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Risk assessment of a cold argon plasma jet in respect to its mutagenicity

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ABSTRACT

Cold atmospheric pressure plasmas represent a favorable option for the treatment of heat sensitive materials and human or animal tissue. Beneficial effects have been documented in a variety of medical conditions, e.g., in the treatment of chronic wounds. It is assumed that the main mechanism of the plasma's efficacy is mediated by a stimulating dissipation of energy via radiation and/or chemical energy. Although no evidence on undesired side effects of a plasma treatment has yet been presented, skepticism toward the safety of the exposure to plasma is present. However, only little data regarding the mutagenic potential of this new treatment option is available. Accordingly, we investigated the mutagenic potential of an argon plasma jet (*kinpen*) using different testing systems in accordance with ISO norms and multiple cell lines: a HPRT1 mutation assay, a micronucleus formation assay, and a colony formation assay. Moderate plasma treatment up to 180 s did not increase genotoxicity in any assay or cell type investigated. We conclude that treatment with the argon plasma jet *kinpen* did not display a mutagenic potential under the test conditions applied and may from this perspective be regarded as safe for the use in biomedical applications.

1. Introduction

A broad variety of cold plasma sources have been developed and investigated for medical or life science use [1]. Especially the field of plasma medicine experienced a boost in the last few years, fueled by the hope to improve treatment options for skin care, in dentistry, or cancer [2–4]. Recent developments also include the treatment of food to preserve quality or reduce microbial load [5,6]. Consequently, fundamental and applied research leads to a growing body of information on biological effects of cold atmospheric pressure plasmas [7,8]. A common feature of these plasmas is the dissemination of energy; via radiation (ultraviolet and visible light, radio waves etc.) and via chemical entities (electrons, ions, molecules), showing synergistic behavior [9].

Two plasma sources widely used in fundamental and applied research are the *kinpen* [10] and the similar *kinpen med* with an improved biocompatibility [11,12]. These argon plasma jets are characterized by an emphasis on oxygen derived species production. Among them, hydroxyl radicals ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), ozone (O_3), and superoxide anion radicals ($^{\bullet}O_2^-$) dominate over atomic oxygen (O) [13]. Nitrogen containing species like nitric oxide (NO), nitrite/nitrate (NO_2^-/NO_3^-), are less abundant but present [14]. Its emission of UV radiation (mainly UVB) has been characterized and was regarded as safe [10,15,16]. Some of these chemical entities have the capability to interact with organic molecules or are second messengers (nitric oxide, hydrogen peroxide) [17]. This potential may represent a threat by interacting with biological macromolecules. It has been shown for different sources and cell lines that plasma can indeed affect nucleic acids. For the *kinpen*, DNA single strand breaks have been observed [18]. The DNA alteration appears to be transient and cellular repair mechanism succeeded within 24 h after exposure. In accordance with this, exposure to plasma in vivo did not reveal adverse effect in animals or in small clinical cohorts in humans [19–21].

With respect to limited treatment times and under consideration of the body of evidence existing to the present date it may hence be assumed that the usage of a *kinpen* can be regarded as safe. However, a dedicated study addressing a potential muta-

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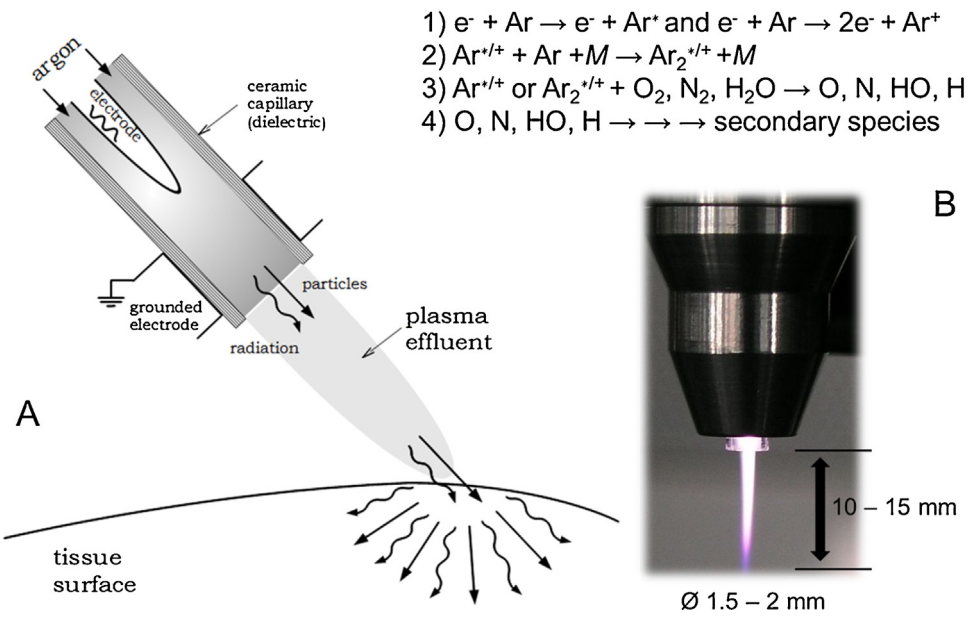

Fig 1. Schematic (A) and side view (B) of *kinpen* plasma source. Close to the pin electrode, the feed gas argon is excited/ionized at the centered pin electrode by high frequent high voltage (1) and forms excimers ($Ar_2^{+/*}$, 2). In the effluent, excited Argon species react with ambient species from the air, forming atomic oxygen and nitrogen (3). In secondary reaction, these create further reactive species (e.g., NO, H_2O_2) which diffuse into tissue or liquids. The tip of the effluent has a bulk temperature of about 30 °C.

genic effect of this plasma source has not been published so far. In accordance with the DIN EN ISO 10993-3:2015-02 recommendations for testing the biocompatibility of medical products, we here investigated the mutagenic potential of the plasma of the *kinpen* by applying the hypoxanthine phosphoribosyltransferase (HPRT1) mutation assay [22,23] and the detection of chromosome breakage (micronucleus assay) [24,25]. Additionally, a 3D-colony forming assay (clonogenicity assay) was applied to estimate the role of plasma on anchorage independent growth of cells in soft agar.

