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The modified HET-CAM as a model for the assessment of the inflammatory response to tissue tolerable plasma

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ABSTRACT

Plasma medicine is a novel, highly interdisciplinary field of research. Although the knowledge is rare concerning plasma based biomedical mechanisms, correct dosages and treatment times, animal experiments have been carried out. To follow the 3Rs (reduction, refinement, replacement), it is necessary to define methods for the screening of plasma parameters.

In order to determine a reliable test and validate the use of tissue tolerable plasma (TTP) for the treatment of chronic wounds, we have selected the modified hen's egg test on the chorioallantoic membrane (mod. HET-CAM) as a model to benchmark the inflammation potential of plasma.

Inflammations of different intensities provoked by using an HF-plasma jet corresponded to the time of plasma–tissue interaction. Additionally, the plasma mode and the gas composition were changed to assess their influence on the efficacy of treatment. Pulsed plasma led to the mildest inflammation, while the addition of 0.1% oxygen to the argon carrier gas led to the most distinct reaction. It was found that the influence of the exposure time was greater than that of the mode and the gas composition. All inflammations were alleviated, when hydrocortisone (HC) was added immediately after plasma treatment.

The results of the study demonstrate that the modified HET-CAM test is suitable for screening plasma sources and for the determination of the optimum parameters for treatment of chronic wounds. To transfer the chronic wound into an acute healing wound without harmful inflammation, the maximum time for plasma–tissue interaction should not exceed 5 s with the tested plasma source.

Furthermore, it is possible to induce defined plasma-inflammations on the chorioallantoic membrane and to determine the anti-inflammatory potential of test substances.

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1. Introduction

Physical plasma as the fourth aggregate state is generated by supplying gases with energy, for instance, by electrical discharge (Moisan et al., 2001). Plasma contains positive and negative ions, free radicals, excited and non-excited molecules as well as photons (Laroussi, 2005). It emits UV light and heat (Lademann et al., 2009) moreover, it has electromagnetic fields of various intensities. Thus, plasma is a highly energetic, extremely reactive state providing a wide range of applications. The surface modifying effects of plasmas have been utilized in technology for decades (Liston et al., 1993). In medicine, plasma has so far been used for the argon plasma coagulation (Raiser and Zenker, 2006), the sterilization of thermolabile materials (von Woedtke and Weltmann, 2008) and the surface modification of medical products by modifying the hydrophobicity and applying drug delivery systems (Maldonado-Codina et al., 2004).

Following the successful generation of TTP (up to now so-called non-thermal plasmas) with temperature spectra in or slightly above the physiological range, new possibilities are opening up for application in the living organism (Weltmann et al., 2010). Considering the wide range of promising potential applications, plasma medicine has recently become an independent interdisciplinary field of research (Fridman et al., 2008). Recent research results show that TTP are opening up new treatment possibilities (Fridman et al., 2007; Kramer et al., 2010; Morfill et al., 2009), for example in oncology (Vandamme et al., 2010), dermatology

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(Daeschlein et al., 2010; Lademann et al., 2010), dentistry (Koban et al., 2010; Rupf et al., 2009), veterinary medicine (Watts et al., 2006), advancing, moreover, the dermal penetration of substances (Kramer et al., 2009; Lademann et al., 2010). Chronic wound treatment is a very promising application when chronic wounds are to be transferred into acute healing wounds (Kramer et al., 2010) in order to interrupt the circulus vitiosus of the persistent inflammatory reaction. It could already be proven that TTP can stimulate the proliferation of endothelial cells (Lloyd et al., 2010). The ability of this new technology to coagulate tissue and its influence on the circulation may add further stimuli to wound healing (Kramer et al., 2010). The positive action of the plasma seems to be caused by the synergy effects of the different physicochemical action mechanisms like UV radiation, oxygen radicals, ozone, nitric oxides and electrical fields, temperature and pH effects (Laroussi, 2005). A positive antiseptic side effect may occur (Hammann et al., 2010).

Although little is known, yet, about the biomedical mechanisms of the therapeutic use of plasmas and the related treatment times necessary, preliminary animal experiments have already been performed (Fridman et al., 2008; Hoene et al., 2009; Vandamme et al., 2010). According to the 3R concept (Russell and Burch, 1959) it is indispensable for ethical reasons, however, to set up models, which provide and widen our understanding of the biomedical effects of plasma, thereby abandoning animal tests to the highest possible extent. At the same time, conclusive screening methods are required to define the properties of TTP. This refers to both the quantification of desired effects and the detection of undesired effects.

