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Safety Implications of Plasma-Induced Effects in Living Cells – a Review of in Vitro and in Vivo Findings

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Review

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Safety implications of plasma-induced effects in living cells – a review of *in vitro* and *in vivo* findings

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Abstract: Cold atmospheric plasma is a versatile new tool in the biomedical field with applications ranging from disinfection, wound healing and tissue regeneration to blood coagulation, and cancer treatment. Along with improved insights into the underlying physical, chemical and biological principles, plasma medicine has also made important advances in the introduction into the clinic. However, in the absence of a standard plasma ‘dose’ definition, the diversity of the field poses certain difficulties in terms of comparability of plasma devices, treatment parameters and resulting biological effects, particularly with regards to the question of what constitutes a safe plasma application. Data from various *in vitro* cytotoxic and genotoxic studies along with *in vivo* findings from animal and human trials are reviewed to provide an overview of the current state of knowledge on the safety of plasma for biological applications. Treatment parameters employed in clinical studies were well tolerated but intense treatment conditions can also induce tissue damage or genotoxicity. There is a need identified to establish both guidelines and safety limits that ensure an absence of (long-term) side effects and to define treatments as safe for applications, where cell stimulation is desired, e.g. in wound healing, or those aimed at inducing cell death in the treatment of cancer.

Keywords: cold atmospheric plasma; cytotoxicity; mutagenicity.

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Introduction

Plasma medicine has rapidly evolved as a field of its own over the past years with experience in the clinic now spanning a decade (Weltmann and von Woedtke, 2017). Research covers a broad range of applications including disinfection, wound healing, blood coagulation, tissue regeneration or cancer treatment in the medical field (Kong et al., 2009). Another complete sector has developed around the uses of plasma in food and agriculture involving reduction of microbial and chemical contamination, pest control and growth promotion (Bourke et al., 2018).

The vast potential for biological applications of the ‘4th state of matter’ lies in the array of reactive species – ions, electrons, radicals, charged particles, metastables – generated as a gas becomes ionised, with temperatures of non-thermal or cold atmospheric plasma (CAP) at close to ambient temperature, thus allowing applications on heat-sensitive biological matter such as tissue (Laroussi, 2018). In particular, the formation of reactive oxygen and nitrogen species (ROS/RNS) such as ozone, atomic oxygen, superoxide anion, hydroxyl radical, hydrogen peroxide, nitric oxide and peroxyxynitrite (Graves, 2012) formed when plasma discharge occurs in or with air is biologically relevant, as many of these are involved in biological signalling pathways or serve as second messengers. Atmospheric cold plasmas may be generated by a range of different plasma devices such as plasma jets, plasma torches, di-electric barrier discharge (DBD), floating electrode di-electric barrier discharge (FE-DBD), surface micro-barrier discharge (SMD), microwave discharge, corona discharge or gliding arc and various device conformations operating with different working gas compositions or input powers have been applied for biomedical investigations (von Woedtke et al., 2013). Table 1 provides an overview of plasma device types most commonly employed in plasma medicine and reference to the working principle. The discovery that longer-lasting reactive species generated in a liquid when it is exposed to plasma discharge

Table 1: Overview of plasma source types frequently used in plasma medicine with reference to publications describing the plasma set-up and discharge principle.

Plasma source	Abbreviations	Gas	Reference
Plasma needle		Argon, helium, nitrogen	(Stoffels et al., 2002)
Plasma torch		Argon, helium	(Shimizu et al., 2008)
Plasma Jet, atmospheric pressure plasma jet	APPJ	Argon, helium (with or without other admixtures)	(Winter et al., 2015)
Dielectric barrier discharge	DBD	Air	(Brandenburg, 2017)
Floating electrode dielectric barrier discharge	FE-DBD	Air	(Fridman et al., 2006)
Surface micro-barrier discharge	SMD	Air	(Klämpfl et al., 2012)

can mediate similar biological effects in cells and tissues as direct exposure to the plasma itself has added another dimension to plasma applications, referred to as plasma-treated or plasma-activated liquids (PAL), with potential advantages in storability, transportability and mode of application (Mohades et al., 2015).

Extensive knowledge is emerging on the physics of the plasma discharge of different plasma devices, on the resulting reactive species chemistry at the biological interface and on the translation into various biological effects. However, comparability and conclusions across the field can be complicated by the vast range of different devices in use – many of which are custom-built – and subsequent differences between their chemical and biological effects and effects.

Discussions are ongoing on how best to define a ‘plasma dose’ or whether such a quantitative definition will ever be possible in the field of plasma, considering the multitude of plasma devices and treatment parameters, and efforts are being made to establish reference methods and systems in order to enable comparison across devices and treatment protocols. Depending on the exposure or treatment intensity, which are influenced by plasma device, treatment and target parameters, plasma can have cell stimulatory or cell inhibitory effects or induce sub-lethal cellular damage (Figure 1), a principle similar to the concepts of ‘oxidative eustress’ promoting physiological and ‘oxidative distress’ causing pathophysiological signalling proposed in redox biology (Sies, 2018). The question whether plasma exposure is safe, particularly with regards to cell toxic, immunogenic or sensitising side effects or genotoxicity and therefore carcinogenic potential, has been key for applications in medicine and healthcare but also for implementations of plasma technology across food and agricultural sectors.