For the *kinpen* and the plasma generated by it, none of these testing systems have yet been applied to investigate its mutagenic potential. The result – no evidence of mutagenicity in any system – thus provides a valuable asset to the risk assessment of this promising argon plasma jet and its derivatives.

2. Materials and methods

2.1. Cell culture

Chinese hamster lung fibroblasts (V79) were obtained from CLS (Eppelheim, Germany), HaCaT human keratinocytes and MRC5 fibroblasts from DSMZ (Braunschweig, Germany). SK-Mel-147 cells were a kind gift from Dr. M. Soengas (Dept. of Dermatology, University of Michigan). All cell culture reagents were purchased from Lonza (Verviers, Belgium) except fetal calf serum (FCS, Sigma Deisenhofen, Germany). Cell lines (V79, HaCaT, MRC5) were cultivated under standard conditions in Roswell Park Memorial Institute 1640 medium (RPMI) containing L-glutamine (2 mM), antibiotics (100 IU/mL penicillin; 100 μg/mL streptomycin), and FCS (V79 5%, HaCaT 8%, MRC5 12%). SK-Mel-147 cells were cultivated in DMEM under elsewise identical conditions.

2.2. Plasma source and treatment

In this work, an atmospheric pressure argon plasma jet (*kinpen*; neoplas, Germany) was used (Fig. 1). It houses two electrodes: a rod electrode (1 mm diameter) in the center of a ceramic capillary (1.6 mm inner diameter) and a grounded electrode surrounding the dielectric capillary. The plasma is generated by high frequency sinusoidal voltage of around 1 MHz which is applied to the centered electrode. As feed gas, 3 standard liters per minute (slm) argon (argon N50, Air Liquide) was used.

Indirect plasma treatment was achieved by the following routine: five milliliters of complete cell culture medium were placed into a 60 mm Petri dish and treated with the plasma device moving in meanders, the effluent just reaching the liquids surface. After treatment the medium was immediately (within 30 s) transferred to cell monolayers prepared in advance in appropriate cell culture vessels. In some instances, a direct treatment was achieved by treating a cell suspension in RPMI medium (2×10^5 cells mL^{-1}). After treatment, this cell suspension was used to seed and cultivate the cells for experiments.

2.3. Detection of ROS and RNS deposited by the plasma treatment

Most plasma generated species are short lived and hard to assess. As a representative, hydrogen peroxide deposition in cell culture medium under experimental conditions (see Section 2.2) was measured using titanium(IV) oxysulfate reagent according to the manufacturers protocol (Sigma, Deisenhofen, Germany). Nitrite and Nitrate as stable products of the reactive nitrogen species were quantified using ion chromatography. Briefly, anion chromatography was performed on an ICS5000 system (Thermo, Dreieich, Germany) using a Dionex IonPac A623 column (2 mm × 250 mm) and microbore isocratic flow with AS23 sodium carbonate eluent. Ions were detected by conductivity and UV absorption (206 nm). Calibration was performed using Thermo's 7-ion standard and dilutions thereof.

2.4. HPRT1 mutation assay and seeding efficacy assay procedure

To find applicable treatment times (plasma jet, UV light) or concentration (H_2O_2) a toxicity assay was performed. Into 60 mm Petri dishes 300 V79 cells were seeded using complete RPMI. After 24 h, cells were washed and incubated with plasma treated FCS-free RPMI (10–300 s), FCS-free RPMI containing H_2O_2 (5–80 μM), or irradiated by broadband UVB (20–160 $J_{eff}\,m^{-2}$,

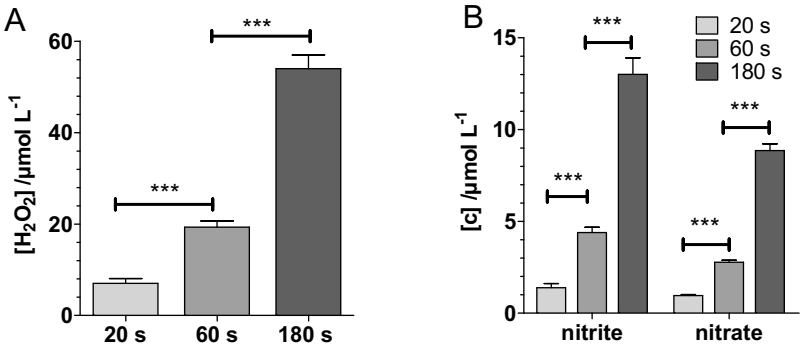

Fig. 2. Deposition of hydrogen peroxide (A) or nitrite/nitrate ions (B) by the plasma device in liquids under the experimental conditions used throughout the study. Data obtained by (A) colorimetric assay or (B) ion chromatography. Data represent mean of two independent experiments with three technical replicates each + SD. Statistical analysis was performed by Student's *t*-test (two tailed). *p*-value <0.001 in all tested cases (***).

Table 1
Micronucleus frequencies detected in HaCaT cells under the respective treatment and cytochalasin B cytokinesis block. Four independent experiments with 2 replicates each. Data show mean + SD. Statistical analysis was performed by means of two-tailed Student's *t*-test.