The chorioallantoic membrane (CAM) of hen eggs had already been successfully as a wound model (Ribatti et al., 1996), as a model for inflammatory responses (Krenn and Paper, 2009; Zwadlo-Klarwasser et al., 2001) and as model for tolerability testing of eye antiseptics (Kramer and Behrens-Baumann, 1997). Investigating the irritancy of TTP in the HET-CAM we found that plasma induced irritations depended on the dosage applied and led, moreover, to time-delayed effects (Bender et al., 2010). In the present study, the HET-CAM was modified to investigate the inflammatory effects of TTP, based on the hypothesis that an induced inflammation would activate chronic inflammations to start the healing process (Huggenberger et al., 2010; Gonzalex-Rubio et al., 1996; Sundaresan et al., 1995). Applying TTP with a high-frequency (HF) plasma jet, dosage-dependent inflammations at different severities were induced and evaluated according to a scoring system.

2. Materials and methods

2.1. Plasma

The plasma was generated with the atmospheric pressure plasma jet kINPen09 (Neoplas GmbH, Greifswald, Germany, CE certification No. 609.003.1) (Fig. 1) described in Weltmann et al. (2009), with argon being used as a carrier gas. The plasma was used both in continuous and in pulsed mode with the following settings:

- Continuous mode: gas flow: 4 standard liter per minute (slm), voltage: Upp = 170 V, power: P = 18 W_f, 14 W_r, frequency: f = 1.82 MHz.
- Pulsed mode: gas flow: 3 slm, voltage: Upp = 310 V, power: 9 W_f, 7 W_r, frequency: f = 1.82 MHz; pulse: 35 cycles à 0.55 μs = 19.25 μs on,−100 μs off (oscilloscope measurement).

In continuous mode, the plasma temperature was 45 °C, in the continuous mode with added oxygen, it was 49 °C (length of the effluent plasma 11 mm from the nozzle), in pulsed mode it was 47 °C (length of the effluent plasma 7 mm from the nozzle) (Figs. 2 and 3).

2.2. Eggs

The eggs (Vakzine Lohmann specific pathogen free eggs; VALO SPF, Lohmann GmbH, Cuxhaven, Germany) were incubated for 6 days at 37 ± 1 °C and 65 ± 7% relative humidity in a small-motored breeder (KMB F/2, Ehret GmbH, Emmendingen, Germany) containing an automatic rotating mechanism and an automatic humidity regulation. On the 4th day of incubation, 6 ml albumin was removed from each egg by injecting a 22-gauge needle at the cuspid pole of the egg. The hole was recovered with a self-adhesive tape. The eggs were placed with the blunt pole containing the air space facing upwards, and incubated for two additional days without further rotation. The experiments were conducted on day 6. Unfertilized eggs were excluded from the study. After evaluation on day 7, the eggs were frozen at −20 °C, killing the embryos.

2.3. Test procedure

All in all, 186 CAMs were tested spread over 10 plasma groups and six control groups (Table 1), with a minimum of six CAMs per group being investigated. The tests were documented before and after the plasma application on the 6th day of incubation and 24 h later, i.e., on day 7, using a photo camera (Canon powerShot G9, Canon Deutschland GmbH, Krefeld, Germany).

2.3.1. Control group

Positive control was by point treatment with the inflammatory agent sodium dodecyl sulfate (SDS; Sigma–Aldrich, Steinheim, Germany). For this purpose, an SDS containing UltraPure® Low-Melting-Point-Agarose (LMPA) pellet (Invitrogen GmbH, Darmstadt, Germany) was applied to the CAM to induce a mild

