The Irritation Potential of Nonthermal Atmospheric Pressure Plasma in the HET-CAM

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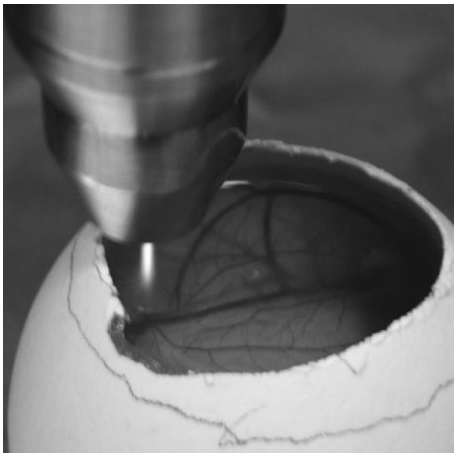
To determine a reliable test and validate the use of nonthermal atmospheric pressure plasma on wounds, we selected the Hen's Egg Test–chorioallantoic membrane (HET-CAM) as an alternative method to the Draize rabbit test to determine the irritation potential of plasma. Irritations of varying intensities provoked by using a dielectric barrier discharge (DBD) and an HF Plasma Jet corresponded to treatment frequencies and exposure times. Pulsed plasma led to the mildest irritations. Depending on the intensity of the irritations, the effects were partially or completely reversible. In the latter case, it may be assumed that this plasma mode can be applied to living tissue without harm. To conclude, the HET-CAM is suitable for screening plasma sources and parameters for the medical applications of plasma.



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Introduction

As a partially ionized gas with free electrons and radicals, low temperature plasma is highly reactive.[1,2] For many years, plasma has been used in technical applications, for instance, for the surface modification of materials.[3] The antimicrobial effects of plasma have been verified[4–6] and are being implemented technically.[7,8] In the past, it has been used medically in surgery as argon plasma coagulation,[9] and more recently the options for plasma application in medicine have been summarized as plasma medicine.[10] One fundamental advantage of plasma treatment is its noncontact mode of application.[11] The well-known plasma characteristics (antimicrobial efficacy, influence on coagulation, surface modification) in addition to the homogeneous applicability with lateral and axial

Plasma Process. Polym. **2010**, *7*, 318–326

DOI: 10.1002/ppap.200900119

spread of the plasma, no disadvantageous evaporation effects, and low penetration depth form the basis for promising biomedical applications.[9,10] Application is conceivable in implantology for the treatment of infected implant wounds, chronic wounds, infectious skin diseases, periodontitis, and in veterinary medicine, i.e., for otitis externa.

Further research on the effects of plasma on the living organism is necessary for the implementation of plasma for those medical applications. Studies have been conducted on the effects of plasma of different technical and physical constitutions on blood coagulation,[12] apoptosis,[13] and cytotoxicity.[14] Prior to the medical application of plasma, its potential for irritation must be known in order to avoid any harmful side effects.

In the present study, the irritancy of plasma was tested with an internationally established standard test for the first time. For the primary screening of various plasma sources, the Hen's Egg Test–chorioallantoic membrane (HET-CAM)[15] as a replacement method for the Draize Test[16] was chosen for ethical reasons. The Draize rabbit test continues to be the method of choice for the regulatory assessment of eye irritation potential (OECD Guideline no. 405). The vascular system of the CAM reacts to harmful substances immediately and is highly sensitive, which can be evaluated visually. The CAM is a vascularized membrane, which surrounds the chorion and the allantois of the chick embryo, and which is responsible for the embryo's gas exchange. During embryogenesis, the CAM can be found at the blunt pole of the egg, near the air space. At the time of the experiments, pain perception provoked by manipulation of the CAM was not yet developed in the embryo.[17]

The risks of further effects of plasma, such as UV radiation and the production of ozone, are being examined in the interdisciplinary research network of Campus PlasmaMed, located in Greifswald, supported by the German Ministry of Education and Research of Germany.