The following review aims to provide an overview of findings to date on the safety of plasma treatment based on available *in vitro* and *in vivo* data. The range of applications of cold atmospheric plasma in the biomedical

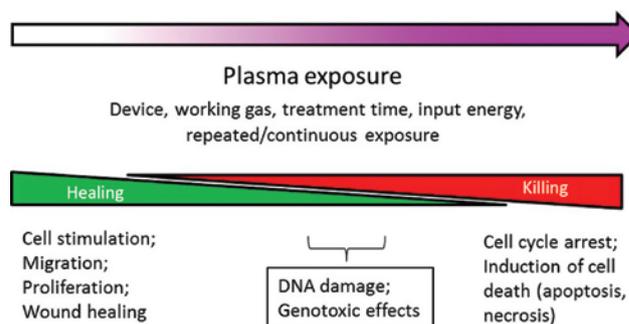


Figure 1: The balance between cell stimulatory and cell inhibitory effects is dependent on the plasma exposure/treatment intensity, which is determined by device and treatment parameters, as well as characteristics of the target cells and their environment. Genotoxic effects can occur when DNA damage exceeds or escapes the capacity of cellular repair mechanisms at sub-lethal plasma treatments.

field and their efficacies have been discussed in detail elsewhere (Weltmann and von Woedtke, 2017) and we therefore focus only on those aspects of studies pertaining to safety implications. By highlighting experimental differences and particularities of various approaches and methodologies, this analysis aims to reconcile apparent conflicting experimental outcomes and conclusions between some studies, as well as provide a broader picture of the current state of knowledge towards safe implementations of plasma in medicine.

Biological effects of plasma

Plasma is able to kill cells – an effect that is being exploited in a range of applications including its use for the inactivation of microorganisms (Bourke et al., 2017) or induction of cell death in cancer cells (Schlegel et al., 2013). The cytotoxic effects of plasma result predominantly from reactive species generated in the plasma discharge and their subsequent transfer to the liquid environment of the target cells.

ROS/RNS formed in the plasma discharge or administered to the cellular environment through plasma activated liquids can result in increased oxidative stress in the cells. While many of these species are involved in the regulation of normal cellular mechanisms, their concentrations are tightly regulated through antioxidants, scavengers and detoxifying enzymes as excessive amounts result in cellular damage and can cause cell death (Görlach et al., 2015). Oxidative stress has been linked to the development of diseases such as cancer, diabetes or cardiovascular disease and many chronic inflammatory disorders are characterised by elevated levels of intracellular oxidative stress (Pisoschi and Pop, 2015).

The effects of plasma on the molecular building blocks of cells include changes in protein structure (Sakudo et al., 2013), oxidation of lipids (Tero et al., 2016), and denaturation of nucleic acids (Arjunan et al., 2015). The susceptibility of amino acids to plasma-induced modification depends on the amino acid side chains with sulfur-containing (cysteine and methionine) and aromatic amino acids being particularly sensitive to plasma treatment (Takai et al., 2014). Plasma treatment can result in changes to protein secondary and tertiary structure and loss of protein/enzyme function (Zhang et al., 2015a). The lipid bilayer of the cell membrane is particularly susceptible to oxidation by plasma derived ROS and can lead to the formation of pores in the cell membrane (Van der Paal et al., 2016). Damage to a cell's genetic material can have profound effects on cell proliferation and transcription mechanisms, where faulty processes can result in the generation of proteins and ultimately cells with altered function, factors which also play a role in carcinogenesis. A range of cell cycle checkpoints prevent the progression of cell division prior to the correction of erroneous genetic material and an inability to repair the damage can result in the induction of the apoptotic pathway leading to programmed cell death and the elimination of the cell.

Genotoxicity

DNA damage

The ability of plasma reactive species to induce damage in DNA molecules has been shown in a number of studies using different types of plasma devices, treatment parameters and gas types.

Plasmid DNA solution treated directly with different helium plasma jets for 10–60 s showed single and

double strand breaks with increasing treatment times and percentage of oxygen admixture indicating that the reactive species produced in the plasma plume were able to directly damage DNA material (Ptasinska et al., 2010; Alkawareek et al., 2014). Ptasinska and colleagues estimated that DNA strand-scission induced in plasmid DNA by a helium plasma jet was predominantly caused by excited and reactive species (>60%) with electrons, positive ions and UV radiation playing minor roles (Ptasinska et al., 2010). Whether short-lived reactive species are able to reach nuclear material and directly effect DNA damage inside a cell is a matter of debate due to the limited penetration depth and lifespan of many of these species on the one hand and their possible reaction with other cellular components (cell membrane, endoplasmic reticulum (ER), mitochondria, antioxidants) encountered as they traverse the cell interior on the other. Yet, some plasma ROS have shown substantial penetration depth (Szili et al., 2014) and longer lived secondary reactive species or products of local reactions such as lipid peroxidation may reach the nucleic acids and result in genetic damage.

Plasma has in fact been used as a potent mutagenesis tool in bacteria for the generation of microbial mutant libraries and may find application as a breeding platform for the bioengineering of industrial microbial strains (Zhang et al., 2014). Quantitative evaluation of the mutation rate of a plasma based mutagenesis tool compared to three conventional mutagenesis systems – ultraviolet radiation (90 $\mu\text{W}/\text{m}^2$, 5–15 min), 4-nitroquinoline-1-oxide (4-NQO) (0.2–1.0 $\mu\text{g}/\text{ml}$), and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (10–30 $\mu\text{g}/\text{ml}$) – showed that the plasma system (helium plasma torch, 10 slm) used at 30–105 s caused greater DNA damage and higher mutation rates (Zhang et al., 2015b).