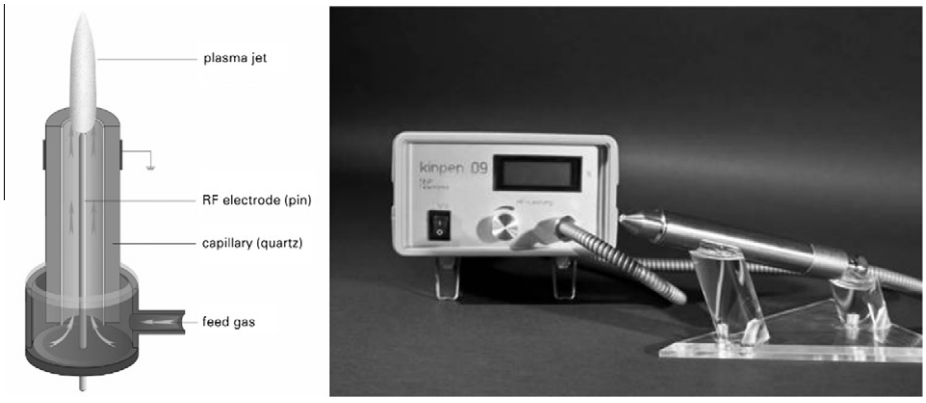

Fig. 1. Schematic (left) and photo (right) of the plasma source kINPen09.

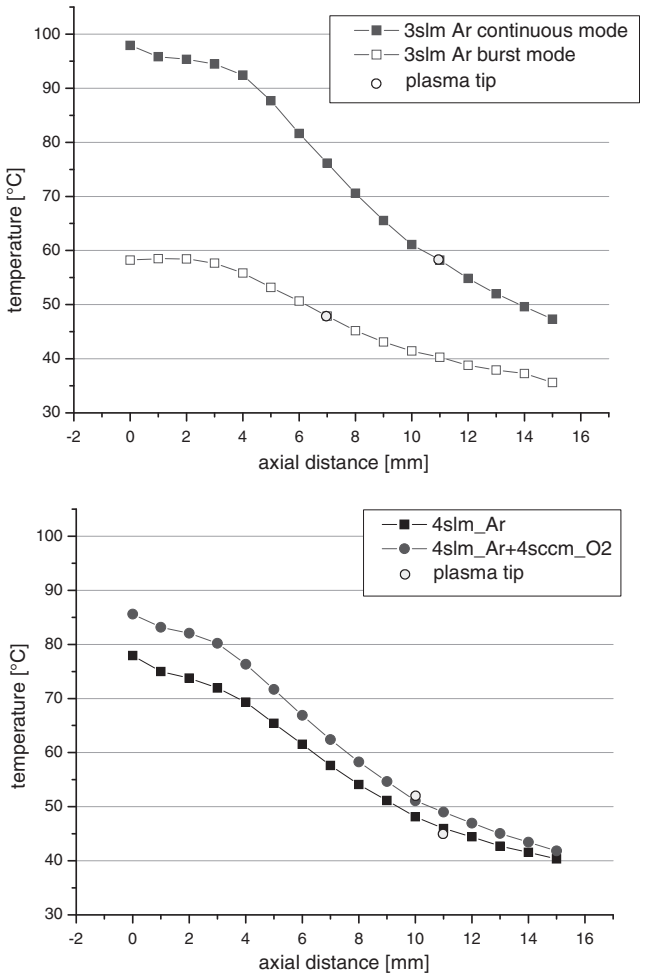

Fig. 2. Temperature progression curve of the plasma effluent (fluor optical measurement at the axial distance of the nozzle), depending on gas flow (left: 3 slm, right: 4 slm), the mode (left: continuous and burst mode, right: continuous mode) and gas composition (left: argon, right: argon and argon with 4 sccm oxygen added).

Fig. 3. Point by point treatment of the chorioallantoic membrane using argon plasma.

inflammatory reaction. For the production of the SDS pellets according to (Bürgermeister et al., 2002), 50 µg SDS were suspended in 10 µL LMPA.

In addition, a control group was formed, in which Hydrocortisone pellets (HC; Caesar & Loretz GmbH, Hilden, Germany) (50 µg HC suspended in 10 µL LMPA analogously to the SDS pellets) were applied to the CAMs as an antiphlogistic agent (Dannhardt et al., 1996).

In another control group, 50 µg, each, of SDS and HC were suspended in the 10 µL LMPA together as pellet and applied to the CAM (SDS+HC). For negative control, pure LMPA pellets were applied to the CAMs.

All the pellets remained on the CAMs until evaluation after 24 h according to Bürgermeister et al. (2002).