Experimental Part

Test Protocol

Vakzine Lohmann Spezifisch Pathogen Frei eggs (VALO SPF, Lohmann GmbH, Cuxhaven, Germany) were used in this test. The eggs were incubated for 10 d at 37 + 1 °C und 55 + 7% relative humidity in a commercial small-motored breeder (Typ KMB F/2, Ehret GmbH, Emmendingen, Germany) with an automatic rotating mechanism and automatic humidity regulation. On the 9th day, the eggs were examined using an egg candler (Powerflash, J. Hemel Brutgeräte GmbH & Co.KG, Verl, Germany) to determine the viability of the embryo. Unfertilized eggs were excluded from the study. Fertilized eggs were placed with the blunt pole containing the air space facing upwards, and incubated for an additional day without further rotation. The experiments were conducted on day 10.

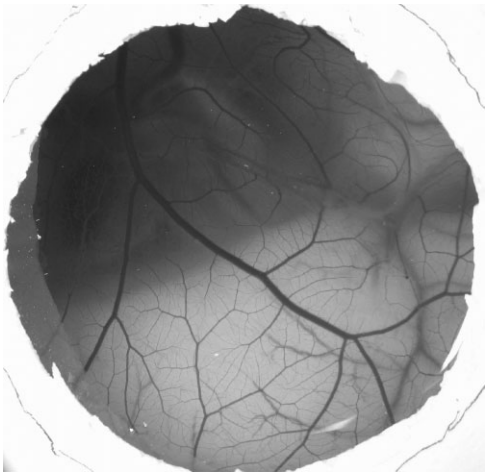

Figure 1. Chorioallantoic membrane (CAM), untreated, vessels unharmed.

The eggshell was carefully removed from the region over the air space using fine scissors and tweezers. After wetting with 0.5 ml 0.9% sodium chloride solution, the inner shell membrane was peeled away to dissect the CAM (Figure 1). Three eggs were treated for each experimental setting. The sample sizes of eggs with stronger initial reaction or discrepant reaction patterns were increased to four to six eggs. 300 μl of a 0.9% sodium chloride solution were applied to the CAM as a negative control. 300 μl of a 1% sodium dodecyl sulfate (SDS) solution (Figure 2) or 300 μl 0.1 N NaOH solution (Figure 3) were applied to the CAM to induce a standardized irritation response as a positive control.

The CAM was photographed (Canon PowerShot G9) before being treated with plasma to document the lack of irritation (Figure 1). The reaction of the CAM to the plasma was photographed 1 and 5 min after being treated with plasma and evaluated using a score (Table 1).

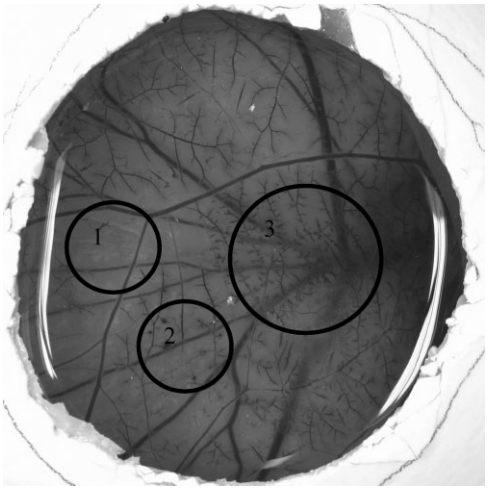

Figure 2. Chorioallantoic membrane (CAM), positive control: 5 min after application of 300 μl 1% SDS solution, (1) lysis of vessels (discoloration), (2) isolated hemorrhages, (3) multiple hemorrhages.

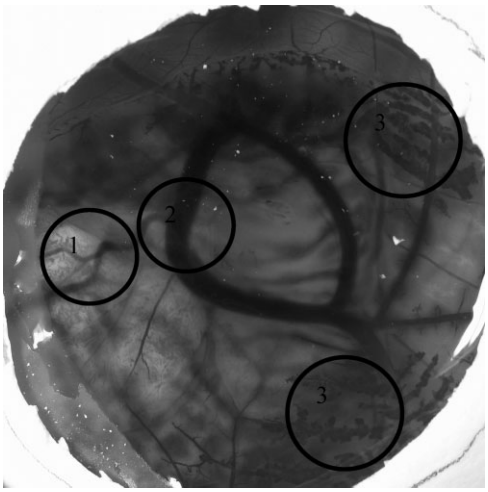

Figure 3. Chorioallantoic membrane (CAM), positive control: 5 min after application of 300 μl 0.1 N NaOH solution, (1) lysis of vessels (discoloration), (2) intravasale coagulation, (3) multiple hemorrhages.