	No. of MN/1000 binucleated cells	Standard deviation (S.D.)	*p*-Value/significancelevel	Cytokinesis block proliferation index (CBPI)
Control	41	11.5	–	1.87
Plasma 20 s	38	7.2	0.68 ns	1.91
Plasma 60 s	43	8.9	0.76 ns	1.8
Plasma 180 s	40	7.4	0.94 ns	1.71
UV 10 mJ cm^{-1}	56	4.6	0.04*	1.67
UV 20 mJ cm^{-1}	63	4.5	0.006**	1.61
UV 30 mJ cm^{-1}	49	1.5	0.45 ns	1.3
Mitomycin 1.5 µM	135	10.3	<0.0001***	1.67

Philips TL12 fluorescent lamps). UVB irradiation was normalized to biological effective irradiation according to the published method from International Commission on Non-Ionizing Radiation Protection (ICNIRP) [26]. After one hour, medium was exchanged for complete cell culture medium and cell left undisturbed for 6 days. Then, number of colonies was counted using Giemsa stain solution. The re-seeding efficacy of cells from expression phase was estimated similarly.

For mutation assay, V79 cells (3×105) were seeded into 60 cm^2 Petri dishes using complete RPMI. After 24 h, medium was exchanged with the treated RPMI medium as described above. Hydrogen peroxide (25 µM or 80 µM), 15 mM 1-methylsulfonyloxyethane (EMS), or 20–60 $J_{eff}\,m^{-2}$ broadband UVB (Philips TL12 fluorescent lamps) served as experimental controls. After one hour of incubation, treated/supplemented RPMI was removed and cells were cultivated in complete DMEM for 7 days (expression phase). After counting, cells were seeded at 1×106 cells per 75 cm^2 cell culture flask in selection medium (complete DMEM containing 9 µg mL^{-1} 6-thioguanin; selection phase). Medium was exchanged every 3–4 days. After 10 days, cells were washed, fixated, stained, and counted. A maximum of 5 passages after initial seeding were allowed in order to keep spontaneous mutation rate low as described in the standard procedure of the HPRT1 assay [27].

2.5. Micronucleus assay

The micronucleus assay detects acentric DNA fragments and chromosomes lost during mitosis [25]. An epithelial cell line was used instead of peripheral blood leukocytes [28]. This procedure has been used previously and with good results while avoiding the use of mitogens [29]. Briefly, HaCaT cells were seeded at 9000 cells cm^{-2} in complete medium. After 48 h, cells were treated with plasma (20–180 s), broadband UVB (20–60 $J_{eff}\,m^{-2}$, Philips TL12 lamps), or mitomycin C (Alexis, Lausen/A, 1.5 µM) in culture medium containing 4 µM cytochalasin B (Axxora, Lörrach, D). After 24 h incubation cells were fixated and stained by 5% Giemsa (Merck, Darmstadt, D) in PBS for 30 min. After drying, cells were embedded in DPX mount (Sigma–Aldrich, Steinheim, D). Cover slides were randomized and 1000 binucleated cells per sample were analyzed for the presence of micronuclei (Observer Z.1; Carl Zeiss, D). Four independent experiments were performed and data were analyzed using the two tailed Student's *t*-test.

2.6. Soft agar assay for colony formation (CFA)

Colony formation in soft agar indicates the ability of anchorage independent growth. SK-MEL-147, HaCaT, and MRC5 cells were incubated with plasma-treated medium as described above for two hours. Then, 3000 cells of each sample were transferred to an agar-medium mixture (0.35% agar) as a sandwich between bottom agar mixture (1%) and top agar (0.5%). After 30 days of incubation at 37 °C the cell-agar mixtures were stained with 500 µL of 0.005% crystal violet and the number of colonies was evaluated microscopically.

3. Results

3.1. ROS and RNS species determination

As indicated in Fig. 1, nonthermal plasmas generate reactive species with usually short life times. As a representative stable product, hydrogen peroxide deposition during treatment was measured. The amount of deposited H_2O_2 correlated with the treatment time. After 3 min of treatment, up to 58 µmol L^{-1} H_2O_2 was detected (Fig. 2A). Nitrite and nitrate ions are frequently found as an indicator for reactive nitrogen species chemistry. Using ion chromatography, treatment time dependent concentrations of up to 12.5 µmol L^{-1} (nitrite ions) and 8.5 µmol L^{-1} (nitrate) were found (Fig. 2B).

