear. Blood vessels are affected during plasma treatment of many tissues and may be an important potential target for clinical plasma therapy. Porcine aortic endothelial cells were treated *in vitro* with a custom non-thermal plasma device. Low dose plasma (up to 30 s or 4 J cm⁻²) was relatively non-toxic to endothelial cells while treatment at longer exposures (60 s and higher or 8 J cm⁻²) led to cell death. Endothelial cells treated with plasma for 30 s demonstrated twice as much proliferation as untreated cells five days after plasma treatment. Endothelial cell release of fibroblast growth factor-2 (FGF2) peaked 3 h after plasma treatment. The plasma proliferative effect was abrogated by an FGF2 neutralizing antibody, and FGF2 release was blocked by reactive oxygen species scavengers. These data suggest that low dose non-thermal plasma enhances endothelial cell proliferation due to reactive oxygen species mediated FGF2 release. Plasma may be a novel therapy for dose-dependent promotion or inhibition of endothelial cell mediated angiogenesis.

Keywords—Angiogenesis, Plasma medicine, Reactive oxygen species, Apoptosis, Wound healing.

INTRODUCTION

Non-thermal dielectric barrier discharge (DBD) plasma has recently emerged as a novel tool in medicine. DBD occurs at atmospheric pressure in air or other gases when high voltage of sinusoidal waveform or short duration pulses is applied between two

electrodes, with at least one electrode being insulated.^{10,35} The insulator prevents current build-up between the electrodes, creating electrically safe plasma without substantial gas heating. This approach allows direct treatment of biological systems without the thermal damage observed in conventional thermal plasma.³⁸ Non-thermal plasma can kill bacteria or induce apoptosis in malignant cells.^{2,15,17,27} It can be applied in sub-lethal doses for gene transfection,^{7,8} cell detachment,^{26,27} wound healing,^{2,21,34} and blood coagulation.^{2,25} In recent studies of plasma blood coagulation^{2,25} and bacteria deactivation,^{2,15} plasma did not demonstrate measurable toxicity in the surrounding living tissue.^{2,7}

Non-thermal plasmas can be used in medicine for either *direct* or *indirect* treatment.¹⁵ Plasma is composed of charged particles (electrons, ions), electronically excited atoms and molecules, radicals, and ultraviolet photons. Both direct and indirect plasma expose cells or the tissue surface to short and long lived neutral atoms and molecules, including ozone (O₃), NO, OH radicals, and singlet oxygen (O₂¹Δ_g). However, direct plasma allows a significant charged particle flux, including electrons and positive and negative ions like superoxide radicals O₂⁻, to reach the surface. Non-thermal plasma temperature and composition can be changed to control plasma products.

Non-thermal plasma interaction with the vasculature must be understood prior to treating vascularized tissue. We hypothesize that plasma can grow or regress blood vessels in a dose-dependent manner. Endothelial cells control many aspects of the vasculature from vascular tone to coagulation to inflammation. Endothelial cells also play a guiding role in angiogenesis.¹³ Endothelial cells produce and secrete angiogenic growth factors such as fibroblast growth factor-2 (FGF2), which in conjunction with many other signals

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80 induces cells to invade the surrounding tissue, proliferate, and develop into new blood vessels.³² Angiogenesis can be both helpful and harmful. In wound healing, angiogenesis is required at the wound site for healing, whereas in cancer, angiogenesis blockade may prevent tumor growth.¹²

86 Using an *in vitro* model, we investigated the effect of non-thermal DBD plasma on endothelial cells. Endothelial cell proliferation and death following plasma were measured. FGF2 release from endothelial cells and its effect on cell proliferation were quantified. Finally, mechanisms of non-thermal plasma effects were explored. We now show that while high dose non-thermal plasma induces endothelial cell death, lower doses induce endothelial cell proliferation. This proliferative effect is likely related to FGF2 release due to plasma-produced reactive oxygen species.

97 METHODS

98 *Endothelial Cell Culture*

99 Porcine aortic endothelial cells (PAEC) were isolated from porcine aortae by the collagenase dispersion method and used between passages 4 and 9.⁴⁰ PAEC were maintained in low glucose Dulbecco's Modified Eagle's Medium (DMEM) (Mediatech) with 5% fetal bovine serum (Hyclone), 1% L-glutamine, and 1% penicillin-streptomycin (Invitrogen). Medium was changed every two days. For plasma treatment, cells were washed with phosphate buffered saline, detached with 0.1% trypsin (Invitrogen), and seeded near confluence (4×10^5 cells per well) on 18 mm uncoated glass cover slips (VWR) in 12-well plates (Corning). PAEC adhere well to bare glass, with close to 100% seeding efficiency. Cells were incubated for 24 h prior to plasma treatment in 1.5 mL supplemented medium at 37 °C and 5% CO₂.

115 Porcine tumor necrosis factor- α (TNF- α) was from R&D Systems. Recombinant human FGF2 was from Peprotech, and neutralizing FGF2 antibody was from Upstate Biotechnology. *N*-Acetyl-L-cysteine (NAC, Sigma), an intracellular reactive oxygen species (ROS) scavenger and sodium pyruvate (Sigma), an extracellular ROS scavenger, were used to block plasma-produced ROS.

123 *Endothelial Cell Plasma Treatment*

124 Non-thermal DBD plasma was produced using the device in Fig. 1 (Fig. 1a shows the device schematic and Fig. 1b shows the actual device).^{2,17} Plasma was generated by applying alternating polarity pulsed (500 Hz to 1.5 kHz) voltage of ~20 kV magnitude

(peak-to-peak) between the insulated high voltage electrode and the sample using a variable voltage and frequency power supply (Quinta). A 1 mm thick clear quartz was the insulating dielectric barrier covering the 1 in. diameter copper electrode. The discharge gap between quartz and the sample was fixed at 2 mm. The pulse waveform was 20 kV, 1.65 μ s width, with rise time 5 V ns⁻¹ (Fig. 1c). Discharge power density was 0.13 W cm⁻² (500 Hz) and 0.31 W cm⁻² (1.5 kHz) using electrical characterization and a custom calorimetric system.¹ The plasma treatment dose in J cm⁻² was calculated by multiplying the plasma discharge power density by the plasma treatment duration. The non-thermal DBD plasma has a g-factor (number of ROS generated per electron volt or eV) of between 0.3 and 0.5.¹⁴ For a plasma dose of 3.9 J cm⁻², 7.32×10^{16} – 1.22×10^{17} ROS are generated. The values for specific plasma parameters are provided in Fig. 1d.