To test reversibility, the plasma-treated CAM of 38 eggs was covered with an adhesive polyethylene film and incubated for another 24 h. The CAM of all eggs was then re-evaluated using photo documentation.

After evaluation, the eggs were frozen at −20 °C, killing the embryos.

Plasma Sources

An HF Plasma Jet (INP Greifswald) (Figure 4) using argon gas[18,19] and a dielectric barrier discharge (DBD, INP Greifswald) (Figure 5)

Table 1. Hen's Egg Test–chorioallantoic membrane (HET-CAM) score.

Irritancy	Occurrence	HET-CAM score
No		0
Hyperemia	Slight	1
	Moderate	2
	Intense	3
Hemorrhages	Single	4
	Multiple	5
	Mass	6
Lysis/discoloration	Vessels no longer visible	7
Coagulation	Thrombus (intravascular coagulation)	8
	Increased opacity (extravascular coagulation)	8

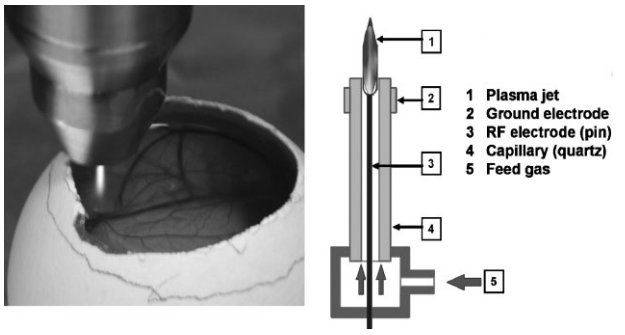

Figure 4. Left: argon-plasma of an HF Plasma Jet application on a CAM, right: schematic of an HF Plasma Jet.

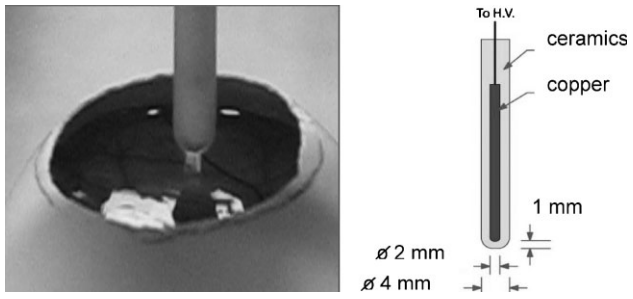

Figure 5. Dielectric barrier discharge (DBD)-plasma application on a CAM, right: schematic of the DBD.

operating in air[20] were implemented as plasma sources. The HF Plasma Jet can be applied in a continuous and in a pulsed mode. In the continuous mode, plasma is generated continuously at a frequency of 1.82 MHz, while in the pulsed mode, plasma is produced with a duty cycle of 19% (19 μs at 1.82 MHz-on, 81 μs-off, total duration of cycle 100 μs) (Figure 6). The temperature (calorimetric measurement) of the plasma in the continuous mode was between 54 and 79 °C, depending on the gas flow. The temperature in the pulsed mode was between 29 and 37 °C (Figure 7).

Plasma Treatment

The test protocol for the HET-CAM test was applied with the following changes: plasma was investigated instead of fluids or solids.[21] The plasma source was clamped into a computer-driven xyz table, under which the egg was positioned. The distance of the electrode was adjusted to the CAM in such a way as to enable a

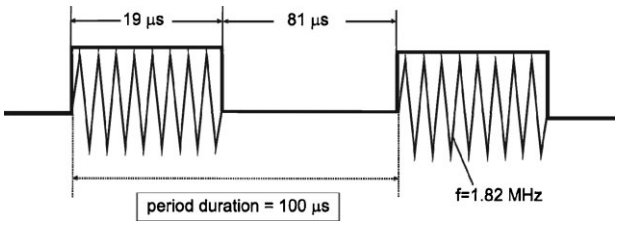

Figure 6. HF Plasma Jet: schematic of the voltage pulse regime for the used pulsed plasma, argon flow: 1, 2, or 3 slm, $f = 1.82$ MHz.