Various different methods have been employed to assess DNA damage in both pro- and eukaryotic cells in response to their exposure to plasma discharge or to plasma-activated liquids, including the comet assay, the micronucleus formation assay or the detection of phosphorylated histone H2AX (Table 2).

Phosphorylation of histone variant H2AX (to γ -H2AX) is seen as an indication of DNA double-strand breaks (DSB) and detectable through immunoblotting or immunostaining. An important role of γ -H2AX is believed to be the retention of DNA repair factors near the DSB. However, a range of other roles independent of DNA damage and repair have been found including chromosome inactivation, cell differentiation and senescence (Turinetti and Giachino, 2015), which suggest that phosphorylation is not only a sign of DNA DSB. Furthermore, γ -H2AX can be also generated during DNA replication, as a consequence

Table 2: Investigation of structural DNA damage caused by plasma exposure.

Plasma source (working gas)	Exposure	Cell/tissue type	Assay type	DNA damage	Reference
Direct plasma treatment					
Helium plasma needle (He)	>10 s	HeLa	Comet	Y	(García-Alcantara et al., 2013)
DBD (air)	1–20 min	HaCaT	Comet	Y	(Blackert et al., 2013)
MimiFlatPlaSter (air)	5–120 s	Mucosal cells	Comet	N	(Welz et al., 2013)
Helium plasma needle (He)	3 s (1–3 times)	Mouse leucocytes	Comet	Y	(Morales-Ramírez et al., 2013)
Helium plasma needle (He)	>10 s	<i>Escherichia coli</i>	Chromo-test	N	(García-Alcantara et al., 2013)
Helium plasma (He)		Human fibroblast, keratinocyte	8-OHdG	Y	(Tarricone et al., 2012)
(AP-DBD) jet, μ APP1 (He+0.5% O ₂)	Distance from nozzle (0–3 cm)	<i>Staphylococcus aureus</i> ,	DNA damage diffusion assay	Y	(Privat-Maldonado et al., 2016)
SMD (air)	60–480 s	<i>Salmonella typhimurium</i>	γ -H2AX	Y for >120 s	(Isbary et al., 2013)
DBD (air)	0.13–7.8 J/cm ²	Human ex vivo skin samples	γ -H2AX	Y	(Kalghatgi and Azizkhan-Clifford 2011)
Surface type air plasma source (air)	10–50 s	MCF10A	γ -H2AX	Y	(Kim et al., 2010)
FE-DBD (air)	30 s to 15 min	B16F10 mouse melanoma cells	γ -H2AX	Y	(Wu et al., 2013)
DBD (air)	30–240 s	Porcine skin	γ -H2AX	Y	(Kaushik et al., 2012)
Argon plasma jet (kINPen 09 or kINPen MED) (Ar)	90–150 s,	T98G brain cancer cell line	Micronucleus formation	Y	(Kluge et al., 2016)
Argon plasma jet (kINPen) (Ar)	180–300 s	Hen egg	Micronucleus formation	N	
Argon plasma jet (kINPen) (Ar+O ₂ /N ₂ admixtures)	20–180 s	HaCaT	Micronucleus formation	N	(Wende et al., 2016)
	60–300 s	Human lymphocyte TK6 cell line	Micronucleus formation	N	(Bekeschus et al., 2018b)
PAL					
He plasma jet (He)	DMEM treated	HCT116 tumour spheroids	γ -H2AX	Y	(Judee et al., 2016a)
	60–240 s				
He plasma jet (He)	DMEM treated	HCT116 tumour spheroids,	γ -H2AX	Y (HCT116),	(Judee et al., 2016b)
	60–240 s	GM637 fibroblast spheroids		N (GM637)	
Micro-second pulsed plasma (air)	DMEM	H460 lung cancer cells	8-OHdG	Y	(Kumar et al., 2016)
Argon plasma jet (Ar)	RPMI-1640	WIL2-NS B lympho-blastoid cells	Micronucleus formation	Y	(Hong et al., 2017)

DBD, dielectric barrier discharge; AP-DBD, atmospheric pressure dielectric barrier discharge; APP1, atmospheric pressure plasma jet; SMD, surface micro discharge; FE-DBD, floating electrode dielectric barrier discharge; DMEM, Dulbecco's Modified Eagle's Medium; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; γ -H2AX, phosphorylated histone variant H2AX; PAL, plasma activated liquid; Ar, argon; He, helium.

of apoptosis, or is found associated with residual DNA damage, and it is therefore important to determine the kinetics, number, size, and morphology of γ -H2AX-associated foci (Sharma et al., 2012).

Kim et al. found accumulation of γ -H2AX markers of DNA double strand breaks and p53 expression in mouse melanoma cells in response to plasma exposure (Kim et al., 2010). The expression of γ -H2AX as hallmarks of DNA damage was also detected in plasma-treated MCF10A cells where further investigations indicated that this damage was mediated by neither ozone nor plasma-induced lipid peroxidation and subsequent formation of bulky DNA adducts (Kalghatgi and Azizkhan-Clifford, 2011; Kalghatgi et al., 2012). Investigations on porcine skin treated with a FE-DBD device also showed that γ -H2AX increased over plasma-exposure time (Wu et al., 2013). Evidence for the generation of pre-mutagenic 8-hydroxy-2'-deoxyguanosine (8OHdG) lesions, which are widely used as biomarkers for oxidative damage and carcinogenesis (Valavanidis et al., 2009), and subsequent adaptive DNA repair response were detected in human fibroblast and keratinocyte cells in response to helium plasma (Tarricone et al., 2012).