PAEC on glass cover slips were exposed to plasma for 5–120 s. Each cover slip was removed from the 12-well plate and placed on a microscope slide, which was positioned on the plasma device grounded base. A 50 μ L serum free medium was added to the sample to prevent drying. Following plasma, the cover slip was immediately placed in a new 12-well plate, 1.5 mL supplemented medium was added, and the samples were returned to the incubator.

Three approaches were used for plasma-treatment of cells: direct, indirect and separated. In direct treatment, the sample was one of the electrodes creating the plasma (Figs. 1a and 1b). Plasma discharge occurred between the quartz and the sample, which exposed the sample directly to neutral reactive species and charged particles. For indirect treatment, a grounded mesh was placed between the high voltage electrode and the sample to eliminate charged particles. In separated plasma treatment, medium was plasma treated separately from cells and then immediately applied to cells. In this case, cells were not in direct contact with any plasma component.

170 *Non-Thermal Plasma Induced Cell Death*

171 Non-thermal plasma endothelial cell cytotoxicity was measured via cell counts and Live/Dead assay. For cell counts, PAEC were plasma treated as described. Three and twenty-four hours following plasma treatment, attached (live) cells were trypsinized and counted using a Coulter counter (Beckman Coulter). These time points were selected to examine immediate and medium-term plasma toxicity effects. Since no change was detected between 3 and 24 h, no longer time points were investigated. For the Live/Dead assay, 3 and 24 h post treatment cells were labeled with 1 μ M ethidium

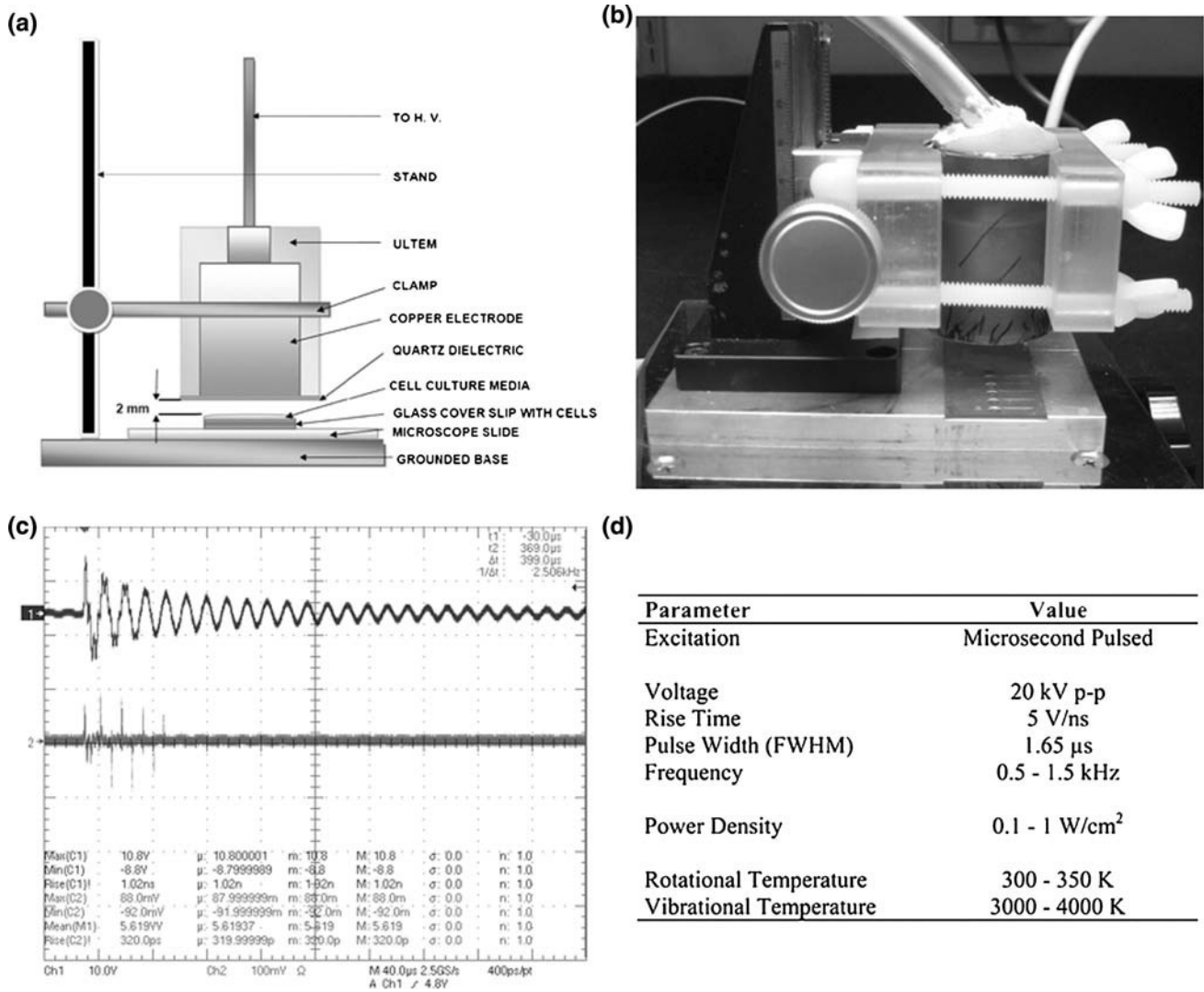


FIGURE 1. DBD plasma (a) schematic and (b) device. (c) Voltage (top) and current (bottom) waveforms. (d) DBD plasma operating parameters.