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DOI: 10.1002/ppap.200900119

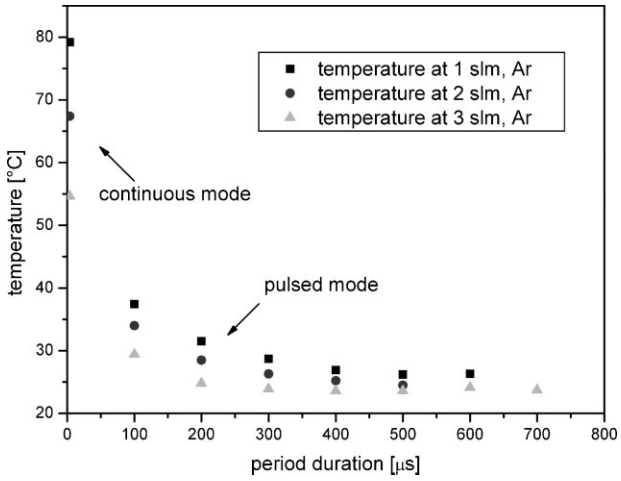

Figure 7. Gas temperature in dependence of the period duration (19 μs on-time).

direct application of plasma. When using the Plasma Jet, the distance from the nozzle to the CAM was 7 ± 1 mm. The nozzle was individually adjusted from CAM to CAM, to ensure that the plasma was always just barely in contact with the CAM, but slight variations (about + 0.5 mm) were allowed, because the CAM, being a biological surface, is not always completely even. The DBD electrode necessitated a distance of only 2 ± 1 mm. The variation of the distance was caused by the surface of the CAM, which sometimes had a convex adherence at the contact areas to the eggshell. At distances > 3 mm, no discharge occurred. In the center of the CAM, a uniform distance of 2 + 0.2 mm could be realized. Both the Plasma Jet and the DBD electrode meandered over the CAM with a speed of 30 mm · s^{-1}. In some experimental settings, points on the CAM were targeted (10 and 40 s). The plasma treatments were repeated one to three times. The following settings were used:

(i) HF Plasma Jet, continuous mode: gas flow, 2 slm (standard liter per min); frequency, $f = 1.82$ MHz; 2–3 W system power.
(ii) HF Plasma Jet, pulsed mode: gas flow, 2 slm; frequency, 1.82 MHz; pulse, 19 μs on; 81 μs off; 0.4–0.6 W system power.
(iii) DBD electrode: $U_{electrode} = 13\ kV_{pp}$ $f = 31$ kHz; 0.4–1.6 W system power; the CAM functioned as the counter electrode, grounding was achieved with a metal egg holder.

Results and Discussion

Assessment of the Results in Order of Excitability

Excitability is the response of the CAM to a noxious chemical or physical stimulus. In terms of the applicability of plasma to sensitive tissues such as wounds, scores up to 5 are acceptable, deduced from test results with wound antiseptics in the HET-CAM[22] (Table 1). Beyond that, responses such as thrombosis and increase in opacity are critical, because they lead to permanent tissue damage.

Control Group

Besides the negative control, the effects of three treatments on the CAM using the argon gas flow with and without application of 0.5 ml 0.9% sodium chloride solution were examined to evaluate mechanical and drying effects of the gas flow. Both were tolerated without leading to irritations, indicating that argon gas flow is not harmful for the CAM.

The SDS positive control group showed intense hemorrhagic reactions after 1 min and lysis after 5 min (Figure 2). Additionally, in the NaOH positive control group, massive thromboses were observed after 5 min (Figure 3). Two eggs treated with argon gas flow/0.9% sodium chloride solution and one egg treated only with argon gas flow did not show any symptoms after another 24 h of incubation (Table 2). It has to be noted that with the HF Plasma Jet, the gas flow is limited. A gas flow of more than 3 slm led to an upward spraying of the gelatinous mass, which mechanically harmed the CAM.

Deviating from the test protocol of HET-CAM, the reactions were evaluated 1 and 5 min after the application, because it is not possible to measure the time (s) between the start of the application of plasma and the excitability during the exposure.[21] Consequentially, the irritation score of the HET-CAM must be modified on an ordinal scale.

Table 2. Scores of the CAM controls: control NaCl.