The comet assay or single cell gel electrophoresis is employed to determine DNA strand breaks through electrophoresis of lysed single cells on a microscope slide, with DNA breaks leading to altered migration behaviour visible as a 'comet tail' (Collins, 2004). The comet assay indicated DNA damage in HaCaT cells exposed to a DBD plasma system (Blackert et al., 2013) and in mouse leukocytes treated by a helium plasma needle (Morales-Ramírez et al., 2013), where DNA breakage was proportional to plasma exposure time, but showed no evidence of damage when mucosal cells were treated with the MiniFlatPlaSter (Welz et al., 2013).

DNA damage induced by a helium plasma needle was assessed in both prokaryotic and eukaryotic cells using the chromotest and comet assays, respectively. No genotoxic effects were detected in the *Escherichia coli* model while HeLa cells showed increasing DNA breakage with treatments from 10 s and complete fragmentation after 30 s (García-Alcantara et al., 2013).

The *in vitro* micronucleus assay is a genotoxicity assay for the detection of micronuclei in interphase cells resulting from chromosome loss or chromosome breakage (Fenech, 2000) and forms part of the OECD guidelines for testing of chemicals.

Plasma treatment of human brain cancer cells between 30 and 240 s with a DBD plasma system showed a treatment time dependent increase in micronuclei formation along with loss in cell viability and clonogenicity (Kaushik et al., 2012).

In an *in vivo* system using the hen's egg test for micronuclei induction (HET-MN) Kluge and co-workers found no increase of micronuclei formation in the red blood cells of chicken embryos for any plasma treatment time between 90 s and 150 s, and 180 s and 300 s using the kINPen 09 or kINPen MED plasma systems, respectively, and concluded that an application of these argon plasma jets did not pose mutagenic risks (Kluge et al., 2016).

A high-throughput image cytometry micronucleus assay was developed by Bekeschus and co-workers to investigate mutagenic effects of cold atmospheric plasma on mouse lymphocytes. Using the kINPen plasma jet, 10 different feed gas compositions with varying RONS profiles were tested at treatment times leading to less than 50% reduction in cell metabolic activity, and no increase of frequency of micronuclei formation was observed in any of the conditions (Bekeschus et al., 2018b).

A micronucleus assay was also used by Hong and co-workers to investigate the genotoxic effects of argon-plasma jet treated cell culture medium on WIL2-NS B lymphoblastoid cells incubated with the medium for 24 h (Hong et al., 2017). Increasing volume fractions of treated medium led to elevated levels of necrosis and the occurrence of chromosomal damage including micronuclei, nucleoplasmic bridge and nuclear bud formation which were correlated to the medium's H_2O_2 concentrations.

Overall, plasma is capable of causing damage to nucleic acids in isolation and in the cellular context. Differences in the detection of DNA damage or lack thereof (Table 2) can be results of the different plasma devices used, differences in treatment parameters, cell targets or experimental methodology. Beyond the parameters such as input power, gas flow rates (for plasma jets), and treatment times, factors such as the distance between target and plasma device, the cell densities used, or their liquid environment (medium, buffer) can introduce further variability, whilst some cell types are inherently more susceptible to the treatment than others.

Functional DNA damage/mutagenicity

Structural DNA damage will not necessarily translate into functional phenotypic effects either due to an effective DNA repair machinery, occurrence in a non-transcribed genetic region or because the damage does not translate to changes in protein expression, structure or altered function. Should DNA damage exceed cellular repair capacities, the induction of the apoptotic pathway leading to cell death may be triggered, ensuring that the damage is not propagated during future cell divisions. Assays based

Table 3: Investigation of functional genetic damage.

Plasma source (working gas)	exposure	Cell type	Assay type	Evidence of genetic damage	Reference
MiniFlatPlaSter (air)	30–240 s, 30 s every 12 h for 5 days	V79	HPRT	N	(Boxhammer et al., 2013)
DBD (air)	Plasma-treated FBS 10%, continuous 3–39 days	CHO-K1	HPRT	Y	(Boehm et al., 2016)
Argon plasma jet (kINPen) (Ar)	Plasma-treated RPMI, 1 h	V79	HPRT	N	(Wende et al., 2016)
MicroPlaster β (Ar)	2–10 min, 5 × 2 min	V79	HPRT	N	(Maisch et al., 2017)
Argon plasma jet (kINPen) (Ar)	Plasma-treated medium, 2 h	MRC5, HaCat, SK-Mel-147	Clonogenic assay	N	(Wende et al., 2016)

HPRT, hypoxanthine phosphoribosyl transferase assay; DBD, dielectric barrier discharge; FBS, foetal bovine serum.

on phenotypic changes to a cell system such as the HPRT assay, clonogenic assays or the AMES bacterial mutagenesis test can provide insight into functional manifestations of potential DNA damage in response to plasma treatment (Table 3). The hypoxanthine phosphoribosyl transferase (HPRT) assay, which is an OECD approved method of assessing mutagenic potential in mammalian cells, was employed by several groups to investigate the effects of direct plasma treatment or treatment with plasma treated liquids. The assay relies on lack-of-function mutations at the HPRT locus resulting in a lack of functional hypoxanthine phosphoribosyl transferase expression, which enables colony formation in the presence of the otherwise toxic nucleoside analogue 6-thioguanine (Johnson, 2012).