182 homodimer and 0.25 μ M calcein (Invitrogen), incu- 200
 183 bated at room temperature for 45 min, and imaged 201
 184 by fluorescent microscopy (Olympus, USA) with a 202
 185 digital high performance CCD camera (Diagnostic 203
 186 Instruments). Live cells convert cell-permeant calcein 204
 187 to a FITC fluorescent form via intracellular ester-
 188 ases, whereas cell impermeant ethidium homodimer
 189 binds nucleic acids in membrane damaged dead cells to
 190 enhance TRITC fluorescence. Dead cells were manu-
 191 ally counted in five distinct sample areas.

192 Endothelial cell apoptosis was measured via
 193 annexin V-propidium iodide labeling. Annexin V
 194 binds phosphatidylserine translocated from the inner
 195 to the outer cell membrane. Cells in early apoptosis
 196 are identified as annexin V-positive and propidium
 197 iodide-negative. PAEC were prepared by combining
 198 floating and trypsin-released attached cells. Samples
 199 were centrifuged to pellet cells, washed thoroughly,

resuspended in annexin binding buffer, and labeled 200
 with annexin V-fluorescein and propidium iodide as 201
 per manufacturer instructions (BD Pharmingen). 202
 Samples were analyzed immediately by flow cytome- 203
 try (BD FACScanto). 204

Endothelial Cell Membrane Damage and FGF2 Release 205

Endothelial cell membrane damage following 206
 non-thermal plasma was quantified through lactase 207
 dehydrogenase (LDH) release. PAEC were plasma 208
 treated as described, however DMEM without sodium 209
 pyruvate was used since sodium pyruvate interferes 210
 with the LDH assay. TNF- α (10 ng mL⁻¹) was the 211
 positive control. A 0.5 mL conditioned medium was 212
 collected 2, 4, 6, 8, 12, and 24 h after plasma, and LDH 213
 was quantified using the CytoTox-ONE Homoge- 214
 neous Membrane Integrity Assay (Promega) as per 215

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216 manufacturer instructions. FGF2 release from plasma
 217 treated cells was measured in collected medium 0.5 to
 218 24 h after plasma treatment. FGF2 levels were quan-
 219 tified via FGF ELISA (R&D Systems).

220 *Non-Thermal Plasma Induced Cell Proliferation*

221 Endothelial cell proliferation was measured through
 222 cell counts and BrdU incorporation on treated cells or
 223 using conditioned medium. For cell counts, 10,000
 224 PAEC were seeded on coverslips and plasma treated as
 225 described. Cell number was quantified on days 1, 3, 5,
 226 and 7 by counting trypsin-detached cells using a
 227 Coulter counter, with medium changes on days 2, 4,
 228 and 6. For directly treated cells, fold proliferation was
 229 determined by comparing cell number on day five to
 230 day one. For conditioned medium, confluent PAEC
 231 were plasma treated as described and incubated for 3 h
 232 in 1 mL serum-free DMEM. Conditioned medium was
 233 collected and centrifuged to remove dead cells. A
 234 0.5 mL conditioned medium, along with 1 mL sup-
 235 plemented medium, was added to subconfluent PAEC
 236 (10,000 cells per well) on days 2, 4, and 6 and cell
 237 proliferation was assessed. Conditioned medium from
 238 untreated cells and serum-free medium were controls.
 239 FGF2 effects were blocked by pre-incubating condi-
 240 tioned medium with FGF2 neutralizing antibody
 241 ($1 \mu\text{g mL}^{-1}$) for 30 min prior to adding it to cells.

242 DNA synthesis induced by plasma-treated cell
 243 conditioned medium was determined by BrdU incor-
 244 poration. Thymidine analogue 5-bromo-2-deoxyuri-
 245 dine (BrdU) is incorporated instead of thymidine into
 246 newly synthesized DNA. 10,000 cells per well were
 247 seeded in a 96-well plate in supplemented medium.
 248 Conditioned medium was collected from plasma-
 249 treated cells as described and added to each well in a
 250 1:2 ratio with supplemented medium. After 18 h,
 251 20 μL BrdU labeling solution (Chemicon) was added

to each well for 3 h. Cells were fixed and incubated
 with anti-BrdU conjugated with peroxidase. The
 optical density (450/570), which was directly propor-
 tional to DNA synthesis level, was determined using a
 microplate reader (TECAN).

257 *Statistical Analysis*

258 Statistical analyses were performed with Prism
 259 software (Graphpad). Data were normally distributed
 260 and expressed as the mean \pm SD. Comparisons
 261 between two groups were analyzed by Student's *t* test,
 262 and comparisons between more than two groups
 263 were analyzed by ANOVA. A value of $p \leq 0.05$ was
 264 considered statistically significant and is indicated
 265 with a pound sign (#). $p \leq 0.01$ is indicated with an
 266 asterisk (*).

267 **RESULTS**

268 *Endothelial Cell Proliferation in Response* 269 *to Non-Thermal Plasma*

270 Endothelial cell proliferation is enhanced by low
 271 dose non-thermal plasma treatment. Five days after
 272 treatment, cells treated with plasma showed greater
 273 viable cell number than control up to 30 s of plasma.
 274 PAEC exposed to 30 s of plasma demonstrated twice
 275 as many viable cells as untreated controls (Fig. 2a).
 276 However, plasma beyond 30 s decreased cell number.
 277 A similar increase in cell number with 30 s of plasma
 278 treatment was observed for cells growth on collagen
 279 coated coverslips (data not shown). To determine if
 280 increased cell number was related to a cell-secreted
 281 soluble factor, PAEC were incubated in conditioned
 282 medium from untreated or plasma treated cells
 283 (3.9 J cm^{-2} , 30 s) (Fig. 2b). Serum-free media, which
 284 does not contain soluble growth factors, was the