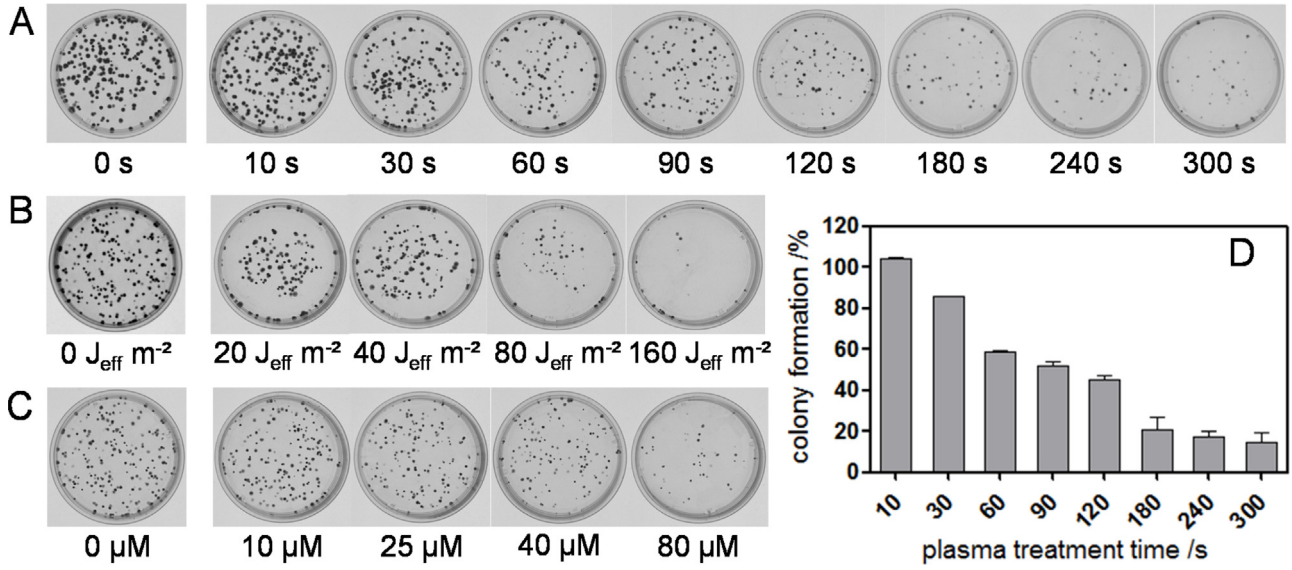

Fig. 3. Survival and colony forming efficacy reveals susceptibility of V79 cells against indirect plasma treatment (A), broadband UVB (B), and hydrogen peroxide (C). Bar chart (D) displays percentage (arithmetic mean + S.D.) of cell survival after indirect plasma treatment (A).

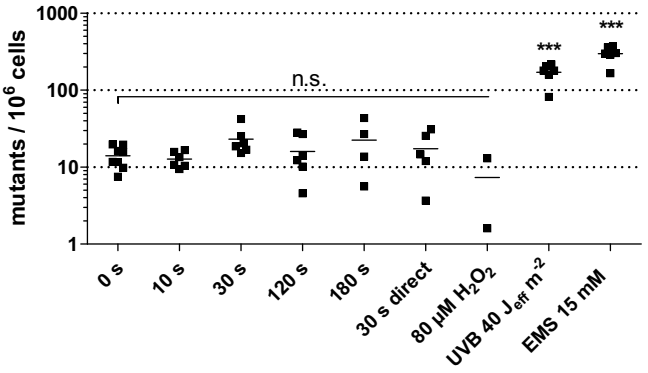

Fig. 4. Results of HPRT mutation assay. V79 cells were exposed to various agents, incubated for 3 weeks, and mutated cells were enumerated. Both, positive control with chemical agents (EMS) and UV-light treatment showed a significantly ($p < 0.001$; ***) increased mutation rate. The number of mutants in cells exposed to plasma or H_2O_2 did not differ significantly from untreated controls ($p = 0.786$). Data represent mean of two to four independent experiments with three to four technical replicates each. Statistical analysis was performed by ANOVA (Tukey HSD).

3.2. HPRT1 assay in V79 cells

Preliminary to the HPRT1 mutation assay cell survival and proliferation were investigated after a one-hour challenge with the respective agents (Fig. 3). Plasma treatment decreased the total numbers of colonies with increasing exposure time (Fig. 3A and D). Treatment times greater than 300 s completely prohibited colony formation (data not shown). H_2O_2 concentrations above 25 µM reduced cell proliferation and above 100 µM completely inhibited colony formation. Hence, 80 µM H_2O_2 was chosen as positive control for the HPRT1 assay (Fig. 3B). UVB irradiation between 20 $J_{eff}\,m^{-2}$ and 160 $J_{eff}\,m^{-2}$ increasingly reduced cell proliferation. Consequently, 40 $J_{eff}\,m^{-2}$ UVB was used as positive control (Fig. 3C).

In the HPRT1 assay, untreated cells were found to have a spontaneous mutation frequency of 14×10^{-6} (Fig. 4), a typical result [30,31]. With 1-methylsulfonyloxyethane (EMS), the number of mutated cells significantly increased to 288×10^{-6}. When cells were challenged with 40 $J_{eff}\,m^{-2}$ UVB radiation, a mutation frequency of 170×10^{-6} was observed. These results clearly indicated the genotoxic nature of both control treatments. Hydrogen peroxide did not show an increase in mutation frequency (7.3×10^{-6}). Likewise, the number of mutants after exposure to indirect plasma treatment ($12.7–22.0 \times 10^{-6}$) did not differ significantly from untreated control. For 30 s of direct plasma treatment the same observation was made (17.3×10^{-6}).

3.3. Micronucleus assay

Micronuclei, chromosomal fragments lacking a centromere unit were detected as small, DNA containing particle (Fig. 5B and C). Mostly, one micronucleus per binucleated cell was observed (Fig. 4A; Table 1). Under harsh conditions, number of micronuclei per cell increased. In our treatment regimen, indirect jet plasma (between 20 s and 180 s) did not increase the number of nuclei as compared to untreated control cells (4.0% vs. 4.1%). After exposure to UVB (20–60 $J_{eff}\,m^{-2}$) significantly more mutations were detected (up to 6.3%). Treatment with mitomycin C significantly increased percentages of cells displaying various numbers of micronuclei (13.5%).

3.4. Clonogenic assay

Finally, the clonogenic capacity of three different cell lines was tested after plasma treatment (Fig. 6). Exposure to plasma did not increase colony formation or colony size of non-cancer cell lines (MRC5, HaCaT) which indicates that anchorage independent growth has not been triggered by the treatment. In contrast, colony formation of SK-Mel-147 cells was distinctive even under control conditions (0 s plasma treatment). Importantly, plasma did not support but diminished growth of these cells which is in line with previous findings [32].