Plasma treatment applied to V79 cells after removal of culture medium using the MiniFlatPlaSter for 30 s–240 s or repeated treatments of 30 s every 12 h for 5 days did not induce mutagenicity at the HPRT locus beyond naturally occurring spontaneous mutations (Boxhammer et al., 2013).

Boehm and co-workers found increased mutation rates upon prolonged continuous exposure of CHO-K1 cells to plasma activated foetal bovine serum (FBS) or plasma activated PBS over a time period of 6 weeks, where cells were supplemented at every sub-culturing with 10% of FBS (or PBS) which had been treated with a DBD source for 0, 1, 5 or 10 min (Boehm et al., 2016).

Wende et al. performed a detailed mutagenicity study of the kINPen plasma jet using micronuclei testing of HaCaT cells exposed to direct plasma exposure, colony forming assays of SK-MEL-147, HaCaT, and MRC5 cells after 2 h exposure to plasma-treated medium and HPRT assay of V79 cells exposed to plasma-treated medium for 1 h. None of these assays indicated an increased mutagenic potential in response to the direct or indirect plasma treatments (Wende et al., 2016).

The safety of the MicroPlaster β plasma torch was also assessed using V79 hamster cells, where confluent

cells were treated for 2, 5 or 10 min in a single treatment or for 2 min every day for five consecutive days. Cells were covered by PBS for the treatment with subsequent medium replacement. No significant increase in mutation rate was recorded for any of the treatment times while control UVC treatment at 0.01 J/cm² showed a more than 3-fold increase of mutations compared to controls (Maisch et al., 2017).

Of these four studies employing the HPRT assay, only one observed increases in the mutation rate in response to plasma activated liquids. Besides some variances in methodology, the most important difference was the continuous exposure of cells to plasma-treated liquids over a duration of 3–39 days while the other studies assessed once off or repeat exposure of limited duration of either direct plasma exposure or plasma activated medium with subsequent change of culture medium and hence removal of plasma reactive species. This specific study therefore presents an excessive treatment regime exposing cells to continuous oxidative stress and is thus more likely to generate DNA damage over time and to exceed the stressed cells' ability for DNA repair. In a similar way, continuous exposure to H₂O₂ in the concentration range detected in the plasma activated liquids increased mutations at the HPRT locus, indicative of the ability of hydrogen peroxide to induce single and double-strand DNA breaks, and which has been shown to induce mutations in other HPRT models (Ziegler-Skylakakis and Andrae, 1987; Diaz-Llera et al., 2000).

While the potential of plasma to induce DNA damage such as double strand-breaks has been established, a lack of manifestation of such DNA damage in some cell systems suggests that the cellular repair machinery may be well capable of dealing with such damage when occurring at low rate, as would occur with other effectors.

Key factors influencing the scale of damage are likely to be the duration of exposure to plasma or plasma-treated liquid, the removal of plasma-reactive species

post-treatment, as well as the liquid environment of the cells as certain antioxidant media components can provide cell protective effects (Adachi et al., 2014). However, cases where the cellular repair machinery has limited functionality or where the appropriate cell cycle check-points are dysfunctional should be analysed in greater detail as these may be more prone to mutagenic effects.

Cytotoxicity

Direct plasma treatment or the exposure to plasma activated liquids can inhibit cell metabolic activity, reduce cell proliferation and decrease viability in a wide range of mammalian cells (Kalgatgi et al., 2011). Hydrogen peroxide has been shown to be a major, albeit not sole, effector of these cytotoxic effects, with cytotoxicity showing dose-response to the concentration of H_2O_2 detected in the cell environment (Winter et al., 2014; Boehm et al., 2017).

Plasma generated RONS lead to increases in intracellular oxidative stress, loss of mitochondrial membrane potential, caspase activation, cell cycle arrest, apoptosis, senescence or necrosis (Hirst et al., 2016). Such processes are important in the application of plasmas to the cells of living tissues, as they present either unwanted effects in wound healing or disinfection applications or indeed desired effects in cancer treatment.

Extensive work has been performed particularly on the effects of plasma on skin cells such as keratinocytes or fibroblasts in light of the range of applications in dermatology. Plasma treatments at short treatment times particularly on dermal cell types have shown no cell inhibitory effects but were able to stimulate cell migration and wound healing (Arndt et al., 2013). Plasma exposure was furthermore shown to affect gene expression, particularly the differential regulation of cytokine and chemokine molecules such as interleukins and growth factors, involved in wound healing both *in vivo* and *in vitro* (Arndt et al., 2013, 2015). A large range of different plasma devices have been tested for dermatological applications (reviewed in Gay-Mimbrera et al., 2016) using *in vitro* and *in vivo* models. Studies using topical application of plasma in *ex vivo* cell or tissue models suggested good tissue tolerability at the plasma doses and exposure times needed to achieve microbial reduction and/or improve wound healing.