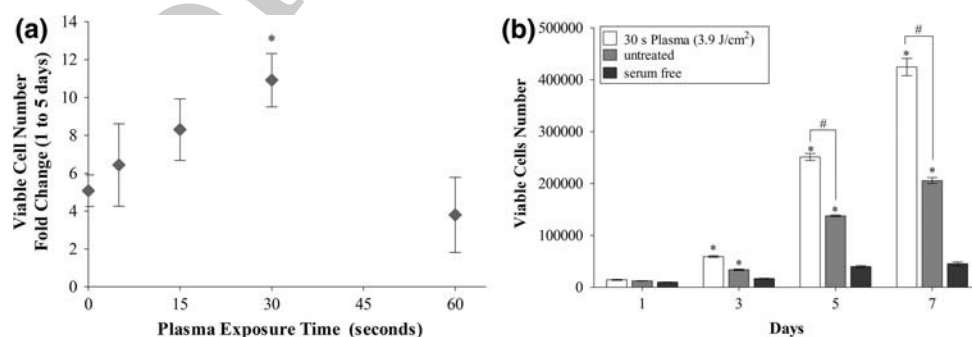


FIGURE 2. Plasma induces endothelial cell proliferation by direct treatment and through conditioned medium from treated cells. (a) PAEC were plasma treated on day 0, and counted on days 1 and 5, with medium changes on days 1 and 3. Data are presented as fold change, since plasma leads to some cell death on day 1. * $p < 0.01$ compared to untreated cells. (b) Conditioned medium was collected after 3 h from untreated or plasma-treated cells and applied to untreated PAEC. Serum-free media, which does not contain soluble growth factors, was the negative control. Cell number was counted with a Coulter counter. * $p < 0.01$ compared to day 1; # $p < 0.01$ comparing untreated cells with 30 s plasma.

285 negative control. Plasma dose was selected based on
 286 maximal observed effect. Viable cell number was
 287 twice as high in cells incubated with plasma-treated
 288 cell conditioned medium on days 3, 5, and 7 compared
 289 to cells incubated with untreated cell conditioned
 290 medium.

291 *Endothelial Cell Death in Response*
 292 *to Non-Thermal Plasma*

293 Decreased viable cell number was observed at high
 294 non-thermal plasma levels; therefore we investigated
 295 endothelial cell death in response to plasma. Plasma
 296 was relatively non-toxic to PAEC up to 60 s. While the
 297 number of live, attached cells decreased as plasma
 298 exposure increased, more than 75% of cells remained
 299 viable up to 60 s plasma (Fig. 3a). There was no sig-
 300 nificant difference in cell viability 3 and 24 h following
 301 plasma exposure, suggesting no long term plasma
 302 toxicity effects. A Live/Dead assay was used to confirm

cell count results. Endothelial cells treated with plasma 303
 for short exposure times (up to 30 s) showed few dead 304
 cells (Fig. 3b, quantified in Fig. 3c), confirming that 305
 plasma is relatively non-toxic at short exposures. Dead 306
 cell number increased with increasing plasma exposure 307
 time ($p < 0.01$ by ANOVA). While dead cell number 308
 increased slightly with 60 s plasma, at 120 s a signifi- 309
 cant number of dead cells and few live cells were evi- 310
 dent. This extensive cytotoxicity may be related to 311
 sample drying. Therefore, 120 s plasma was not used 312
 for subsequent assays. 313

To determine the endothelial cell death mechanism 314
 induced by plasma, PAEC were analyzed 24 h post- 315
 plasma for apoptosis (Fig. 3d). Apoptosis increased 316
 with plasma treatment ($p < 0.01$ by ANOVA). At 30 317
 and 60 s plasma, 20% of cells were apoptotic com- 318
 pared to 10% of untreated cells. At 120 s, nearly 60% 319
 of cells were apoptotic. These data confirm that shorter 320
 plasma exposures are non-toxic, and apoptosis is one 321
 mechanism of plasma-induced cell death. 322