Mode	Egg (no.)	Score after 1 min	Score after 5 min	Score after 24 h
Control NaCl	70	0	0	
	71	0	0	
	72	1	0	
	73	0	1	
Control SDS	74	5	7	
	75	7	7	
	76	5	7	
	77	5	7	
Control NaOH	78	6	8	
	79	6	8	
	80	6	8	
	81	6	8	
	82	6	8	
3 × argon	83	1	1	
	84	0	1	
	85	0	1	0
3 × argon + NaCl	86	0	0	0
	87	0	1	
	88	0	1	0
	89	0	1	

HF Plasma Jet, Pulsed Mode

The temperature of plasma was physiological in the pulsed mode with a period duration of 100 μs and a gas flow of 2 slm (Figure 7). Thermal effects on the CAM can therefore be excluded.

After a single application ($n = 3$), two of the CAMs reacted with hyperemia, and one CAM with isolated hemorrhages. For the double and triple treatments, the sample number was doubled to six. The double treatment led to multiple hemorrhages in one case. After three treatments, irritations were intensified (in two of six CAMs discolorations were observed, in one CAM vascular thromboses).

A dose-response relationship was identified; three applications appear unsuitable for wound antisepsis. Based on the thrombotic effect, however, applicability is conceivable for haemostasis.

The triple application of pulsed plasma was additionally tested on seven CAMs treated with 0.9% sodium chloride (Figure 8). Sodium chloride was used to wet the samples, as it is possible that generated radicals can be dissolved or formed in it, which may lead to an increased reaction. The CAMs wetted with 0.9% sodium chloride solution showed hemorrhages more often (four of seven), of which one case was massive. In three of seven CAMs, vascular lysis and/or discoloration was observed. Of five of the eggs incubated for 24 h, two demonstrated increased opacity, while in the others, the reaction was reversed partially or completely (Table 3). The result suggests that by radical formation in the solvent, the irritation is slightly increased, albeit too slight to be relevant.

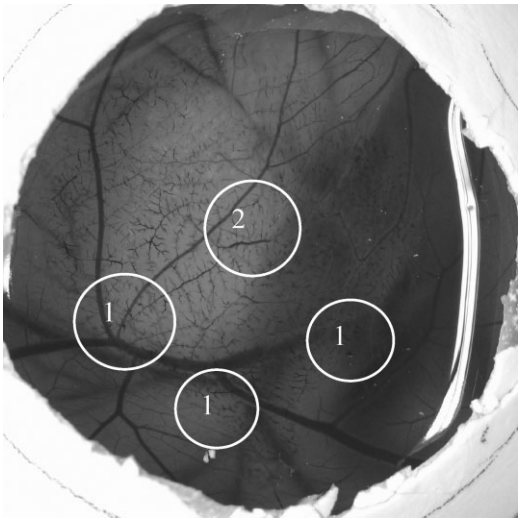

Figure 8. Chorioallantoic membrane (CAM), 5 min after triple treatment with burst argon plasma (wetted with 0.5 ml 0.9% sodium chloride solution) (1) multiple microhemorrhages areas, (2) hyperemia.

Table 3. Scores of the CAM after single to triple treatment with plasma in the pulsed mode.

Mode	Egg (no.)	Score after 1 min	Score after 5 min	Score after 24 h
1 × pulsed	1	1	1	
	2	1	2	
	3	1	4	
2 × pulsed	4	0	4	
	5	0	4	
	6	4	4	
	7	5	5	
	8	4	4	
	9	2	4	8
3 × pulsed	10	1	2	
	11	1	4	
	12	2	4	
	13	4	1	
	14	4	8	
	15	7	7	
3 × pulsed + NaCl	16	4	4	
	17	1	1	
	18	5	5	
	19	4	7	4
	20	4	5	8
	21	4	5	0
	22	5	7	0

HF Plasma Jet, Continuous Mode

After one meandering treatment ($n = 5$), three CAMs displayed symptoms of mild irritation and individual hemorrhages, while two CAMs showed multiple hemorrhages. The double treatment led to thromboses in two of six CAMs. One CAM had massive hemorrhages, three CAMs only showed isolated hemorrhages (Figure 9). After three treatments in the continuous mode, five of six CAMs displayed thromboses. One CAM demonstrated isolated hemorrhages. Wetting with 0.9% sodium chloride and triple treatment led to isolated lysis and discoloration in one CAM, to thromboses in another, and to isolated hemorrhages in the third CAM. The continuous treatment led to hemorrhages and thromboses within 5 min. The targeted treatment over the length of 40 s provoked burns, while these could not be observed when the treatment was only applied for 10 s. The incubation for an additional 24 h led to an increase in opacity in all eggs treated two or three times (Figure 10, Table 4). In the continuous mode the irritancy is more pronounced. In terms of application as a wound