4. Discussion

Cold plasma possesses a therapeutic potential in various pathologies but its safety is not fully established yet. We therefore tested the mutagenicity of a widely used argon plasma jet (*kinpen*) according to official normative DIN EN ISO 10993-3:2015-02. The *kinpen* (Fig. 1A) is a pre-clinic device designed for the treatment of biological samples. It is intensively studied and is similar in construction and plasma generation to the *kinpen med* (e.g.,

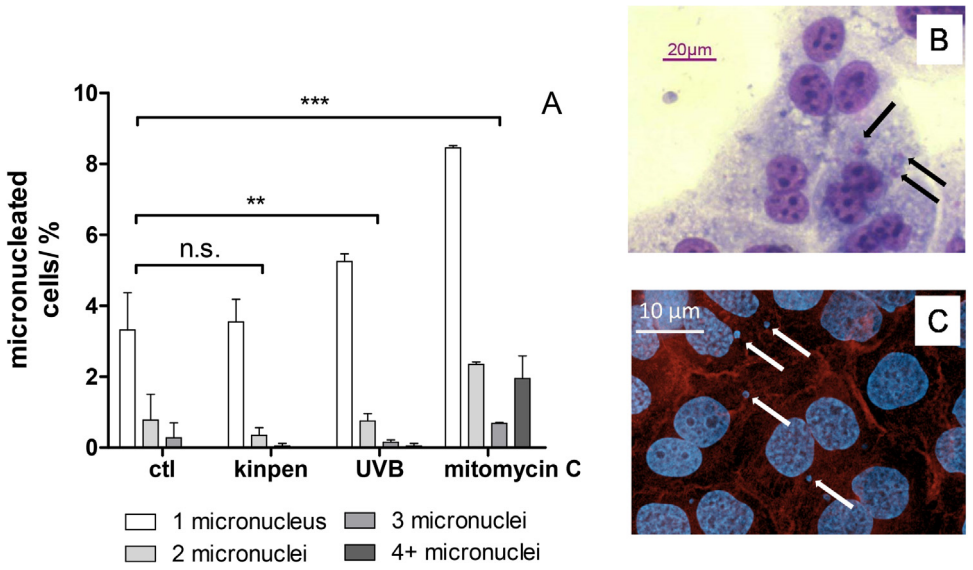

Fig. 5. Micronucleus assay using HaCaT keratinocytes. UVB treatment (40 J_{eff} m^{-2}) and mitomycin C (1.5 µM) but not plasma (180 s) showed a significant increased number of micronuclei compared control (A). Images show microphotographs of binucleated cell containing micronuclei (arrows) using Giemsa stain (B) or DAPI (C). Two independent experiments with 4 replicates each. Data show mean + SD. Statistical analysis was performed by means of two-tailed Student's *t*-test. $^{**}p < 0.01$; $^{***}p < 0.001$.

	SK-Mel-147	MRC5	HaCaT
0 s	+++	+	-
20 s	++	+	-
60 s	+	+	-
180 s	+	-	-
240 s	+	-	-
300 s	+	-	-

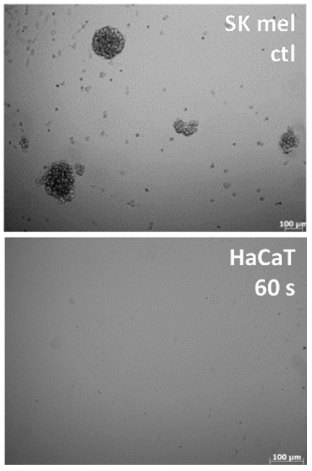

Fig. 6. Clonogenicity assay. Formation of clones (+++), small clone (++), or single cell survival (+) was followed for 30 days. Microphotographs show clones of SK-Mel-147 cells (control, top) and no detectable clones in HaCaT cells (60 s plasma, bottom) after 30 d of incubation. Results were evaluated by scoring of 2 independent investigators (no visible cells/clones (−), slightly visible clones (+), visible clones (++), clearly visible clones (+++)). No evidence for anchor independent growth was observed. All experiments were performed at least twice.

dimensions, antimicrobial efficacy, energy dissipation, and effluent composition), which is specifically designed for clinical applications.

In this work and using different assays and cell lines, we neither found a mutagenic action of the *kinpen* plasma nor liquids treated by it. Presumably, comparable results would be obtained with the *kinpen med* due to the almost identical plasma properties. A similar result was published for a different plasma device (a dielectric barrier discharge plasma in air), indicating the general plausibility of the results presented [33]. Others however reported an increase of micronucleus formation after a dielectric barrier discharge treatment of TG98 glioma cells in air [34]. The plasma source used for this study operates at higher power and creates huge amounts of reactive species which then led to apoptotic nucleus fragmentation potentially interfering with micronucleus detection as is suggested by the figures published. Further, no experimental controls had been performed as recommended by the micronucleus assay working group [35].

Various reactive components are being generated by the ignited plasma jet. In the gaseous effluent of the *kinpen* especially oxygen dominated reactive species prevail, including hydroxyl radicals ($^{\bullet}OH$) and the corresponding hydrogen peroxide (H_2O_2), ozone (O_3), superoxide anion radicals ($^{\bullet}O_2^-$), and traces of atomic oxygen (O) [13,36–38]. In open systems as described here, ozone is almost insoluble and failed to show an impact even at high concentrations [39,40]. The other species contribute to the presence of oxygen species in the liquid, e.g., H_2O_2. Less abundant but present in the effluent are nitrogen reactive species like nitric oxide (NO) which lead to the formation of nitrite/nitrate (NO_2^-/NO_3^-) in the liquid [14]. They do have effects in their own right in signaling but do not directly interfere with DNA fidelity [41].