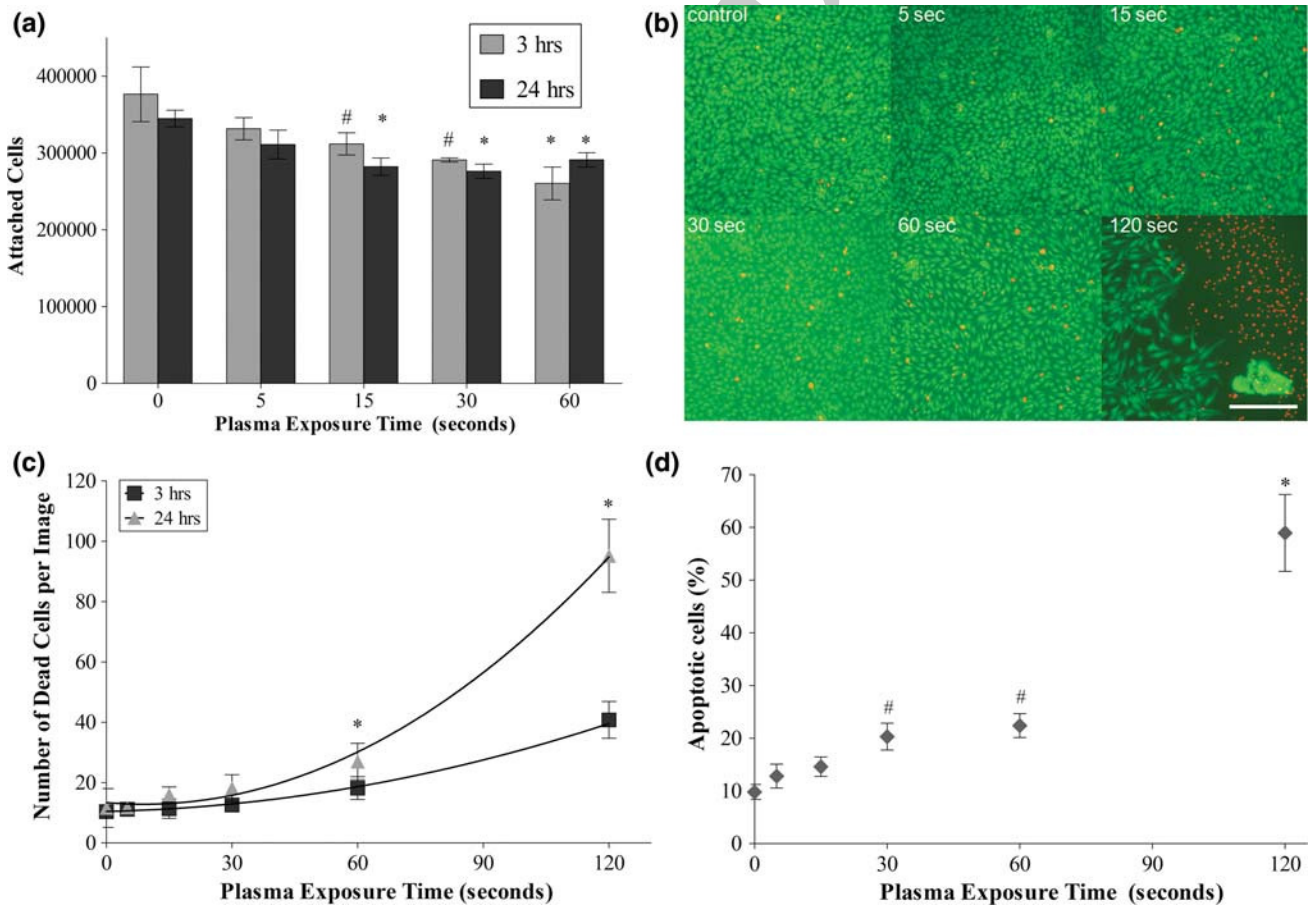


FIGURE 3. Low dose plasma is relatively non-toxic to cells, but high dose induces apoptotic cell death. (a) Attached cells, confirmed as alive by Trypan blue, were counted 3 and 24 h after plasma ($p < 0.01$ by ANOVA). (b) Endothelial cell death was measured by Live/Dead assay. Live cells appear green, whereas dead cells appear red. Scale bar is 200 μm . (c) Quantified Live/Dead images ($n = 5$). (d) Apoptosis was measured by Annexin V-propidium iodide 24 h after plasma.

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Endothelial Cell FGF2 Release Post Plasma

324 We next considered whether FGF2 was released
 325 from endothelial cells following plasma, and whether
 326 released FGF2 contributed to enhanced cell proliferation.
 327 FGF2 has no signal sequence for secretion, and
 328 therefore is primarily thought to be released during
 329 sub-lethal cell membrane damage. Cell-released FGF2
 330 increased up to 3 h after plasma treatment (3.9 J cm^{-2} ,
 331 30 s) and then rapidly decreased up to 24 h after
 332 plasma (Fig. 4a). In contrast, FGF2 levels for cells
 333 treated with 10 ng mL^{-1} TNF- α as a positive control
 334 rose more slowly but continued to rise up to 24 h.
 335 Endothelial cell membrane damage was assessed
 336 by LDH release following plasma. Medium LDH
 337 increased significantly by 4 h after plasma and con-
 338 tinued to rise throughout the first 24 h (Fig. 4b,
 339 $p < 0.01$ by ANOVA), comparable to TNF- α positive
 340 control.

The role of released FGF2 in plasma-enhanced
 endothelial cell proliferation was investigated by
 treating conditioned medium from plasma-treated cells
 with an FGF2 neutralizing antibody to block FGF2
 effects. Serum free media, which does not contain
 FGF2, was the negative control. The FGF2 neutral-
 izing antibody significantly suppressed proliferation in
 PAEC exposed to plasma-treated cell conditioned
 medium (Fig. 4c). Viable cell number for samples
 with FGF2 blocked was similar to untreated cell
 conditioned medium. These data were confirmed
 with a BrdU assay (Fig. 4d). BrdU incorporation was
 enhanced for cells incubated in plasma-treated cell
 conditioned medium, but the FGF2 neutralizing
 antibody abrogated the effect. These data suggest
 that plasma leads to FGF2 release, which contributes
 to enhanced endothelial cell proliferation following
 plasma.