DOI: 10.1002/ppap.200900119

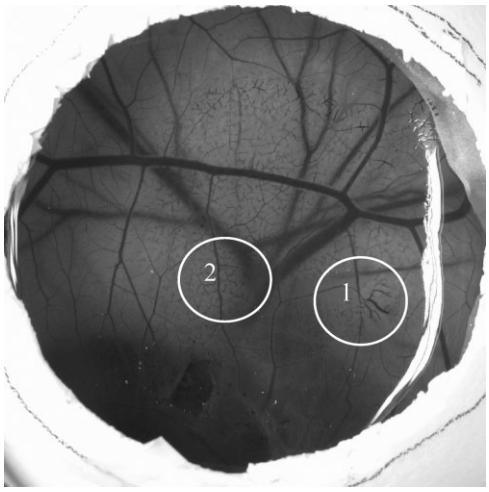

Figure 9. Egg 30, CAM, 5 min after double treatment with continuous argon-plasma, (1) hyperemia, (2) isolated hemorrhages.

antisepsis, only a single application seems to be acceptable. Because of the small number of samples the above-named influence of wetting with 0.9% sodium chloride solution could not be verified.

As temperatures between 54 and 79 °C are reached in the continuous mode, the results of the targeted treatment must have been influenced by thermal effects. The intensity of the effects correlated with the treatment length. At a speed of 30 mm · s^{-1}, no burns were provoked; however, an increase in the irritation due to local temperature increases may be responsible. Pretreatment with sodium chloride led to milder irritations as a result of the lower temperatures.

Because the energy impact of argon plasma in the tested setting has an antimicrobial effect, the results are relevant for wound antisepsis.[23]

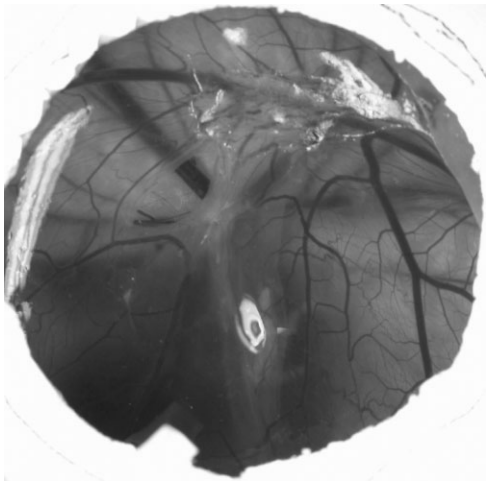

Figure 10. Egg 30, CAM, increased opacity 24 h after double treatment with continuous argon-plasma.

Table 4. Scores of the CAM after single to triple treatment with plasma in the continuous mode.

Mode	Egg (no.)	Score after 1 min	Score after 5 min	Score after 24 h
1 × continuous	23	4	4	
	24	4	5	
	25	4	5	
2 × continuous	26	5	6	
	27	4	8	
	28	4	8	
	29	4	4	
	30	4	4	8
	31	8	8	8
3 × continuous	32	8	8	
	33	8	8	
	34	8	8	
	35	4	4	
	36	4	8	8
	37	7	8	8
3 × cont. + NaCl	38	5	7	
	39	8	8	
	40	4	4	
3 spots (3 × 40 s)	62	8	8	
	63	8	8	
	64	8	8	
3 spots (3 × 40 s) + NaCl	65	8	8	
	66	4	4	
5 spots (5 × 10 s)	67	4	4	
	68	5	5	
	69	5	5	