Selectivity

In contrast to applications in wound healing, the use of plasma reactive species to kill cancerous cells is aimed

at inducing cell toxic phenomena – DNA damage, loss of mitochondrial membrane potential, cell cycle arrest, apoptosis or necrosis (Schlegel et al., 2013). A selectivity of plasma for affecting cancerous cells over normal has been found in some studies (Keidar et al., 2011; Tanaka et al., 2011; Song et al., 2014; Kim and Chung, 2016) while others reported higher resistance or comparable sensitivity of malignant cells (Hirst et al., 2015; Dezest et al., 2017). Increased sensitivity of cancerous cells to plasma redox species could be linked to their elevated metabolic activity (Hirst et al., 2016) or a higher percentage of actively dividing cells (Schlegel et al., 2013) and thus bears similarities in mechanism of action to other cancer therapeutics such as certain chemo- or radiotherapies involving an increase in oxidative stress (Hirst et al., 2016). A further theory points to the higher expression of aquaporins on the cell membrane of cancerous cells as cause for the selective toxicity, where these membrane channels enable greater influx of ROS such as H_2O_2 (Yan et al., 2015). An advantage of non-thermal plasma in cancer treatment could lie in the range of generated RONS affecting multiple cellular targets and pathways. However, by-stander effects on non-malignant surrounding tissues need to be considered and similar to side effects observed for radio- and chemotherapy, impacts on actively dividing cells could be more pronounced.

Stimulation of the immune system

Recent research focus has turned to the ability of non-thermal plasma to induce ‘immunogenic cell death’, resulting in active recruitment of host immune cells to the tumour site and their involvement in tumour cell elimination through the recognition of damage associated molecular patterns (DAMPs) which are expressed by the plasma treated cancer cells (Miller et al., 2016). Plasma treatment has been shown to result in the release of ATP (as so-called ‘find me’ signal) and in the localisation of calreticulin to the cell-surface in cancer cells (as ‘eat me’ signal) (Lin et al., 2015; Bekeschus et al., 2018a). While the penetration depths of more stable plasma reactive species have been estimated in the μm to mm range (Szili et al., 2017), immunogenic effects could explain much deeper penetration of plasma-induced effects and the reduction of subcutaneous tumours. From a safety point of view, the immunogenic potential of plasma treatment needs to be considered where undesirable immune reactions against benign host cells could potentially be triggered or inflammatory responses elicited. A more detailed understanding of plasma-induced expression of DAMPs on the one hand and the stimulation and migration of immune cells on the other will be needed.

In vivo studies

Animal

Both direct plasma treatment and the use of plasma activated liquids have been studied in a number of animal, and particularly rodent, models and only a selection of studies pertinent to plasma tolerability and side effects are referenced here.

The MicroPlaster β plasma device was assessed in an *in vivo* wound model in mice with regards to wound healing, inflammatory response and cytokine expression. Following a 2 min treatment wound healing was accelerated at days 3 and 5 post wounding and a range of pro-inflammatory cytokines and collagen and alpha-SMA (α smooth muscle actin) were upregulated in the dermal tissue while no negative side effects such as infection, swelling, oozing or erythema were observed (Arndt et al., 2013).

A murine model was also used to determine skin sensitisation in response to repeated cold atmospheric plasma treatment of 25 s with the kINPen MED device (on days 1, 2, 3 and 7). This study showed local short-lasting hyperemia in response to the treatment but no longer lasting irritation, histological skin damage or other illness were detected. The OECD murine local lymph node assay protocol indicated no local inflammation as sign of skin sensitisation (van der Linde et al., 2017).

Low dose non-thermal plasma of 5 min per day over a 4-week period generated with a plasma jet was found to accelerate wound healing in a rat model. Treated tissues showed increases in 4-HNE (4-hydroxynonenal) and E-cadherin but no toxicity to vital organs or significant differences in blood markers were observed (Hung et al., 2016).

Kos and co-workers evaluated the safety of a helium plasma jet on mouse skin *in vivo* for different flow rates of 1–5 l/min and treatment times of 0.5–4 min using fluorescence microscopy, histology and IR measurements (Kos et al., 2017). Evidence of both early direct skin damage and late indirect skin damage were observed and progressed with increasing flow rate and treatment time. Increasing flow rates led to increases in surface temperature of up to 96°C and increases in concentrations of gaseous RONS.

The treatment of both intact and wounded skin of pigs using a floating electrode-discharge barrier discharge (FE-DBD) at low and high power indicated that at lower power (20.4 J/cm²) plasma could be safely applied for up to 2 min without inducing microscopic skin damage, while wounded skin sustained 5 min of treatment at higher power (39 J/cm²) without damage due to protective effects of blood clotting (Wu et al., 2013).

An experimental study of cold plasma treatment of ulcerative colitis in a mouse model indicated no damage to the colon tissue in terms of tissue integrity or histological damage following 4 s, 30 s or 60 s treatment with a Pin-to-Hole Spark Discharge (PHD) Plasma (Chakravathy et al., 2011). Utsumi and colleagues used plasma activated medium to treat subcutaneous xenograft tumours of ovarian cancer cells in nude mice. Administration of the plasma-activated medium via sub-cutaneous injection of 200 μ l was rated as nontoxic by observing mouse weights, survival, and behaviour, and no complications such as anaphylaxis and skin necrosis were detected (Utsumi et al., 2013).

In vivo experiments evaluating the effect of plasma treatment on chicken embryos at early and late developmental stages, showed treatments with a DBD device to be lethal above 4 min, with higher voltages leading to earlier onset of embryonic death and a higher sensitivity of the early stage embryos (Zhang et al., 2017).