2.3.2. Plasma resp. gas exposure of the CAM

The plasma treatment was carried out point by point in continuous mode for different application times (cont5s, cont10s, cont20s). In pulsed mode, the plasma–tissue interaction time took 20 s (puls). For one test group, oxygen at a concentration of 0.1% was added to the argon carrier gas by means of a flow controller (was equalent of 4 sccm oxygen in 4 slm argon). This test group was then treated point by point for 20 s (cont+O_2).

To reduce the development of the expected inflammatory response, HC pellets (50 µg HC suspended in 10 µL LMPA analogously to the SDS pellets) were applied to other test groups, both in continuous (cont+HC) and pulsed (pulse+HC) mode, as well as in the group with the admixing oxygen (cont+O_2+HC), immediately after the plasma treatment.

To document the isolated argon gas effect on the CAM, the CAMs of one group were treated with pure argon gas for a period of 20 s without plasma generation.

For all test groups, the distance of the jet nozzle to the CAM was chosen, so that the plasma directly contacted it (pulsed plasma: 7 mm, continuous plasma: 10–11 mm) (Fig. 3).

2.4. Evaluation

The response patterns – granuloma development (with associated spoke wheel-like angiogenesis), hemorrhages, coagulation and contracture – were classified by two calibrated evaluators according to a scoring system, rating the response of each CAM depending on the degree of severity (from degree 1 – no response to degree 5 – heavy response) (Table 2). The non-directional increase in the capillary density as an enhanced angiogenesis was evaluated additionally to the spoke wheel-like angiogenesis within the scope of the inflammation. Taking into consideration that the macroscopically interpretable non-directional angiogenesis had been mild, only three from five scores were reached (Table 2). The spoke wheel-like angiogenesis was included in the granuloma evaluation, because it developed as a direct result of the inflammation (Bürgermeister et al., 2002). Here, a score of five was achieved.

The individual values were then added to form a score sum (SS), and the mean value was calculated from the score sums of all CAM of one treatment mode (score sum mean, SSM) (Table 1). A CAM, which had remained unchanged after 24 h was scored SS five (minimum), the maximum SS being 23.

The score allocated to the plasma tolerance towards sensitive tissues like wounds is described in Table 3. The allocation is based on the reaction of the CAMs in line with Kramer and Behrens-Baumann (1997). Five different and clearly visible tolerance categories can be distinguished. Allocating the SSM, we tried to take this classification into account.

3. Results

3.1. Control effects

The CAM remained unchanged after 24 h of exposure to the LMPA pellets (negative control), which had been applied.

Table 1
Treatment modes, number of samples, mean value of score sum, confidence interval and average values of the response patterns.

Treatment	*n*	SSM	Confidence interval	Granuloma	Haemor-rhage	Coagula-tion	Contacture	Angioge-nesis
cont5s	18	10.22	1.968	2.33	2.22	2.39	2.28	1.00
cont5s+HC	10	7.2	1.568	2.52	2.80	2.82	2.16	1.00
cont10s	27	12.89	1.230	3.01	3.14	3.24	2.47	1.00
cont10s+HC	8	7.75	0.811	1.25	2.38	2.00	1.13	1.00
cont20s	20	13.8	1.657	3.60	3.20	3.40	2.60	1.00
cont20s+HC	12	11.92	2.082	2.42	3.08	3.08	2.33	1.00
puls20s	6	13.17	2.112	3.33	2.50	3.00	3.33	1.00
puls20s+HC	6	9.67	1.872	1.83	2.50	2.17	2.17	1.00
cont20s+O_2	6	14	2.993	3.33	2.83	3.33	3.50	1.00
cont20s+O_2+HC	9	12.11	1.802	2.44	3.00	3.00	2.67	1.00
Gas	10	10.4	1.965	2.10	2.40	3.00	1.90	1.00
Gas+HC	10	7.8	1.568	1.40	2.10	1.70	1.60	1.00
HC	11	6.27	0.840	1.05	1.73	1.27	1.05	1.18
SDS+HC	9	6.89	0.947	1.22	1.33	1.33	1.11	1.89
SDS	15	7.73	1.139	1.47	1.40	2.27	1.60	1.00
LMPA	9	5	0	1.00	1.00	1.00	1.00	1.00

Table 2
Scoring system to evaluate the inflammatory response of the chorioallantoic membrane.

