Clinical Plasma Medicine 7–8 (2017) 58–65

Contents lists available at ScienceDirect

Clinical Plasma Medicine

journal homepage: www.elsevier.com/locate/cpme

Original research article

Platelets are key in cold physical plasma-facilitated blood coagulation in mice

Sander Bekeschus[a,*,1], Janik Brüggemeier[b,1], Christine Hackbarth[b], Thomas von Woedtke[a,c], Lars-Ivo Partecke[b], Julia van der Linde[b]

[a] Leibniz-Institute for Plasma Science and Technology, ZIK plasmatis, Felix-Hausdorff-Str. 2, 17489 Greifswald, Germany
[b] University Medicine Greifswald, Department of General Surgery, Visceral, Thoracic and Vascular Surgery, Ferdinand-Sauerbruch-Str., Greifswald 17475, Germany
[c] University Medicine Greifswald, Institute of Hygiene and Environmental Medicine, Walther-Rathenau-Str. 49 A, 17475 Greifswald, Germany

ARTICLE INFO

Keywords:
Anticoagulants
kINPen MED
Hemostasis
Plasma medicine

ABSTRACT

Purpose: Surgical interventions inevitably lead to destruction of blood vessels. This is especially dangerous in anticoagulated patients. Electrocauterization is a frequently used technique to seal incised tissue. However, leading to a superficial layer of necrotic tissue, the treated area evolves a high vulnerability to contact, making it prone to detachment. As a result, dangerous postoperative bleeding may occur. Cold physical plasma was previously suggested as a pro-coagulant treatment method. It mainly acts by expelling a delicate mixture of oxidants. We therefore tested the suitability of an atmospheric pressure plasma jet (kINPen MED) as a new medical device for sufficient blood coagulation in a murine model of liver incision.

Methods: Plasma treatment of murine blood ex vivo induced sufficient coagula. This effect did not affect any tested parameter of plasmatic coagulation cascade, suggesting the mechanism to be related to cellular coagulation. Indeed, isolated platelets were significantly activated following exposure to plasma, although this effect was less pronounced in whole blood. To analyze the biological effect of plasma-on blood coagulation in vivo, mice were anticoagulated (clopidogrel inhibiting cellular and rivaroxaban inhibiting plasmatic hemostasis) or received vehicle only. Afterwards, a partial resection of the left lateral liver lobe was performed. The quantification of the blood loss after liver incision followed by treatment with kINPen MED plasma or electrocauterization revealed a similar and significant hemostatic performance in native and rivaroxaban but not clopidogrel-treated animals compared to argon gas-treated controls. In contrast to electrocauterization, kINPen MED plasma treatment did not cause necrotic cell layers.

Conclusion: Our results propose a prime importance of platelets in cold physical plasma-mediated hemostasis and suggest a clinical benefit of kINPen MED plasma treatment as coagulation device in liver surgery.

1. Introduction

Hemorrhage (blood loss) is a major issue during surgical intervention especially in anticoagulated patients [1]. Artrial fibrillation is the most frequently occurring cardiac arrhythmia [2–4], potentiating the risk of cardioembolic strokes [5]. For prevention, new oral anticoagulants (NOAC, e.g. rivaroxaban), are more convenient in comparison to former anticoagulative therapy using vitamin K antagonists (e.g. warfarin) [6]. Therefore, NOAC prescriptions increase. Due to the lack of proper antidotes [7], there is demand for pro-coagulant therapy [8]. Carbonizing and thus necrotizing tissues during endoscopy or surgery in anticoagulated patients, thermal cauterization is successfully in use facilitating hemostasis [9]. However, contact vulnerability of necrotic tissue after cauterization treatment may result in gradual bleeding, putting patients at risks to haematoma, infection, and abcess formation [10].

Hemostasis is important for sealing of wounds at trauma sites [11]. First, vasoconstriction of damaged vessels is initiated to limit blood loss [12]. Second, primary (cellular) hemostasis is initiated in which platelets are activated and release for example ADP binding to ADP receptors on other platelets to set platelet aggregation in motion [13]. This process is counteracted by the anticoagulant clopidogrel [14]. Third, the extrinsic and intrinsic pathway of secondary (plasmatic) hemostasis become activated. Both pathways merge upon factor X (FX) activation in a common pathway with degradation of fibrinogen to fibrin, effectively solidifying the clot [15]. The anticoagulant

* Corresponding author.
E-mail address: sander.bekeschus@inp-greifswald.de (S. Bekeschus).
[1] authors contributed equally to this work.

http://dx.doi.org/10.1016/j.cpme.2017.10.001
Received 7 March 2017; Received in revised form 12 October 2017; Accepted 16 October 2017

rivaroxaban directly inhibits the active form of FX called factor Xa (FXa) [16].