Immediately after the treatment the presence of ROS was detected as hydrogen peroxide (Fig. 2A) and the presence of RNS as nitrite/nitrate ions (Fig. 2B). The results presented suggest that within the given exposure time their reactivity, half-life time, and/or concentration in the liquid was not sufficient to have muta-

genic effects. We could not detect remaining ROS after one-hour incubation with mammalian cells under experiment conditions. For HaCaT cells we recently showed the activation of the antioxidant Nrf2 signaling pathway, indicating an improved anti-oxidant potential after plasma treatment [42]. In parallel, 80 μM H_2O_2 failed to show a mutagenic effect in the HPRT1 assay, a concentration which is generated by the *kinpen* in 240 s of treatment [43]. However, at this concentration cell proliferation rate is reduced markedly (Fig. 3A). Higher concentrations of H_2O_2 (>750 μM) have been shown to be mutagenic [44] but are unlikely to be present even after a clinically-irrelevant plasma treatment of tens of minutes with the jet described here. Data indicate, that within a certain concentration range, H_2O_2 in itself may not be a DNA damaging item. In contrast, highly reactive hydroxyl radicals may be generated by Fenton reaction from H_2O_2. When created in the bulk (cell culture medium, wound fluids) the lifetime of hydroxyl radicals is too low the reach the intracellular DNA [45]. It was reported that H_2O_2 can access intracellular compartments by diffusion across the cell membrane or through specific membrane pores, allowing a Fenton reaction within the cell [46]. Yet, its intracellular concentration is tightly controlled by dedicated proteins to maintain intrinsic signaling functions of this molecule [47]. The observed activation of the Nrf2 pathway way further improves this control and reduces the intracellularly available H_2O_2 [42]. Another possibility which may lead to an overestimation of H_2O_2 mutagenicity in some experiments originates from the use of lymphatic cells [48]. The then present myeloperoxidase gives rise to hypochlorite, a strong DNA damaging agent. Also, common lymphatic cell stimulants like phorbol myristate acetate trigger an oxidative burst interfering with the external ROS [28]. By using cells of non-lymphatic origin in our study this issue was avoided. Accordingly, with the moderate H_2O_2 amounts produced by the *kinpen* we did not find an acute mutagenic effect in the HPRT1 assay. Prolonged treatment with this chemical in vitro and ex vivo rather led to apoptosis [49].

Beside reactive species, radiation is another matter. The *kinpen* creates UV radiation, mainly in the UVB-range with an considerably low radiation output ($0.35\,W_{eff}\,m^{-2}$) [10]. Hence, if a $1\,cm^2$ areal is treated for the maximal recommend 60 s, only $1\,J_{eff}\,m^{-2}$ UV is delivered which is well below the ICNIRP recommendations ($30\,J_{eff}\,m^{-2}$) [8]. In both the micronucleus assay and the HPRT1 assay, 20–40 $J_{eff}\,m^{-2}$ were necessary to show a mutagenic effect. Thus, the role of UV radiation generated by the *kinpen* is expected to be negligible in vivo in living systems. Accordingly, the direct treatment in the present study did not reveal a mutagenic potential and studies using plasma generated UV radiation without reactive species showed that the efficacy of plasma treatment mainly relies on reactive species [9,50].

5. Conclusions

In this work, we demonstrated that exposure to the plasma of the *kinpen* was not genotoxic to human cells in vitro. The experiments were carried out using standard procedures for the evaluation of the mutagenic potential of medical products as suggested by a directive of the European Union. In addition to previous risk assessment, these results provide further evidence for the safe application of *kinpen* plasma in clinics. On the other hand, these results also suggest that oxygen and/or nitrogen radicals generated by the plasma may not directly interact with DNA or show only low concentrations which allow DNA repair mechanisms to clear the damage. Work is underway to observe and quantify the long-term effect of plasma in an animal based study to complement this study and to obtain a general risk evaluation.