Reaction pattern	Manifestation	Score
None		1
Hemorrhage	Slight, point	2
	Moderate, centered	3
	Massive, centered	4
	Massive, laminar	5
Contracture	Slight, point	2
	Moderate, centered	3
	Massive, centered	4
	Massive, laminar	5
Coagulation	Slight, point	2
Increased opacity	Moderate, centered	3
(extra vascular)	Massive, centered	4
	Massive, laminar	5
Granuloma	Become visible, up to 2 mm	2
	>2–3 mm	3
	>3–4 mm	4
	>4 mm	5
Angiogenesis	Slight increase in capillary density	2
	Distinct increase in capillary density	3

Table 3
Assignment of score sum (SS) to the compatibility.

SS	Compatibility
5–7	Very good, without inflammation
7.1–9	Very good, with mild inflammation
9.1–11	Moderate, with medium inflammation
11.1–13	Poor, with severe inflammation, applicable to limited extent, only
>13	Insufficient, with high-grade inflammation, application is not recommended

For the positive control, SDS was used to induce mild inflammations after 24 h (SSM 7.73; Table 1). The SDS effect was most distinct as coagulation in the CAM (score mean value of 2.27). A non-directional angiogenesis was not observed (score mean value of one). The development of mild granuloma was observed (score mean value of 1.47).

In the SDS HC group, a non-directional angiogenetic effect was observed. In addition, this effect, although very weak, was also found in the HC group. Apart from the angiogenesis, the effects in the SDS HC group were weaker compared to those of the SDS group (Table 1).

The strongest effect caused by the control using gas was coagulation in the form of an enhanced opacity outside the vessels (SSM 10.4; Table 1; Figs. 4 and 5).

3.2. Plasma effects

All plasma treatments caused macroscopically visible inflammations (Figs. 6–9). Compared to the pure gas treatment, all types of plasma treatments induced stronger inflammatory responses (Fig. 4). The response to treatment with continuous plasma for 5 s (SSM 10.22; Table 1, Fig. 9), for example, was almost as strong as treatment with argon for 20 s (SSM 10.4; Table 1, Fig. 5). A distinct time–effect relationship became evident among the groups subjected to plasma treatment in continuous mode (Table 1, Fig. 4).

Plasma treatment was carried out for 20 s, each, in continuous and in pulsed mode, as well as in continuous mode adding 0.1% oxygen to the argon carrier gas. While the group treated with the oxygen additive showed the strongest inflammatory response (SSM 14.0; Fig. 8), treatment in pulsed mode induced the mildest reaction (SSM 13.17; Fig. 6), with an SSM of 13.8, treatment in continuous mode ranked in between (Table 1, Fig. 4).

Although the group subjected exclusively to HC treatment showed an insignificant reaction of the CAM (SSM 6.27), all groups, in which HC was used in combination with another mode of treatment, showed distinctly alleviated inflammatory responses (Table 1, Figs. 4 and 7).

4. Discussion

The HET-CAM is successfully established as a predictive model for eye tolerance of chemical agents and is also used as a wound model. The only problem is that there is no validation of the HET-CAM against an appropriate wound model in animals. The HET-CAM predicts the suitability of agents for wound treatment. The old surgical aphorism "do not apply anything to a wound that you would not put in your eye" remains pertinent (Leaper et al., 2010). As a consequence, eye antiseptics are used as wound antiseptics and vice versa (Kramer and Rudolph, 2002), so that an established wound antiseptic was introduced as the standard for preoperative eye antisepsis (Hansmann et al., 2004). Because up to now there has been no acceptable animal model for chronic wounds, in vitro results on cytotoxicity and from the HET-CAM have been extrapolated to test potential wound antiseptics (Kramer et al., 2004), e.g., on human skin grafts (Reimer et al., 2000). Furthermore, cold atmospheric pressure plasma was already used in a trial to decrease bacterial load on chronic wounds in patients (Isbary et al., 2010). For this reason and according to the results of the explant test on skin and peritoneum explants of neonatal rats (Kramer et al., 1998), we chose the HET-CAM for this study, because this model reacts more sensitively than do the