The use of cold physical plasmas has been suggested to mediate hemostasis [17–19]. Cold physical plasma is an ionized gas and some sources, such as the kINPen MED, can be applied to tissues without creating thermal damage [20]. Besides some intrinsic physical properties such as electrical fields, charged particles, and light of different wavelengths and intensity, the expulsion of a delicate mix of reactive oxygen and nitrogen species has been found to be mainly responsible for mediating biological effects [21–23]. Although initial studies in plasma medicine seemed promising regarding cold physical plasmas in pro-coagulative medicine, detailed studies investigating the mechanisms and biological relevance of such an effect are scarce.

In the present work, we utilized an argon plasma jet (kINPen MED) that is accreditated as medical device class IIa for dermatological applicatons as with the directive 93/42/EEC of the European Union [24–26]. Its ability to induce hemostasis was investigated *ex vivo* and *in vivo* in mice. The aim was to investigate i) the ability of plasma to induce hemostasis in murine blood, ii) parameters of platelet and fibrin activation, and iii) the biological relevance of both in a murine liver incision model. *In vivo*, anticoagulants and thermal cauterization were used as controls. We demonstrate that cold physical plasma mediates blood clotting in a platelet-dependent manner without necrotizing any tissue.

2. Material and methods

2.1. Blood collection and platelet separation

The local ethics committee (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern, Rostock, Germany) approved blood collection and animal experiments (approval number: LALLF M-V/TSD/7221.3-1-056/14). 81 female, 12 weeks old C57BL/6N mice were obtained from Charles River Laboratories (Sulzfeld, Germany). Mice were kept for 2 weeks in conventional animal facilities before intervention. During experiments, mice weighted 20 g to 25 g. All substitutions of anesthetics or anticoagulans were done adapted to the individual weight. Prior to blood collection, mice were anesthetized via intraperitoneal injection with 100 mg Ketamin / 10 mg Xylazinhydrochlorid per kg bodyweight using 0.9% NaCl solution as vehicle. Blood collection was done in by retroorbital puncture with a glas capillary tube of 1 cm length. The collected blood was pooled from different animals for each experiment to reach enough volumen. Citrate (3.2%) or heparin (25 IU) was used as anticoagulant for 800 µl of murine whole blood. For downstream experiments, blood was either used directly or centrifuged in modified Tyrode's buffer to retrieve isolated platelets resuspended in phosphate buffered saline with divalent cations. Samples were kept at 37 °C in a water bath to minimize platelet and coagulation factor activation [27].

2.2. Cold physical plasma treatment ex vivo

As cold physical plasma source, an atmospheric pressure argon plasma jet (kINPen MED; neoplas tools, Greifswald, Germany) was utilized. It was operated with three standard liters per minute of argon gas (99.999% purity; Air Liquide, France) at 1 MHz frequency with a pulsed frequency of about 2.5 kHz. The plasma of the kINPen MED was previously shown to be safe without inducing genotoxic effects [28–30]. In 12 well plates (Greiner Bio One, Austria), 500 µl of blood or isolated platelets were directly exposed to the kINPen MED plasma, argon gas alone (Ar), or left untreated. The nozzle of the plasma jet was in about 1 cm distance to the fluid layer which resulted in a direct contact style of plasma jet treatment. As the kINPen MED was mounted to a plotter the plasma was applied perpendicular to the solutions and meander-like movements were run in a standardized manner for either 60 s, 120 s, or 180 s. Argon gas controls were used in the same manner with the plasma being turned off.