In light of a predominance of skin-based applications of cold plasma, a majority of animal model studies investigated topical plasma treatments and most of these found no adverse effects on the skin tissue or the animal in general for different plasma devices and treatments times ranging from several seconds up to 5 min. Notable exceptions were skin damage observed by Kos et al. which showed treatment time and gas flow rate dependency. This study found local thermal damage to be a major contributor to the effects observed whilst most other plasma devices have been shown not to increase temperatures above 42°C. The lethal effects on chicken embryos at treatment times above 4 min (Zhang et al., 2017) point to a higher susceptibility of developmental processes to plasma reactive species. In summary a range of plasma treatments are well tolerated at treatment times below 5 min in animal tissues and systemic adverse effects have not been observed. Yet sensitivity to longer treatments suggests that treatment parameters need to be well controlled and monitored as oxidative damage can be induced.

Human

A number of clinical studies or patient pilot studies have been conducted in the dermatological field where plasma is used for microbial reduction and to assist wound healing. Most studies to date noted no evidence of adverse side effects, sensitisation or allergic reaction and suggest that the treatments were well tolerated and did not induce relevant side effects (Table 4). A number

Table 4: Overview of patient pilot studies and clinical trials using cold atmospheric plasma.

Objective	Plasma device	Number of patients (treatments)	Study outcome	Adverse effects reported	Reference
Alleviation of chronic venous leg ulcers	PlasmaDerm® VU-2010	14 (7 with plasma)	Antibacterial effects	Comparable to standard treatment	(Brehmer et al., 2015)
Effects of CAP on cutaneous micro-circulation	PlasmaDerm® FLEX9060 (1 × 90 s)	20	Enhanced cutaneous microcirculation	No adverse effects; no pain; well tolerated	(Kisch et al., 2016a)
Effects of repetitive CAP on cutaneous micro-circulation	PlasmaDerm® FLEX9060 (3 × 90 s)	20	Enhanced cutaneous microcirculation	No adverse effects; no pain; well tolerated	(Kisch et al., 2016b)
Antimicrobial effects for wound treatment	kINPenMed	10	Microbial reduction in chronic wounds	Without any side-effects	(Ulrich et al., 2015)
Adjuvant antifungal therapy	kINPen MED	8 (split-mouth)	Erythema surface was reduced significantly	No side effects reported	(Preissner et al., 2016)
Treatment of wound healing disorders	kINPen MED	4	Complete wound closure	No side effects; no inflammation or infection	(Hartwig et al., 2017)
Wound healing in skin graft donor sites	MicroPlaSter β	40 (wound split-plasma vs. argon control)	Improved healing, reepithelialisation; fewer fibrin layers, blood crusts	No relevant side effects	(Heinlin et al., 2013b)
Bacterial reduction in chronic wounds	MicroPlaSter α; MicroPlaSter β	14 patients (70 treatments); 10 patients (137 treatments)	Decrease of bacterial load in chronic wounds	No side effects; treatment was well tolerated	(Isbary et al., 2012)
CAP as add-on-therapy in pruritic diseases	MicroPlaSter β	46	Pruritus reduction comparable to treatment with placebo	No relevant side effects; treatment was well tolerated	(Heinlin et al., 2013a)
CAP to decrease bacterial load in wounds	MicroPlaSter α	36 (38 wounds) (291 treatments)	Reduction of bacterial load	No side effects occurred, treatment was well tolerated	(Isbary et al., 2010)
Comparison of direct and indirect CAP for skin treatment	Surface micro-barrier discharge (SMD)	12	Reduction of physiological concentrations of bacteria on skin	No side effects such as pain, heat, uneasiness	(Li et al., 2013)

of clinical studies are furthermore on-going or actively recruiting participants at the current time (<https://clinicaltrials.gov>). Most of the studies to date are based on three types of plasma devices which have obtained CE medical device approval or their precursor devices: the MicroPlaSter (Adtec Plasma Technology Co. Ltd., Fukuyama, Japan), the kINPen Med (neoplas tools GmbH, Greifswald, Germany), the PlasmaDerm (CYNOGY GmbH, Duderstadt, Germany).

MicroPlaSter devices

Isbary and co-workers conducted a number of studies on the application of surface micro discharge plasma devices (FlatPlaSter2.0, MiniFlatPlaSter, MicroPlaSter α , MicroPlaSter β) for topical treatment of human skin, aimed at microbial reduction and wound healing.

Experiments on *ex vivo* human skin using the FlatPlaSter2.0 and MiniFlatPlaSter devices indicated treatments up to 60 s to be safe and tolerable based on histological and imaging analysis (Isbary et al., 2013). Trials conducted on *in vivo* skin showed a 2-min treatment with either MicroPlaSter α or MicroPlaSter β plasma devices to be a safe, painless and effective technique to decrease the bacterial load in chronic wounds of 24 patients without occurrence of side-effects (Isbary et al., 2012). The treatments of chronic wounds with a single application of 3–7 min using the MicroPlaSter α device (Isbary et al., 2012) or 5 min weekday treatments of Herpes Zoster with the MicroPlaSter β for a total of three to nine treatments also indicated no side effects and were well tolerated in all cases (Isbary et al., 2014).

Studies by the same group also showed that direct and indirect plasma treatment based on surface micro barrier discharge compared for decontamination of physiologically contaminated forearms of 12 volunteers demonstrated good tolerability of the plasma treatment without negative side effects including pain, heat or uneasiness (Li et al., 2013).

kINPen devices

Investigations of an atmospheric pressure plasma jet and a DBD-source on the skin of healthy human volunteers showed that plasma was well tolerated in terms of paresthesia, pain and heat, without causing damage to the skin barrier or resulting in skin dryness (Daeschlein et al., 2012a,b). *In vivo* risk assessments of temperature and UV exposure by plasma indicated that UV radiation of the

plasma jet was an order of magnitude below the dose inducing sun burn, and that thermal damage of the tissue by the plasma could be excluded (Lademann et al., 2009).