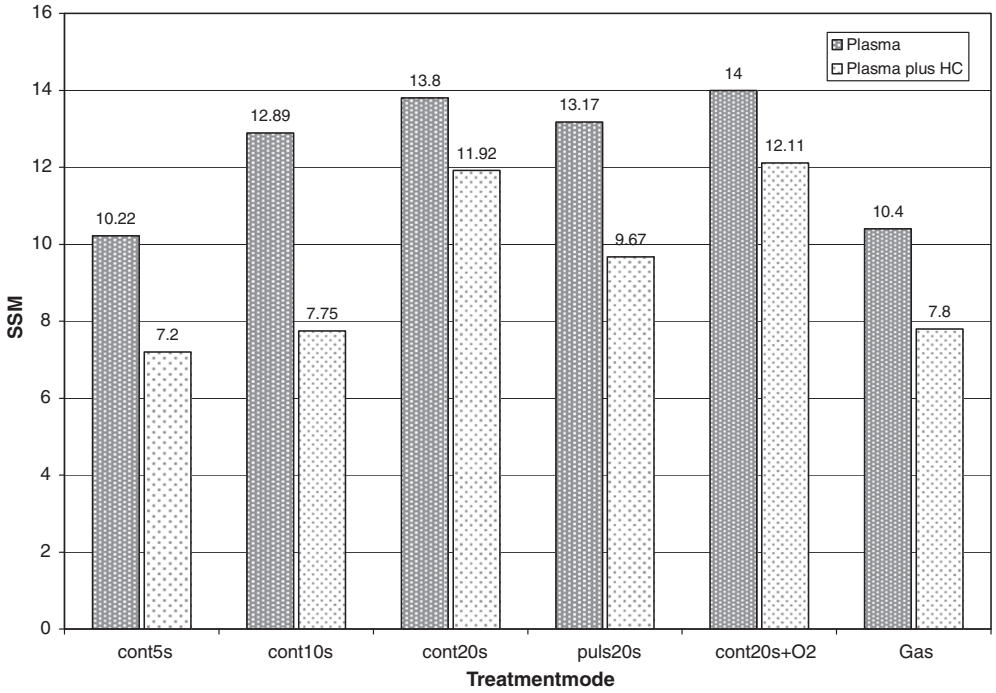

Fig. 4. Mean values of the score sums means (SSM) 24 h after plasma application with and without hydrocortisone.

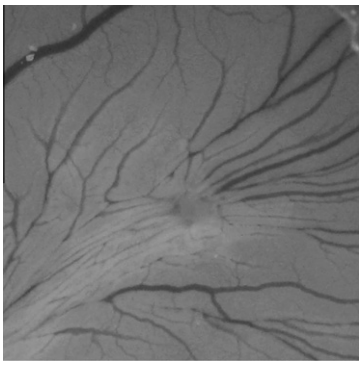

Fig. 5. Chorioallantoic membrane, 24 h after treatment wit argon gas for 20 s.

eye and the explants, and thus provides a margin of safety. Of course, this model will not lead to a reduction in the number of experimental animals, but it will avoid new animal tests.

The study carried out had three objectives. In the first place, it had to be clarified whether the modified HET-CAM test was suitable to evaluate TTP for its inflammatory responses. To verify whether or not the induced responses are inflammatory, test groups were formed. To these test groups one pellet containing HC at an antiphlogistically efficient concentration (0.5%) was applied after plasma treatment, in order to alleviate the response classified as inflammation. The test results confirmed that TTP induced, indeed, inflammations (Fig. 4).

Another objective of the study was to establish to what extent the plasma treatments cause reactions which may be beneficial for application to chronic wounds, for instance, coagulation, contracture and angiogenesis, as these reactions are considered essential for secondary wound healing (Lange-Asschenfeldt, 2009). All three reactions could be successfully induced by TTP, although angiogenesis manifested only in the form of a directional spoke wheel-like angiogenesis as part of the inflammation-related development of granuloma (Fig. 6). In this context, angiogenesis was only subjectively classified by two calibrated evaluators. Closer