2.3. Ex vivo analysis of platelets and clotting parameters

The area of whole blood clot formation in 12 well plates was evaluated by quantitative image analysis of photographs taken immediately following treatment with either kINPen MED plasma or argon gas alone. Fibrinogen in blood plasma of whole blood exposed to either condition was measured using an enzyme-linked immunosorbent assay (ELISA; Affinity Biologicals, Canada) by reading absorbance on a multiplate reader (Tecan, Switzerland). Calcium concentration and pH-values of untreated or treated whole blood was assessed using a blood gas analyzer (Radiometer, Germany). Chemical analysis and calculations were done taking into account evaporation of liquid during the kINPen MED plasma or argon gas treatment. For quantification of liquid evaporation we used an albumin ELISA (Abcam, United Kingdom). Platelet activation was monitored using multicolor flow cytometry. Briefly, treated and control whole blood or platelet isolate was incubated with monoclonal antibodies directed against CD42c (conjugate: fluorescein isothiocyanate) and CD62P (conjugate: phycoerythrin) or respective isotype controls (all Emfret Analytics, Germany). As positive control, convulxin (cvx; Enzo Life Science, USA) was used (final concentration 500 µg/ml) which binds collagen receptors on platelets [31]. This leads to their activation and CD62P expression [32]. Citrated whole blood was subjected to kINPen MED plasma or Ar treatment, or was left untreated before cvx was added immediately or 30 min later to maximally activate platelets. After an initial stimulus, platelet surface expressed CD62P is cleaved from the membrane [33]. Upon a second stimulus, maximal activation (i.e. CD62P expression levels) will be lower if the initial stimulus was activating. At least 10,000 events in the $CD42c^+$ gate were acquired on a Gallios flow cytometer (Beckman-Coulter, USA). Platelet activation in whole blood was done in both citrated as well as heparin anticoagulated blood.

2.4. Liver incision model

All experiments were performed under terms and conditions of a clinical operating room. Throughout the experiements temperature was kept on average to 22.6 °C and humidity to 63.2%. Animals were pretreated with either 50 mg/kg clopidogrel (Plavix; Sanofi, France) or 5 mg/kg rivaroxaban (Xarelto; Bayer, Germany). Tap water was used as vehicle for drugs or as control. The volume of the suspensions matched 10 ml/kg body weight. The application was *intragastral* 50 h, 26 h, and 2 h prior to surgery. Mice underwent laparotomy. A qualified surgeon undertook a partly resection of the left lateral liver lobe resulting in a standardized bleeding area of 10 mm in length. This bleeding area was subsequently treated for one single treatment time of 60 s with either kINPen MED plasma or argon gas alone, or tissue was necrotized using a thermal coagulator (Faromed, Germany). During treatment the kINPen MED was moved manually in a vertical position meander-like along the wound with a speed of 5 mm per second. The distance was kept to about 10 mm as the plasma effluent stayed in contact to the tissue. This simulated terms and conditions of a real operation. Blood was absorbed by a cotton bud during surgery and for up to 30 min after treatment, while swapping cotton buds every 5 min. Each cotton bud was dissolved in 4 ml of 3% acetic acid and absorbance of hemoglobin (405 nm) was measured to quantify bleeding during treatment using a microplate reader (Tecan).

2.5. Histological analysis

One part of the treated liver lobes were fixed in 4% formaldehyd over night and embedded in paraffin. The other part was cryofixated in Tissue-Tec (Sakura Finetek Europe B.V., Netherlands). Paraffin samples were sliced into 2 µm sections and cryofixated tissue was sliced into 5 µm sections. Paraffin sections were stained with hematoxylin and

eosin (HE). Cryosections were stained with FITC-labeled platelet-specific anti-CD42c or anti-fibrinogen antibodies (both Emfret Analytics, Germany). As secondary antibody peroxidase-cojugated anti-rat IgG and as tertiary antibody tetramethylrhodamine (TRITC)-conjugated streptavidin (both Jackson Immuno Research, USA) was used. Images were acquired using a fluorescence microscope BZ-9000 (Keyence, Germany).

2.6. Calculations and statistical analysis

Flow cytometric data analysis was performed using Kaluza 1.5a (Beckman-Coulter). For CD62P expression on platelets, geometric mean fluorescence intensity (gMFI) of positive cells multiplied with the percentage of $CD62P^+$ cells was calculated. Results of fibrinogen ELISA were relative values normalized against a control group of six pooled genetically identical mice. Calculations (e.g., area under the curve), graphing, and statistical analysis was performed using prism 7.02 (GraphPad Software, USA). For intergroup comparison, Mann-Whitney U testing was done with $\alpha = 0.05$ (*); $\alpha = 0.01$ (**); $\alpha = 0.001$ (***).