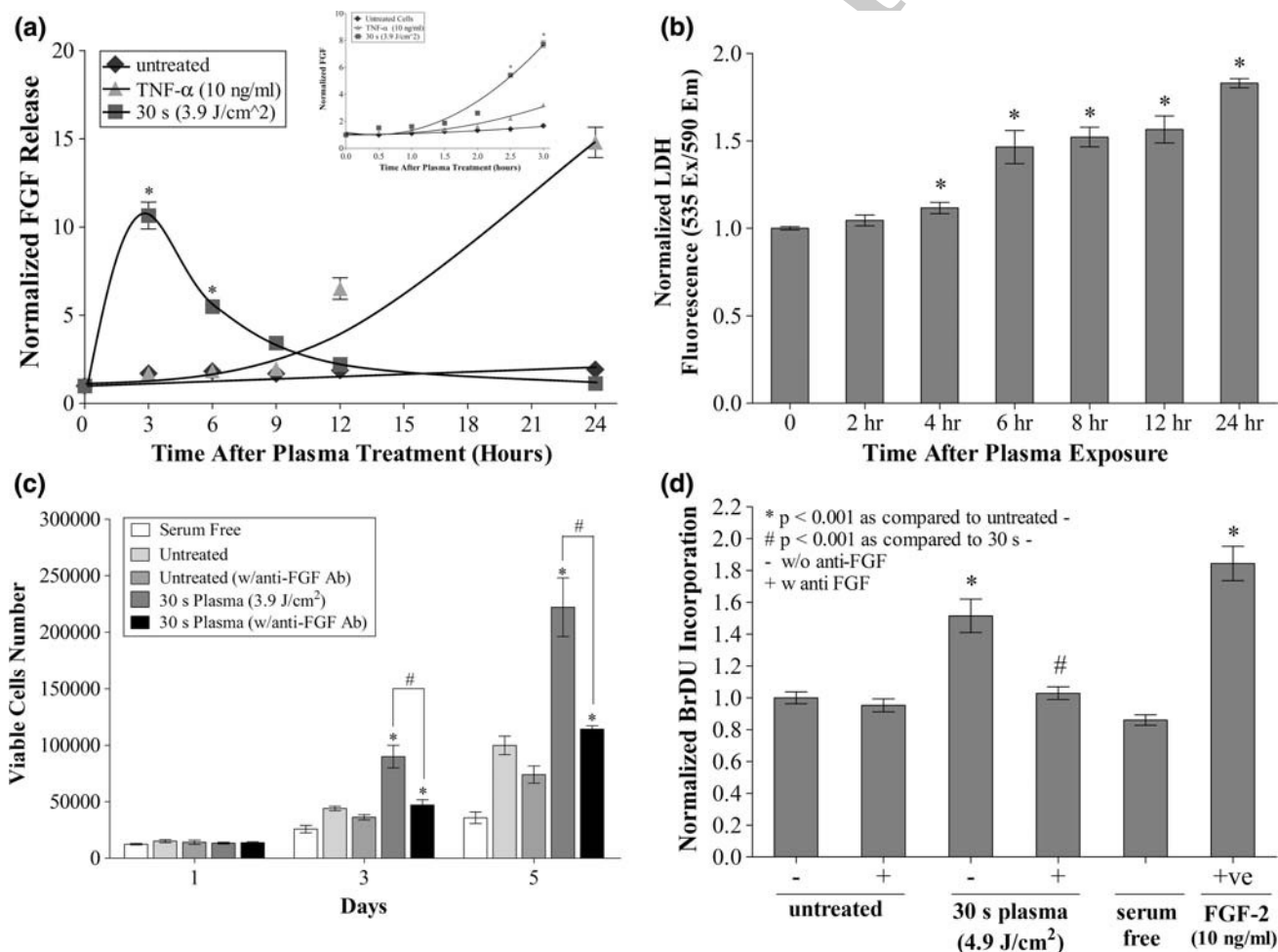


FIGURE 4. Endothelial cells release FGF2 after plasma, and cell-released FGF2 enhances proliferation. (a) FGF2 was quantified in cell medium after plasma by ELISA. Inset shows medium FGF2 up to 3 h after treatment. (b) LDH release was measured in cell medium post-plasma. (c) FGF2 effects were blocked by incubating plasma-treated cell conditioned medium with FGF2 neutralizing antibody. (d) FGF2 blockade reduced DNA synthesis in response to plasma-treated cell conditioned medium, measured by BrdU incorporation. Serum free media, which does not contain FGF2, was the negative control.

359 *Mechanism of Release of FGF2 from Endothelial*
 360 *Cells Following Plasma Exposure*

361 Plasma produces neutral short and long lived reac-
 362 tive species and charged particles like ions and elec-
 363 trons, yet which plasma component led to endothelial
 364 cell FGF2 release was unknown. To better understand
 365 FGF2 release mechanisms, PAEC were exposed to
 366 plasma directly, indirectly, or in a separated configura-
 367 tion. There was no statistically significant difference
 368 in FGF2 release between direct and indirect treatment
 369 (Fig. 5a). Both direct and indirect cell plasma treat-
 370 ment induced significantly greater endothelial cell
 371 FGF2 release as compared to separated treatment,
 372 however separated treatment still produced signifi-
 373 cantly more FGF2 release than untreated control.

374 Non-thermal plasma produces large amounts of
 375 ROS. These ROS may interact with endothelial cells,
 376 leading to FGF2 release. To determine the role of ROS
 377 in plasma-induced cell FGF2 release, endothelial cells
 378 were pre-incubated in 4 mM NAC (intracellular ROS)
 379 and then plasma-treated in supplemented medium with
 380 or without 10 mM sodium pyruvate (extracellular
 381 ROS). Both NAC and sodium pyruvate significantly
 382 suppressed FGF2 release from plasma treated cells
 383 (Fig. 5b), suggesting that intracellular and extracellu-
 384 lar ROS may contribute to plasma effects.

DISCUSSION

385
 386 Non-thermal plasma interacts with the vasculature
 387 during tissue treatment, and plasma may be able to
 388 induce dose-dependent blood vessel growth and
 389 regression. We now show that high dose plasma
 390 induces endothelial cell death, whereas low dose
 391 enhances endothelial cell proliferation. The plasma
 392 proliferative effect is likely related to sub-lethal cell

membrane damage by ROS, which leads to FGF2
 release. FGF2, together with vascular endothelial
 growth factor and other critical signals, induces
 endothelial cell proliferation, migration, and tube
 formation.³² Angiogenesis is a complicated process,
 and endothelial cell proliferation plays only a small
 initial role.¹³ However, our data suggest that low-dose
 plasma could promote angiogenesis to accelerate
 wound healing, whereas high-dose plasma could in-
 hibit angiogenesis to prevent cancer growth. In cases
 where plasma cannot be used directly, fluid from
 plasma treated cells could also be used.

While FGF2 alone will likely not complete the
 angiogenic process, additional subsequent plasma
 treatments could be tuned to induce other angiogenic
 signals. For example, ROS play an important role in
 vascular endothelial growth factor signaling, and
 thermal plasmas that produce nitric oxide could also
 promote angiogenesis. Thus repeated plasma treat-
 ments of different doses or with different plasma types
 could be timed to maximize the angiogenic response.
 During these repeated treatments, plasma effects on
 the surrounding tissue must also be considered. In our
 previous work, high plasma doses did not induce
 gross or histological skin damage in an animal model,
 and malignant epithelial cells were more sensitive to
 plasma-induced apoptosis.^{16,17} These data suggest that
 plasma may induce angiogenesis without harming the
 surrounding tissue, whereas plasma inhibition of angi-
 ogenesis may synergistically kill malignant cells. How-
 ever, since each of these studies were performed under
 slightly different plasma and cell or tissue conditions,
 additional studies should be performed to directly
 compare plasma sensitivity of various cell types.