No side effects or inflammation were registered in trials using the kINPen med for treatment of wounds such as chronic leg ulcers (Ulrich et al., 2015) or wound healing disorders (Hartwig et al., 2017), or when employed as an adjuvant anti-fungal treatment in oral applications (Preissner et al., 2016).

Palliative treatment of advanced, inoperable head and neck cancer with the kINPen med was performed in six patients by Metelmann et al. (2018). Reduced odour, pain and partial tumour remission was achieved in some of the cases with four out of the six patients describing an increase in the quality of life while some mild side effects such as edema, dry mouth, pain and exhaustion occurred.

PlasmaDerm devices

As outlined above, Daeschlein and coworkers investigating the effects of both the kINPen plasma jet and a PlasmaDerm DBD-source on the skin of healthy human volunteers found no evidence of skin damage and demonstrated that plasma was well tolerated (Daeschlein et al., 2012a,b). PlasmaDerm devices were also employed to study the effects of plasma on skin microcirculation as well as antibacterial effects in chronic leg ulcers and were well tolerated by the patients without occurrence of pain or adverse effects (Brehmer et al., 2015; Kisch et al., 2016a,b).

In summary all studies of topical application of cold plasma performed on human patients indicated good tolerability and an absence of significant side effects at the parameters used.

Long-term studies

In vivo studies of the effects of plasma treatment in the long-term or late-stage side effects are scarce to date in light of the relative recent evolution of the field and its gradual introduction to the clinic. Valuable follow-up data would pertain in particular to immunogenic responses, sensitisation and possible malignant transformation of plasma treated sites as well as the response of cells in these areas to a repeated plasma exposure or other stressors such as UV (sunlight) or chemical agents.

A 12-months follow-up study was conducted in five patients of laser lesions treated by plasma for 10 s, 30 s or three repeated treatments of 10 s and did not show any pre-cancerous skin features (Metelmann et al., 2013).

Schmidt and co-workers performed a detailed 1-year follow-up study on mice exposed to 14-day consecutive plasma exposure in an ear wound model. Histological, biochemical and imaging analysis were used to assess long-term side effects of repetitive treatment with the argon plasma jet kINPen 11 for 20 s using a total of 84 study animals (Schmidt et al., 2017). Plasma treated mice did not differ from controls in health state, nutrition or behaviour and displayed no chronic wound inflammation or other side effects while wound healing progressed physiologically. Magnetic resonance imaging (MRI) and computer tomography/positron emission tomography (CT/PET) scans showed no evidence of tumour formation in major organs such as liver, lymph node, spleen, heart, lung, and brain or at the wound site and the absence of neoplastic markers in the blood suggested no other tumour sites.

Though long-term follow-up data is still very limited, the available studies indicate no adverse long-term effects in either animals or humans and importantly, no evidence of tumour formation or pre-cancerous features were observed.

Conclusion

In-depth evaluation of clinically relevant plasma-devices under conditions currently employed, e.g. for the treatment of wounds, suggests that plasma treatment at these conditions is safe and well tolerated. With applications having reached the clinic only in the last decade, truly long-term clinical follow-up data are still lacking but should start to emerge over the next few years. These will pertain in particular to the evaluation of potential late stage adverse effects, sensitisation and cancerous or pre-cancerous progression. Experimental data from more excessive, long term or continuous exposure to plasma reactive species indicated the potential for a manifestation of genotoxic effects, hence, as with any treatment safety limits and correct dosage need to be established and will be paramount to ensuring patient safety. Potential risks for any medical intervention lie in side effects and adverse reactions as well as hazards resulting from incorrect, excessive or non-target specific application, or an interaction with other drugs and environmental factors. In the case of plasma this may pertain in particular to additional sources of oxidative stress. Differences in sensitivity of various cell and tissue types to plasma have been shown and need to be considered in the treatment design.

While a DIN SPEC on 'General requirements of plasma devices in medicine' was published in an attempt

to establish a point of reference for the comparison of different devices and their biological effects, there seems to be only limited uptake of this reference standard in the plasma research community to date (Mann et al., 2016). A lack of comparability therefore remains a major hurdle in reconciling what can be conflicting or divergent findings. In the absence of a useful definition of 'dose' in the plasma context or the establishment of standards, every device and treatment regime will need to be assessed individually to establish efficacy levels and safety margins for the application in question. In light of these limitations, this review does not attempt to draw any overall conclusions on the safety of plasma, where the vast array of devices, parameters and targets make comparability near impossible, but aims to (a) present an overview illustrating this complexity and diversity of the field and (b) enable readers to identify findings most relevant to their own devices and set-up.

While references to mutagenicity or genotoxicity may be seen to fuel 'plasma-scepticism' and could inhibit implementation of a promising new technology in the clinic, open discussions from an early stage can help to make the benefit-risk analysis of plasma medicine transparent and support the uptake of this technology in the long-run. It is important that all stakeholders including endusers are incorporated to this discussion to promote successful outcomes and applications. Plasma and its derived plasma-activated liquids have immense potential across a wide range of applications, however, rigorous evaluation along with open discourse and discussion of its possible risks and limitations are needed – not only to ensure a safe implementation but also to assist uptake of this novel technology by clinicians, healthcare providers and patients and to promote overall public acceptance.

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