Acknowledgements

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References

[1] X. Lu, M. Laroussi, V. Puech, On atmospheric-pressure non-equilibrium plasma jets and plasma bullets, Plasma Sources Sci. Technol. 21 (2012) 034005.
[2] T. von Woedtke, H.R. Metelmann, K.D. Weltmann, Clinical plasma medicine: state and perspectives of in vivo application of cold atmospheric plasma, Contrib. Plasma Phys. 54 (2014) 104–117.
[3] E.M. Liston, L. Martinu, M.R. Wertheimer, Plasma surface modification of polymers for improved adhesion—a critical-review, J. Adhes. Sci. Technol. 7 (1993) 1091–1127.
[4] J. Ehlbeck, U. Schnabel, M. Polak, J. Winter, T. von Woedtke, R. Brandenburg, T. von dem Hagen, K.D. Weltmann, Low temperature atmospheric pressure plasma sources for microbial decontamination, J. Phys. D Appl. Phys. 44 (2011) 013002.
[5] S. Perni, G. Shama, M.G. Kong, Cold atmospheric plasma disinfection of cut fruit surfaces contaminated with migrating microorganisms, J. Food Prot. 71 (2008) 1619–1625.
[6] O. Schluter, J. Ehlbeck, C. Hertel, M. Habermeyer, A. Roth, K.H. Engel, T. Holzhauser, D. Knorr, G. Eisenbrand, Opinion on the use of plasma processes for treatment of foods, Mol. Nutr. Food Res. 57 (2013) 920–927.
[7] D.B. Graves, The emerging role of reactive oxygen and nitrogen species in redox biology and some implications for plasma applications to medicine and biology, J. Phys. D Appl. Phys. 45 (2012) 263001.
[8] T. von Woedtke, S. Reuter, K. Masur, K.-D. Weltmann, Plasmas for medicine, Phys. Rep. 530 (2013) 291–320.
[9] S. Schneider, J. Lackmann, F. Narberhaus, J. Bandow, B. Denis, J. Benedikt, Separation of VUV/UV photons and reactive particles in the effluent of a He/O_2 atmospheric pressure plasma jet, J. Phys. D: App. Phys. 44 (2011) 295201.
[10] K.D. Weltmann, E. Kindel, R. Brandenburg, C. Meyer, R. Bussiahn, C. Wilke, T. von Woedtke, Atmospheric pressure plasma jet for medical therapy: plasma parameters and risk estimation, Contrib. Plasma Phys. 49 (2009) 631–640.
[11] H.-R. Metelmann, T. von Woedtke, R. Bussiahn, K.-D. Weltmann, M. Rieck, R. Khalili, F. Podmelle, P.D. Waite, Experimental recovery of CO_2-laser skin lesions by plasma stimulation, Am. J. Cosmet. Surg. 29 (2012) 52–56.
[12] R. Bussiahn, N. Lembke, R. Gesche, T. von Woedtke, K.-D. Weltmann, Plasmaquellen für biomedizinische Applikationen, Hyg. Med. 38 (2013) 212–216.
[13] K. Wende, P. Williams, J. Dalluge, W.V. Gaens, H. Aboubakr, J. Bischof, T. von Woedtke, S.M. Goyal, K.D. Weltmann, A. Bogaerts, K. Masur, P.J. Bruggeman, Identification of the biologically active liquid chemistry induced by a nonthermal atmospheric pressure plasma jet, Biointerphases 10 (2015) 029518.
[14] S. Reuter, H. Tresp, K. Wende, M.U. Hammer, J. Winter, K. Masur, A. Schmidt-Bleker, K.D. Weltmann, From RONS to ROS: tailoring plasma jet treatment of skin cells, IEEE Trans. Plasma Sci. 40 (2012) 2986–2993.
[15] J. Lademann, C. Ulrich, A. Patzelt, H. Richter, F. Kluschke, M. Klebes, O. Lademann, A. Kramer, K. Weltmann, B. Lange-Asschenfeldt, Risk assessment of the application of tissue-tolerable plasma on human skin, Clin. Plasma Med. 1 (2013) 5–10.
[16] R. Bussiahn, N. Lembke, R. Gesche, T. Von Woedtke, K. Weltmann, Plasmaquellen für biomedizinische Applikationen, Hyg. Med. 38 (2013) 212–216.
[17] P.D. Ray, B.W. Huang, Y. Tsuji, Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling, Cell. Signall. 24 (2012) 981–990.
[18] K. Wende, S. Strassenburg, B. Haertel, M. Harms, S. Holtz, A. Barton, K. Masur, T. von Woedtke, U. Lindequist, Atmospheric pressure plasma jet treatment evokes transient oxidative stress in HaCaT keratinocytes and influences cell physiology, Cell Biol. Int. 38 (2014) 412–425.
[19] H.-R. Metelmann, T.T. Vu, H.T. Do, T.N.B. Le, T.H.A. Hoang, T.T.T. Phi, T.M.L. Luong, T.T.H. Nguyen, T.H.M. Nguyen, T.L. Nguyen, Scar formation of laser skin lesions after cold atmospheric pressure plasma (CAP) treatment: a clinical long term observation, Clin. Plasma Med. 1 (2013) 30–35.
[20] A. Kramer, J. Lademann, C. Bender, A. Sckell, B. Hartmann, S. Münch, P. Hinz, A. Ekkernkamp, R. Matthes, I. Koban, Suitability of tissue tolerable plasmas (TTP) for the management of chronic wounds, Clin. Plasma Med. 1 (2013) 11–18.
[21] G. Daeschlein, S. Scholz, R. Ahmed, A. Majumdar, T. von Woedtke, H. Haase, M. Niggemeier, E. Kindel, R. Brandenburg, K.D. Weltmann, M. Junger, Cold plasma is well-tolerated and does not disturb skin barrier or reduce skin moisture, J. Dtsch. Dermatol. Ges. 10 (2012) 509–515.
[22] J.P. O'Neill, P.A. Brimer, R. Machanoff, G.P. Hirsch, A.W. Hsie, A quantitative assay of mutation induction at the hypoxanthine-guanine phosphoribosyl transferase locus in Chinese hamster ovary cells (CHO/HGPRT system):