Non-thermal plasma can induce endothelial cell
 death via the apoptotic pathway. In our previous work
 on ROS and endothelial cells, we showed that low

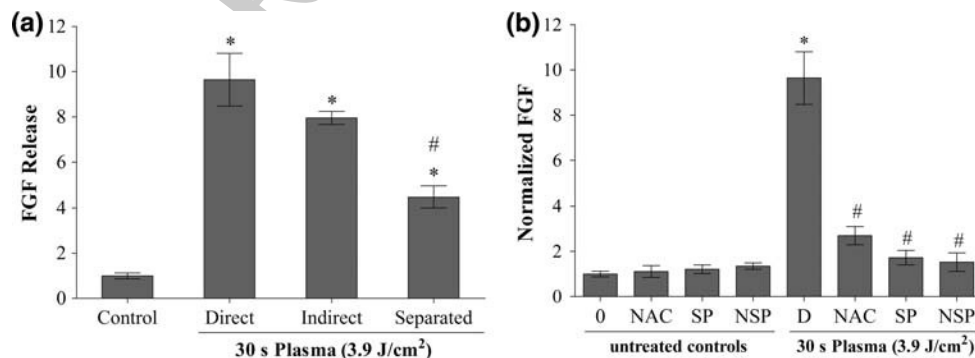


FIGURE 5. Endothelial cell FGF2 release is linked to neutral ROS (a) PAEC were plasma treated directly, with a grounded mesh to remove charged particles (indirect), or medium was plasma treated and then applied to cells (separated). Medium FGF2 was measured by ELISA 3 h after plasma. * $p < 0.05$ compared to direct (b) PAEC were pretreated with 4 mM (NAC, intracellular) or 10 mM sodium pyruvate (SP, extracellular), or both ROS scavengers (NSP). Samples were directly plasma treated, and cells pretreated with ROS scavengers were compared to cells directly treated with plasma alone (D). Medium FGF2 was quantified by FGF ELISA 3 h after plasma.

430 ROS levels induce sub-lethal cell membrane damage,
 431 higher ROS levels induce apoptosis, and extremely
 432 high ROS levels induce non-specific cell death which is
 433 likely necrosis.³⁰ Our data suggest that plasma dose
 434 can similarly be used to modulate the cell death
 435 mechanism, which is an important consideration both
 436 *in vivo* and *in vitro*. Apoptosis is programmed cell
 437 death initiated by physiological or pathological signals.
 438 Apoptotic cells are broken up into apoptotic bodies,
 439 which are engulfed by neighboring cells, leading to
 440 clean cell death without significant inflammatory
 441 response.^{11,29} On the contrary, necrosis is cell death
 442 accompanied by swelling, blebbing and increased
 443 membrane permeability leading to cytosolic content
 444 spillage. This typically leads to inflammation in sur-
 445 rounding tissue.^{11,29} By controlling plasma dose, we
 446 may be able to kill endothelial cells without significant
 447 necrosis and subsequent inflammation.

448 Plasma induces endothelial cell FGF2 release.
 449 FGF2 is thought to be released only at cell injury or
 450 death, since it has no signal sequence for secretion.³²
 451 Since plasma effects occur shortly after treatment,
 452 plasma may induce sub-lethal endothelial cell mem-
 453 brane damage, rendering the cells leaky to intracellular
 454 contents like FGF2. Other stimuli which induce cell
 455 membrane damage lead to FGF2 release. Biochemical
 456 changes, such as high glucose, enhance FGF2 release
 457 also through ROS.³⁰ Transient plasma membrane
 458 disruption by mechanical forces leads to rapid cyto-
 459 solic FGF2 release. This FGF2 release initiates growth
 460 required for tissue integrity maintenance and/or repair
 461 after injury.³¹ Mechanical strain also stimulates a
 462 proliferative response in coronary vascular smooth
 463 muscle cells via FGF2 release, and strain can even
 464 enhance endothelial cell FGF2 mRNA expression.^{28,36}
 465 *In vivo*, FGF2 released into the coronary circulation
 466 after vascular injury promotes human vascular smooth
 467 muscle cell proliferation.⁴

468 Similar to mechanical damage, cell membrane
 469 injury from ionizing radiation induces FGF2 expres-
 470 sion and release in endothelial and epithelial cells
 471 *in vitro*^{5,23,24} and *in vivo*.⁴¹ FGF2 enhances endothelial,
 472 epithelial, and hematopoietic cell survival after ionizing
 473 radiation,^{18,19,22,23} and FGF2 release is critical to
 474 radiation damage repair.²³ Pulsed electromagnetic
 475 fields can stimulate endothelial cell growth, angiogen-
 476 esis, and wound healing through endogenous FGF2
 477 release.^{3,37,42} Non-thermal plasma differs from irradi-
 478 ation and electromagnetic fields in that the latter are
 479 penetrating and injure surrounding tissue, or they need
 480 an expensive setup to be generated safely. Plasma
 481 provides a novel and safer means to induce FGF2
 482 release and angiogenesis since it provides precise con-
 483 trol of treatment area and depth. Non-thermal plasma
 484 devices are also small and relatively simple to produce.