3. Results

3.1. kINPen MED plasma partially affected murine blood coagulation and platelets ex vivo

The aim of this work was to identify the performance and mechanisms of cold physical plasma (kINPen MED) treatment in murine blood *ex vivo* and *in vivo*. *Ex vivo*, treatment of citrated blood with the kINPen MED (120 s) led to a significantly ($p = 0.0009$) greater clotting area (Fig. 1**A**) compared to treatment with argon gas (120 s) alone (Fig. 1**B**). This was similar in heparinized blood (data not shown). During the treatment of whole blood with either kINPen MED plasma oder argon gas, an empty center appears due to the argon gas flow, which pushes the blood aside. The blood coagulates in the surroundings. The blood stiffness after treatment can be recognized as the remaining cavity, as the blood cannot migrate back directly (Fig. 1).

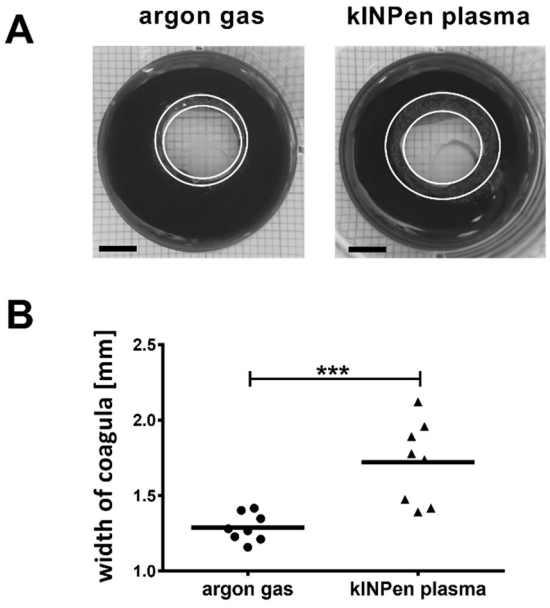

Fig. 1. kINPen MED plasma clots murine blood *ex vivo* Murine blood was added to plastic culture dishes and exposed to argon gas alone (A, left) or kINPen MED plasma (A, right) for 120 s. Empty center was caused by gas flow pushing the blood to the side. Image quantification yielded a significantly enlarged coagula area in the plasma-treated blood (B). Data are presented as one representative (A) and mean of single data points (B) retrieved from pooled blood. Scale bar is 5 mm.

Argon gas treatment caused stiffness of the blood to some degree, presumably due to its drying effect (three standard liters per minute) on liquids. Of note, blood coagula solubilized a few minutes after treatment with both plasma and argon (data not shown). Interestingly, there was no significant ($p > 0.05$) decrease in concentration of fibrinogen in heparinized whole blood (Fig. 2**A**), indicating cellular/corpuscular hemostasis to be important. Divalent calcium is an important coagulation factor as well. Yet, we could not identify a significant ($p > 0.05$) change in its blood concentration following exposure to kINPen MED plasma (Fig. 2**B**). The pH significantly (all $p = 0.0001$) decreased in argon gas and to a greater extend in kINPen MED plasma-treated blood (Fig. 2**C**), suggesting part of the response being related to the drying effect.

Subsequently, flow cytometric analysis of $CD42c^+$ platelets (Fig. 3**A**, left figure) was performed in platelet isolate. Positive controls (convulxin) showed an increase in the platelet activation marker CD62P (Fig. 3**A**, right panel). This effect was also seen with kINPen MED plasma treatment whereas argon gas alone failed to induce an increased CD62P externalization on platelets in platelet isolate (Fig. 3**B**). The kINPen MED plasma gave a graded response, with shorter (60 s) or longer (180 s) treatment times yielding proportionally less or more signal (data not shown). Replicating experiments in murine citrated whole blood, however, yielded no significant (ctrl vs plasma $p = 0.34$; argon vs plasma $p = 0.45$) effects of kINPen MED plasma (Fig. 3**C**). This was independent of the kINPen MED-plasma treatment time or anticoagulant used (data not shown). Convulxin (cvx) was added to whole blood immediately or after 30 min to assess maximal activation of platelets. Platelets were not yet reported to produce CD62P *de novo* [34]. CD62P expression in kINPen MED plasma-treated samples was significantly (ctrl vs plasma $p = 0.01$; argon vs plasma $p = 0.02$) increased compared to argon (120 s) and untreated controls (Fig. 3**D**) when cvx was added immediately after treatment. This increase in maximal activiation argued for a priming activity of kINPen MED plasma. Vice versa, addition of convulxin 30 min after treatment showed a significant (ctrl vs plasma $p = 0.04$; argon vs plasma $p = 0.02$) decrease in CD62P expression in platelets (Fig. 3**E**). This suggested a plasma-induced activation of platelets with a subsequent cleavage of CD62P. Altogether, coagulation was present in kINPen MED plasma-treated anticoagulated murine blood *ex vivo* although lead parameters such as fibrin generation (a marker of fibrinogen degradation) and platelet activation were only partially detected.