development and definition of the system, Mutat. Res./Fundam. Mol. Mech. Mutagen. 45 (1977) 91–101.
[23] J. Duan, L. Nilsson, B. Lambert, Structural and functional analysis of mutations at the human hypoxanthine phosphoribosyl transferase (HPRT1) locus, Human Mutat. 23 (2004) 599.
[24] M. Fenech, The in vitro micronucleus technique, Mutat. Res. 455 (2000) 81–95.
[25] M. Fenech, A.A. Morley, Measurement of micronuclei in lymphocytes, Mutat. Res. 147 (1985) 29–36.
[26] I.C.o.N.-I.R. Protection, Guidelines on limits of exposure to ultraviolet radiation of wavelengths between 180 nm and 400 nm (incoherent optical radiation), Health Phys. 87 (2004) 171–186.
[27] S.H. Swierenga, J.A. Heddle, E.A. Sigal, J.P. Gilman, R.L. Brillinger, G.R. Douglas, E.R. Nestmann, Recommended protocols based on a survey of current practice in genotoxicity testing laboratories, IV. Chromosome aberration and sister-chromatid exchange in Chinese hamster ovary, V79 Chinese hamster lung and human lymphocyte cultures, Mutat. Res. 246 (1991) 301–322.
[28] H. Birnboim, DNA strand breakage in human leukocytes exposed to a tumor promoter, phorbol myristate acetate, Science 215 (1982) 1247–1249.
[29] A. Jaeger, D.G. Weiss, L. Jonas, R. Kriehuber, Oxidative stress-induced cytotoxic and genotoxic effects of nano-sized titanium dioxide particles in human HaCaT keratinocytes, Toxicology 296 (2012) 27–36.
[30] J. O'Neill, L. Sullivan, J. Booker, B. Pornelos, M. Falta, C. Greene, R. Albertini, Longitudinal study of the in vivo hprt mutant frequency in human T-lymphocytes as determined by a cell cloning assay, Environ. Mol. Mutagen. 13 (1989) 289–293.
[31] G.E. Johnson, Mammalian Cell HPRT Gene Mutation Mssay: Test Methods, Genetic Toxicology, Springer, 2012, pp. 55–67.
[32] Anke Schmidt, et al., Cell migration and adhesion of a human melanoma cell line is decreased by cold plasma treatment, Clin. Plasma Med. 3 (1) (2015) 24–31.
[33] V. Boxhammer, Y.F. Li, J. Koritzer, T. Shimizu, T. Maisch, H.M. Thomas, J. Schlegel, G.E. Morfill, J.L. Zimmermann, Investigation of the mutagenic potential of cold atmospheric plasma at bactericidal dosages, Mutat. Res.-Gen. Tox. Environ. 753 (2013) 23–28.
[34] N.K. Kaushik, H. Uhm, E.H. Choi, Micronucleus formation induced by dielectric barrier discharge plasma exposure in brain cancer cells, Appl. Phys. Lett. 100 (2012) 084102.
[35] M. Kirsch-Volders, T. Sofuni, M. Aardema, S. Albertini, D. Eastmond, M. Fenech, M. Ishidate, S. Kirchner, E. Lorge, T. Morita, Report from the in vitro micronucleus assay working group, Mutat. Res./Genet. Toxicol. Environ. Mutagen. 540 (2003) 153–163.
[36] S. Reuter, J. Winter, S. Iseni, S. Peters, A. Schmidt-Bleker, M. Dunnbier, J. Schafer, R. Foest, K.D. Weltmann, Detection of ozone in a MHz argon plasma bullet jet, Plasma Sources Sci. Technol. 21 (2012) 034015.
[37] S. Reuter, J. Winter, A. Schmidt-Bleker, D. Schroeder, H. Lange, N. Knake, V. Schulz-von der Gathen, K.D. Weltmann, Atomic oxygen in a cold argon plasma jet: TALIF spectroscopy in ambient air with modelling and measurements of ambient species diffusion, Plasma Sources Sci. Technol. 21 (2012) 024005.
[38] H. Tresp, M.U. Hammer, J. Winter, K.D. Weltmann, S. Reuter, Quantitative detection of plasma-generated radicals in liquids by electron paramagnetic resonance spectroscopy, J. Phys. D Appl. Phys. 46 (2013) 435401.
[39] B. Haertel, S. Strassenburg, K. Oehmigen, K. Wende, T. von Woedtke, U. Lindequist, Differential influence of components resulting from atmospheric-pressure plasma on integrin expression of human HaCaT keratinocytes, BioMed Res. Int. 2013 (2013) 761451.
[40] S. Kalghatgi, A. Fridman, J. Azizkhan-Clifford, G. Friedman, DNA damage in mammalian cells by non-thermal atmospheric pressure microsecond pulsed dielectric barrier discharge plasma is not mediated by ozone, Plasma Process. Polym. 9 (2012) 726–732.
[41] M.T. Gladwin, A.N. Schechter, D.B. Kim-Shapiro, R.P. Patel, N. Hogg, S. Shiva, R.O. Cannon, M. Kelm, D.A. Wink, M.G. Espey, The emerging biology of the nitrite anion, Nat. Chem. Biol. 1 (2005) 308–314.
[42] A. Schmidt, S. Dietrich, A. Steuer, K.-D. Weltmann, T. von Woedtke, K. Masur, K. Wende, Non-thermal plasma activates human keratinocytes by stimulation of antioxidant and phase II pathways, J. Biol. Chem. 290 (2015) 6731–6750.
[43] J. Winter, H. Tresp, M.U. Hammer, S. Iseni, S. Kupsch, A. Schmidt-Bleker, K. Wende, M. Dunnbier, K. Masur, K.D. Weltmannan, S. Reuter, Tracking plasma generated H_2O_2 from gas into liquid phase and revealing its dominant impact on human skin cells, J. Phys. D Appl. Phys. 47 (2014) 285401.
[44] K. Ziegler-Skylakakis, U. Andrae, Mutagenicity of hydrogen peroxide in V79 Chinese hamster cells, Mutat. Res. 192 (1987) 65–67.
[45] C.C. Winterbourn, Reconciling the chemistry and biology of reactive oxygen species, Nat. Chem. Biol. 4 (2008) 278–286.
[46] G.P. Bienert, A.L. Møller, K.A. Kristiansen, A. Schulz, I.M. Møller, J.K. Schjoerring, T.P. Jahn, Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes, J. Biol. Chem. 282 (2007) 1183–1192.
[47] S.G. Rhee, Cell signaling. H_2O_2, a necessary evil for cell signaling, Science 312 (2006) 1882–1883.
[48] P. Thomas, K. Umegaki, M. Fenech, Nucleoplasmic bridges are a sensitive measure of chromosome rearrangement in the cytokinesis-block micronucleus assay, Mutagenesis 18 (2003) 187–194.
[49] S. Hasse, T. Duong Tran, O. Hahn, S. Kindler, H.R. Metelmann, T. von Woedtke, K. Masur, Induction of proliferation of basal epidermal keratinocytes by cold atmospheric pressure plasma, Clin. Exp. Dermatotol. 41 (March 2) (2016) 202–209.
[50] S. Bekeschus, K. Masur, J. Kolata, K. Wende, A. Schmidt, L. Bundscherer, A. Barton, A. Kramer, B. Broker, K.D. Weltmann, Human mononuclear cell survival and proliferation is modulated by cold atmospheric plasma jet, Plasma Process. Polym. 10 (2013) 706–713.