Dielectric Barrier Discharge (DBD) Plasma

The single treatment ($n = 3$) led to isolated hemorrhages in one case and multiple hemorrhages in another. The third CAM displayed additional thromboses. After prior wetting with 0.9% sodium chloride, mild hyperemia was observed in one CAM. A second case had only mild hemorrhages and a third case thromboses. Of the CAMs treated twice, all specimens showed hemorrhages and thromboses after 5 min. Of those wetted with 0.9% sodium chloride before the treatments, two of three CAMs showed thromboses, while one CAM showed isolated hemorrhages. The triple treatment led to thromboses in three of four cases (Figure 11). One CAM displayed only mild hemorrhages. Those wetted with 0.9% sodium chloride before the treatments all had thromboses after 5 min. The eggs that had previously

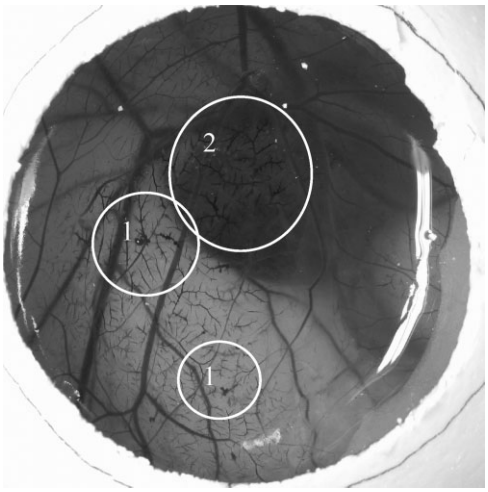

Figure 11. Chorioallantoic membrane (CAM), 5 min after triple treatment with DBD-plasma (wetted with 0.5 ml 0.9% sodium chloride solution) (1) punctiform hemorrhagic (2) intravasale thrombosis.

shown mild irritations had no symptoms after being incubated longer. In the eggs that had shown thromboses, the symptoms disappeared in 4 of 15 CAMs, while hemorrhages remained. Increased opacity could be observed in the remaining eggs (Table 5). These results show that DBD plasma is too harmful when applied to wounds in the test setting. Nevertheless, the dose-response relationship is apparent.

The analysis of the three settings shows that from slight hyperemia up to intravascular thromboses, all types of irritation can be provoked by plasma depending on the exposure time and the type of plasma. DBD plasma causes thrombosis with only one application, while two applications in the continuous mode and three applications in the pulsed mode are necessary to cause the same result. The pulsed treatment provoked the mildest irritations and the irritation increased with the number of treatments (Figure 12). The DBD treatment provoked the most intense irritations on average, which corresponded to the positive control with 1% SDS. Four factors are possibly responsible for the stronger thrombosis of DBD plasma. Calorimetric measurements showed that the thermal output of the DBD is up to 200 mW, while only 150 mW in the HF Plasma Jet (continuous mode) are transformed into heat. Moreover, the DBD operating in air creates a higher amount of radiation in the UVA and UVB sector, caused by nitrogen bands. Furthermore, using DBD, the egg is the counter electrode, so that the electrical current goes through the CAM. Also, compared to Argon plasma, other species of radicals are expected in the

Table 5. Scores of the CAM after single to triple treatment with DBD plasma.

Mode	Egg	Score after 1 min	Score after 5 min	Score after 24 h
1 × DBD	41	5	5	8
	42	5	8	8
	43	4	4	0
2 × DBD	44	8	8	5
	45	8	8	8
	46	4	8	8
	47	8	8	
3 × DBD	48	4	4	
	48	8	8	5
	50	8	8	6
	51	4	8	5
1 × DBD + NaCl	52	8	8	
	53	4	4	0
	54	1	1	0
2 × DBD + NaCl	55	8	8	8
	56	4	4	0
	57	8	8	
3 × DBD + NaCl	58	8	8	8
	58	4	8	8
	60	8	8	8
	61	8	8	