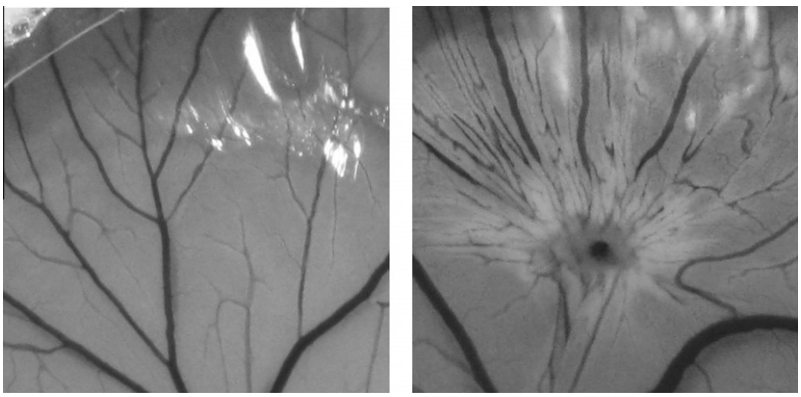

Fig. 6. Chorioallantoic membrane before (left) and 24 h after pulsed argon plasma application for 20 s (right) with granuloma development and spoke wheel-like angiogenesis, contracture and central hemorrhage.

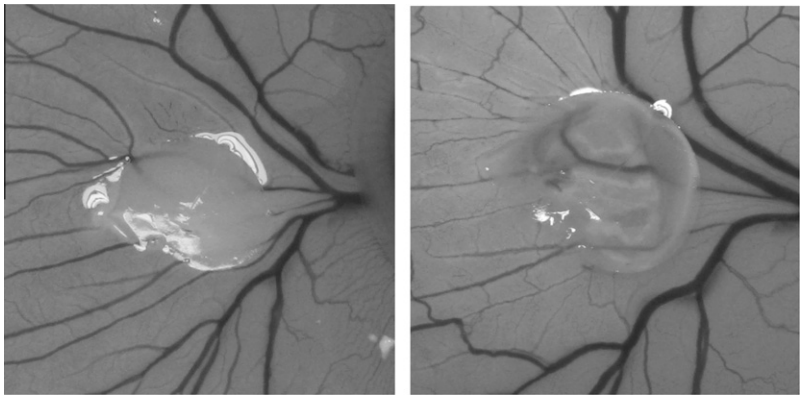

Fig. 7. Chorioallantoic membrane immediately after treatment with continuous argon plasma for 10 s and application of a 50 μg HC Pellets (left) as well as 24 h later (right).

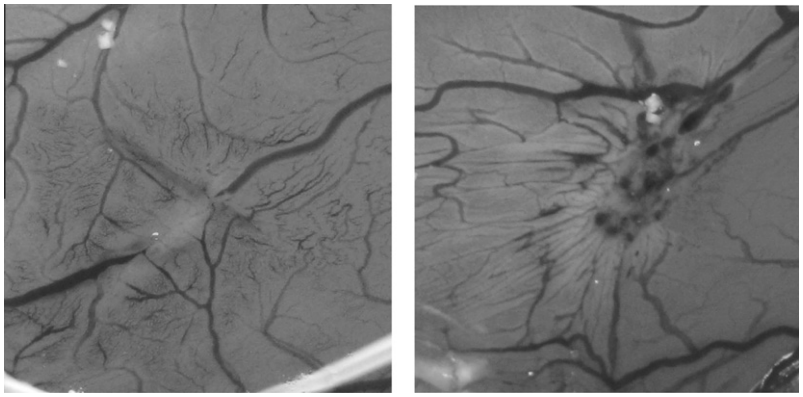

Fig. 8. Chorioallantoic membrane immediately after treatment with continuous argon plasma for 20 s with 0.1% O_2 additive (left) as well as 24 h later (right).

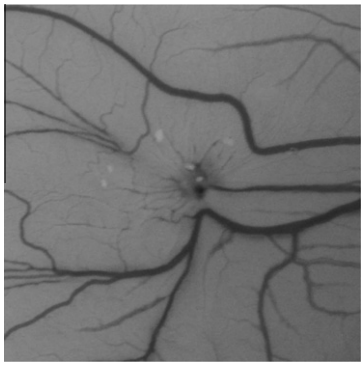

Fig. 9. Chorioallantoic membrane, 24 h after treatment with continuous argon plasma for 5 s.

focus on angiogenesis would require histomorphometric evaluation to increase objectivity (Maas et al., 2001).