485 When endothelial cells are exposed to plasma, the
 486 conditioned medium FGF2 level peaks three hours
 487 after treatment and then declines. In contrast, cells
 488 treated with TNF- α show a gradual increase in med-
 489 ium FGF2. Thus plasma FGF2 release kinetics are
 490 essentially different from TNF- α . One possible reason
 491 is that TNF- α remains in the medium continuously,
 492 whereas plasma treatment occurs over a short, finite
 493 time. An alternative is that while both plasma and
 494 TNF- α likely release FGF2 related to ROS, TNF- α
 495 takes longer to produce ROS. While TNF- α activates a
 496 variety of biochemical signaling pathways in endothe-
 497 lial cells, the most likely path for TNF- α FGF2 release
 498 is cell membrane damage, since FGF2 has no known
 499 signal sequence for secretion. TNF- α must bind the
 500 membrane-bound TNF receptor, which activates
 501 intracellular signaling cascades leading to ROS.^{33,39}
 502 Thus the indirect and extended FGF2 release from
 503 TNF- α cell damage differs greatly from the finite and
 504 direct nature of plasma-induced rapid FGF2 release.
 505 The released FGF2 may then be bound by remaining
 506 viable cells, which explains the drop in medium FGF2
 507 level after 3 h and enhanced endothelial cell prolifer-
 508 ation. This brief and defined FGF2 release may be
 509 critical to angiogenesis, since timing and local bio-
 510 chemical environment play important roles in FGF2
 511 signaling.⁶

512 Both FGF2 and LDH are released from cells due to
 513 cell membrane damage, yet in our experiments, FGF2
 514 and LDH release from plasma treated cells followed
 515 different trends. Whereas FGF2 release peaked 3 h
 516 post-plasma and then declined, LDH release became
 517 significant only 4 h after plasma treatment but then
 518 increased up to 24 h. This difference may be related to
 519 the relative LDH (134 kDa) and FGF2 (18 kDa) sizes.
 520 FGF2 may be released after early sub-lethal plasma
 521 membrane damage, whereas LDH release occurs
 522 gradually after more extensive cell membrane damage.
 523 Additionally the gradual post-plasma LDH release
 524 may indicate that non-thermal plasma does not lead to
 525 immediate irreversible membrane integrity loss nor-
 526 mally associated with severe trauma or cell death.
 527 Viable cells release low LDH amounts without ham-
 528 pering cell function.⁹ LDH also has no extracellular
 529 function, whereas FGF2 binds to cell membrane
 530 receptors, thus cell-released LDH is not metabolized
 531 by other cells whereas FGF2 is. This correlates with
 532 FGF2 decrease up to 24 h after plasma. Non-thermal
 533 plasma may lead to sub-lethal membrane damage
 534 which is gradually repaired as living cells uptake
 535 released FGF2 and remain viable.

536 Plasma-induced FGF2 release is likely related to
 537 neutral ROS. Non-thermal plasmas produce long lived
 538 (O_3 , NO, HO_2 , H_2O_2) and short lived (OH, O, elec-
 539 tronically excited O) neutral particles and charged

540 particles (ions and electrons). Both charged and neu- 595
 541 tral particles can lead to ROS in treated fluid. When 596
 542 endothelial cells were treated directly or indirectly 597
 543 (excluding charged particles), endothelial cell FGF2 598
 544 release was not significantly different. However, FGF2 599
 545 release decreased in separated treatment, in which 600
 546 medium was treated prior to applying it to cells. The 601
 547 time required to collect separately treated medium and 602
 548 apply it to untreated cells eliminated short lived neutral 603
 549 species and direct contact between plasma and cells. 604
 550 Direct plasma effects could include local heating by 605
 551 plasma streamers or UV radiation. Since separated 606
 552 treatment decreased FGF2 release by 50%, and FGF2 607
 553 release remained significantly greater than in control 608
 554 cells, we believe that both short and long lived neutral 609
 555 species play a major role in plasma-induced FGF2 610
 556 release. While a wide variety of plasma-produced ROS
 557 could affect cells, both atomic and singlet oxygen are
 558 short lived and therefore highly likely to recombine
 559 before reaching the sample surface during treatment.
 560 The plasma-produced ROS most likely to contribute to
 561 endothelial cell FGF2 release are OH radicals, hydro-
 562 gen peroxide, and HO₂.

563 Non-thermal plasma produces a large ROS con-
 564 centration in extracellular medium during treatment.
 565 However, it is unclear if these ROS go inside cells.
 566 Both intracellular and extracellular ROS scavengers
 567 decreased FGF2 release following plasma. Combined
 568 scavengers reduced FGF2 release more than either
 569 scavenger alone. ROS produced by plasma extracel-
 570 lularly may move across the cell membrane through
 571 lipid peroxidation, opening transient cell membrane
 572 pores, or signaling pathways which modify ROS inside
 573 cells. Active species produced by plasma may also
 574 modify the cell medium, which in turn interacts with
 575 cells. Since many of active species have a short life
 576 span, they may immediately interact with medium
 577 components including amino acids and proteins,
 578 leading to production of long lived reactive organic
 579 hydroperoxides.²⁰ These hydroperoxides may then
 580 induce lipid peroxidation and cell membrane damage,
 581 or they may bind to cell membrane receptors and
 582 activate intracellular signaling pathways leading to
 583 FGF2 expression and release.

584 We believe that non-thermal plasma could be used
 585 *in vitro* and *in vivo* to stimulate angiogenesis. Potential
 586 plasma applications include vascularizing tissue engi-
 587 neering structures, enhancing transplanted tissue
 588 incorporation, and accelerating wound healing. Our
 589 two-dimensional treatment model—an endothelial cell
 590 monolayer on a glass substrate covered with a thin
 591 medium film (~100 μm)—is likely more severe than
 592 what would be experienced by cells either *in vivo* or in
 593 three-dimensional *in vitro* models. Both sample geom-
 594 etry and the amount of liquid covering the sample are

crucial to plasma treatment efficacy. We previously 595
 showed that increasing media depth over malignant 596
 epithelial cells decreased plasma-induced cell death.¹⁷ 597
 ROS are highly reactive and may be inactivated prior 598
 to reaching cells if the distance between the plasma and 599
 the cells is too great. In these situations, a higher 600
 plasma dose could be used to maintain plasma effects. 601
 In the future, we will examine plasma penetration 602
 depth variation with environmental conditions by 603
 treating endothelial cells within three-dimensional 604
 collagen gels. We observed similar plasma-induced 605
 proliferation results for cells seeded on uncoated and 606
 collagen-coated substrates in two dimensions, sug- 607
 gesting that plasma effects will be similar in a more 608
 tissue-like environment. 609
 610

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 614

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