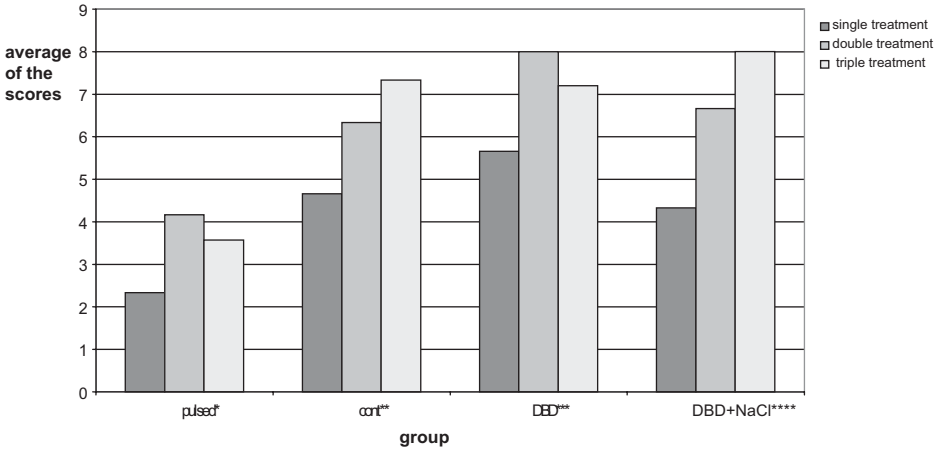

Figure 12. Average of the scores 5 min after single, double and triple treatment with plasma: *argon-plasma, pulsed mode; ** argon-plasma, continuous mode; ***DBD-Plasma; ****DBD + NaCl = DBD-plasma after wedding with 300 µl 0.9% sodium chloride solution.

DOI: 10.1002/ppap.200900119

DBD plasma. Additional studies are necessary to explain these effects.

To examine the effects of the temperature of the DBD-plasma, we chose a static setting with a thermometry with fluoroptic probes inserted in the CAM closely beneath the area of the CAM contacting the plasma. In this static setting, we observed a time-dependent local decrease in the temperature up to 65 °C in 100 s. Therefore, a thermal influence of the DBD-plasma is given.

Survival Rate and Excitability 24 h after Plasma Treatment

The mortality rate was 26.3% on average, independent of the controls and treatments. Death of the embryos after 24 h can be ascribed to the interruption in the incubation for the duration of the experiments. Additionally, uncovering of the CAM leads to a cooling of the egg. Removal of the eggshell may lead to an imbalance in the micromilieu, resulting in a disruption of egg's metabolism. As no differences could be observed between the control and the plasma groups, it can be assumed that toxic reaction products are not produced in amounts that are lethal for the embryo as monitored in this time frame. Of course, later side effects are possible, i.e., genotoxic effects.

It is noteworthy that a reversibility of the symptoms was observed depending on the type of plasma. The effects were completely reversible if only isolated hemorrhages were present 5 min after treatments. Multiple hemorrhages were only partially reversible. Of the CAMs showing intense responses, most displayed increased opacity of the gelatinous mass 24 h after treatment, which led to maximal scores. In order to check whether this delayed reaction resulted from the coagulation of albumin, the egg whites of non-incubated eggs ($n = 10$) were treated with the same experimental settings and incubated at 37° for 24 h. Increased opacity was not evident. An explanation thus does not exist at present. Hypothetically, the extravasation of fibrinogen and plasma-activated coagulation to fibrin may have occurred, leading to increased opacity.[12]

The goal of the plasma treatment must be considered when interpreting scores. Plasma can, e.g., be implemented, depending on the mode and exposure time, to provoke coagulation or to increase circulation. Possibilities include use as an antiseptic for the skin, mucous membranes and wounds, wound debridement, irritation therapy for skin disorders, removal of biofilms from the oral cavity, as well as targeted vascular obliteration.

Conclusion

It was demonstrated that the HET-CAM test can be used to assess the irritation potential of non-thermal plasma treatment. Furthermore, the HET-CAM allows screening different plasma sources for determining tolerable application parameters in regard to irritancy. For application to sensitive tissues such as mucous membranes, wounds, or the eye, the pulsed plasma in the tested setting appears to be the least harmful, possibly due to the negligible thermal effects.

Acknowledgements: This work was realized within the framework of the multi-disciplinary research cooperation "*Campus PlasmaMed*", particularly within the project "*PlasmaBiozid*". The authors acknowledge that this work was supported by a grant funded by the *German Ministry of Education and Research* (BMBF, grant no, 13N9779).

Received: July 21, 2009; Revised: October 16, 2009; Accepted: October 23, 2009; DOI: 10.1002/ppap.200900119

Keywords: dielectric barrier discharge (DBD); HET-CAM; implant; irritancy; membranes; Plasma Jet; plasma treatment

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DOI: 10.1002/ppap.200900119