Finally, the parameters relevant to the strength of the TTP effect were to be determined. The plasma effects are influenced by the type of plasma, the application time and the gas stream. To determine the influence of the application time, various treatment times were selected for evaluating a dosage–effect relationship as contact time–effect relationship. A distinct contact time–effect relationship was established (Table 1, Fig. 4).

In addition, several types of plasma (pulsed and non-pulsed argon plasma; argon plasma enriched with oxygen) were compared. Both the mode and the composition of the gas led to different effects (Table 1, Fig. 4). The mode-related differences may be explained by various causes. Compared to the pulsed mode, the plasma generated in continuous mode proved to be "denser" because reactive species were continuously generated (Walsh et al., 2006). The reduced gas flow in pulsed mode results in a slightly lower mechanical influence. Since the SSM of 13.17 in pulsed mode at 20 s and gas flow of 3 slm deviated only slightly from the SSM of 13.7 in continuous mode at 20 s and gas flow of 4 slm, could be significantly compared to the SSM of 10.22 after 5 s of treatment with continuous plasma and gas flow of 4 slm, thus the treatment time is obviously more important than the gas flow. Moreover, the gas composition influences the effects. The addition of 0.1% oxygen resulted in a slightly stronger inflammatory response compared to the application of pure argon gas, which is supposed to be due to the increased formation of reactive oxygen species like ozone and OH radicals (Nagatsu et al., 2005; Laroussi, 2005). Also, the temperature of the continuous argon plasma enriched with oxygen was higher (Fig. 2). The variations in the effects due to a change from pulsed to continuous mode, or vice versa, or due to the addition of oxygen were, however, smaller than those caused by different time modi. This shows that the treatment time played a much greater role than the mode and the gas composition applied.

Considering the results achieved regarding the effects of plasma it is recommended to apply TTP point by point at 4 slm for 5 s (SSM 10.22), at a maximum, on chronic wounds (Table 3), as this dosage has a stimulating effect due to the mild inflammatory response and is also sufficiently tissue-compatible. If longer application times are required, HC can be used to alleviate the inflammatory response.

The gas stream generates two essential effects. Firstly, it mechanically irritates the CAM and secondly, it intensifies the evaporation of moisture on the surface and, consequently, cooling of the CAM. A combination of these effects could induce a contracture of the smooth muscle cells with the subsequently observed

effects upon the CAM structure. The mechanical pressure could be responsible for the intravascular fluid being transferred to the extra vascular tissue, which could result in extravasale coagulation with an enhanced opacity. The strong responses to the sole point impact of the gas observed distinctly differ from the CAM response when the argon gas is applied on a meandering course. CAMs subjected to meandering gas application proved to be unchanged 24 h after testing in a previous study (Bender et al., 2010). This implies that the gas stream applied point by point to highly sensitive tissues induces a pronounced autoresponse, if the contact time lasts several seconds, while short contact times in a meandering course are tolerated without any consequences.

HC is able to alleviate the gas effects on the CAM as well as the TPP effects. This should be caused by the properties of HC to stabilize the membranes of the capillaries. Moreover, the inhibition of early and late coming reactions, such as dilatation of capillary, progression of edema, fibrination, leukocyte migration, etc. (Auphan et al., 1995).

Contrary to expectations, the application of HC in the HC control group led to a slightly enhanced SSM, caused mostly by the score mean values of hemorrhage and coagulation. This may be due to the CAM showing a more sensitive response than the human eye (Kramer and Behrens-Baumann, 1997).

The antiphlogistic efficacy of the HC against the various degrees of inflammation on the CAM indicates that plasma is also suitable for generating defined aseptic inflammation on the CAM, which could then be used for investigating the anti-inflammatory properties of test substances in further studies. Consequently, it can be used as a model for the treatment of inflamed wounds.

5. Conclusion

The modified HET-CAM lends itself as a model for the evaluation of inflammatory effects by TTP. Plasma can generate inflammations of various severities depending on the time of contact. Already, within seconds, the treatment time has an influence on the effects stronger than the type of plasma and the setting mode. Angiogenesis effects can be regularly generated as part of the inflammatory responses. HC is suitable to alleviate the plasma responses. In addition, the application of plasma to the CAM may permit the development of a wound inflammation model.

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