3.2. kINPen MED plasma was highly coagulative in vivo in a platelet-dependent manner

To evaluate the efficacy of kINPen MED plasma-mediated hemostasis, a murine liver incision model was performed. Treatment time was 60 s im vivo. Blood loss was quantified by collecting excess blood in a cotton swab (Fig. 4**A**). Wound treatment with electrocauterization caused prominent necrotic lesions (Fig. 4**B**) whereas exposure to kINPen MED plasma was non-necrotic (Fig. 4**C**). In non-anticoagulated animals, electrocauterization and kINPen MED plasma showed a faster response compared to argon gas-treated animals (Fig. 4**D**). This effect was similar in rivaroxaban anticoagulated animals (Fig. 4**E**). By contrast, in clopidogrel anticoagulated animals liver wounds did not response to kINPen MED plasma (Fig. 4**F**). As expected, electrocauterization gave the fastest response in clopidogrel pre-treated mice. Calculating the area under the curve (AUC) of each time series, kINPen MED plasma performed significantly ($p = 0.009$) better in native mice compared to argon gas controls, and similar ($p = 0.83$) to electrocauterization (Fig. 4**G**). Comparable results were obtained in the rivaroxaban group ($p = 0.007$ and $p = 0.57$, respectively) (Fig. 4**H**). This suggested plasmatic hemostasis to be of minor importance in kINPen MED plasma-induced blood coagulation. Inhibiting cellular hemostasis by ADP-receptor blockage on platelets using clopidogrel fully abolished coagulation by kINPen MED plasma but not electrocauterization (Fig. 4**I**). Altogether, the results demonstrated that cold physical plasma

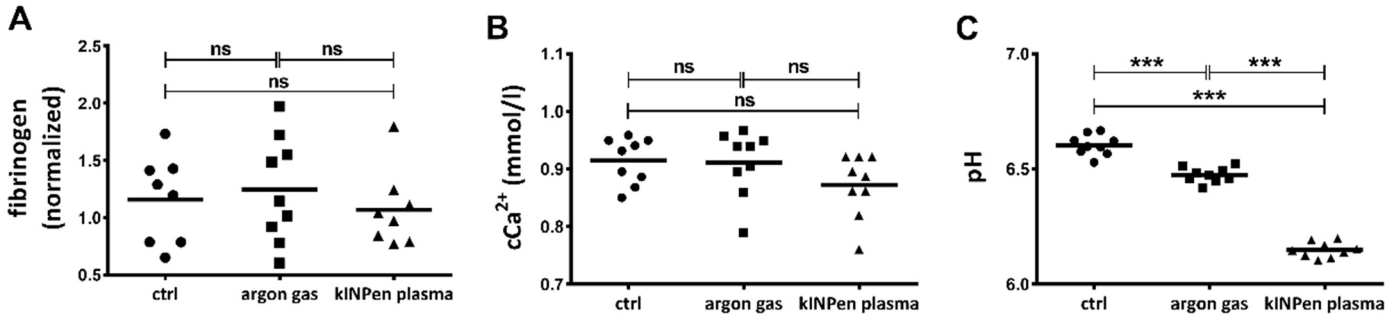

Fig. 2. Murine blood plasma analysis following exposure to kINPen MED plasma In murine whole blood, fibrinogen was not significantly decreased in either argon gas (120 s) or kINPen MED plasma-treated (120 s) samples (A). Similarly, the concentration of ionized calcium was not significantly changed in any sample compared to untreated blood (B). For argon gas and kINPen MED plasma treatment of whole blood, the pH of blood plasma was significantly decreased compared to untreated blood (C). Data are presented as mean and single data points retrieved from eight to nine animals.

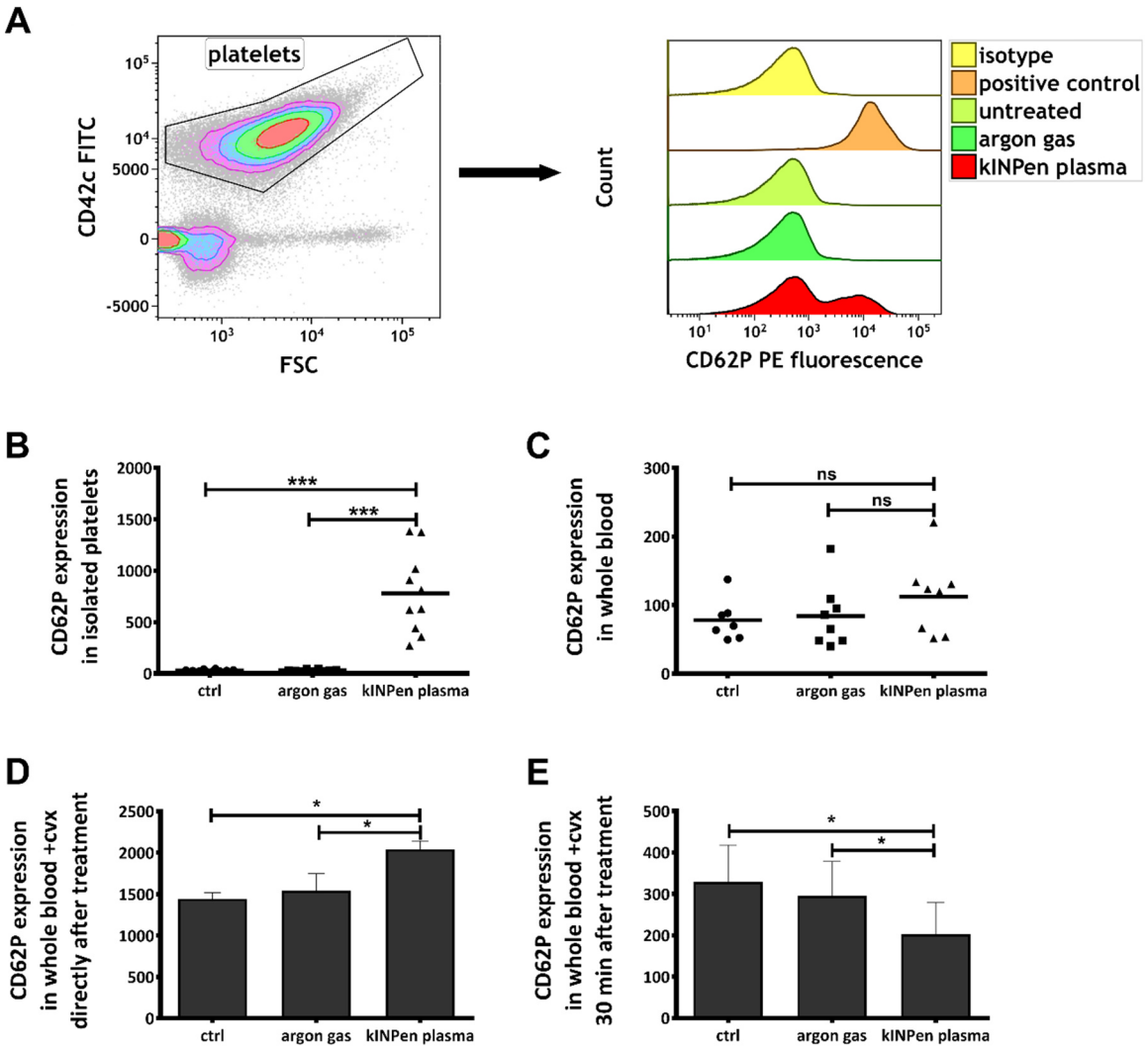

Fig. 3. Flow cytometric analysis of platelet activation Whole blood or enriched platelets were subjected to flow cytometry. $CD42c^+$ events were gated (A, left plot) and their CD62P expression was quantified (A, right plot). The latter was significantly increased in kINPen MED plasma-treated isolated platelets compared to untreated and argon gas treated samples (B). This was not seen with platelets treated in citrated whole blood (C). To maximal activate platelets, convulxin (cvx) was added immediately after treatment to citrated whole blood, and CD62P expression was significantly increased in platelets that had received kINPen MED plasma (120 s) treatment (D). Activation 30 min after kINPen MED showed significantly decreased CD62P receptor expression (E). Data are presented as one representative (A), as mean and single data points retrieved from eight animals (B, C), or as mean +S.E. of three experiments (D, E).

expelled by the kINPen MED is capable of inducing pro-coagulative responses *in vivo* during surgical procedures in a platelet-dependent manner.

3.3. kINPen MED plasma but not thermal cauterization showed similar hemostasis to natural coagulation

Sections of fully coagulated liver tissues were performed to elucidate i) the histological impact of the kINPen MED plasma treatment,

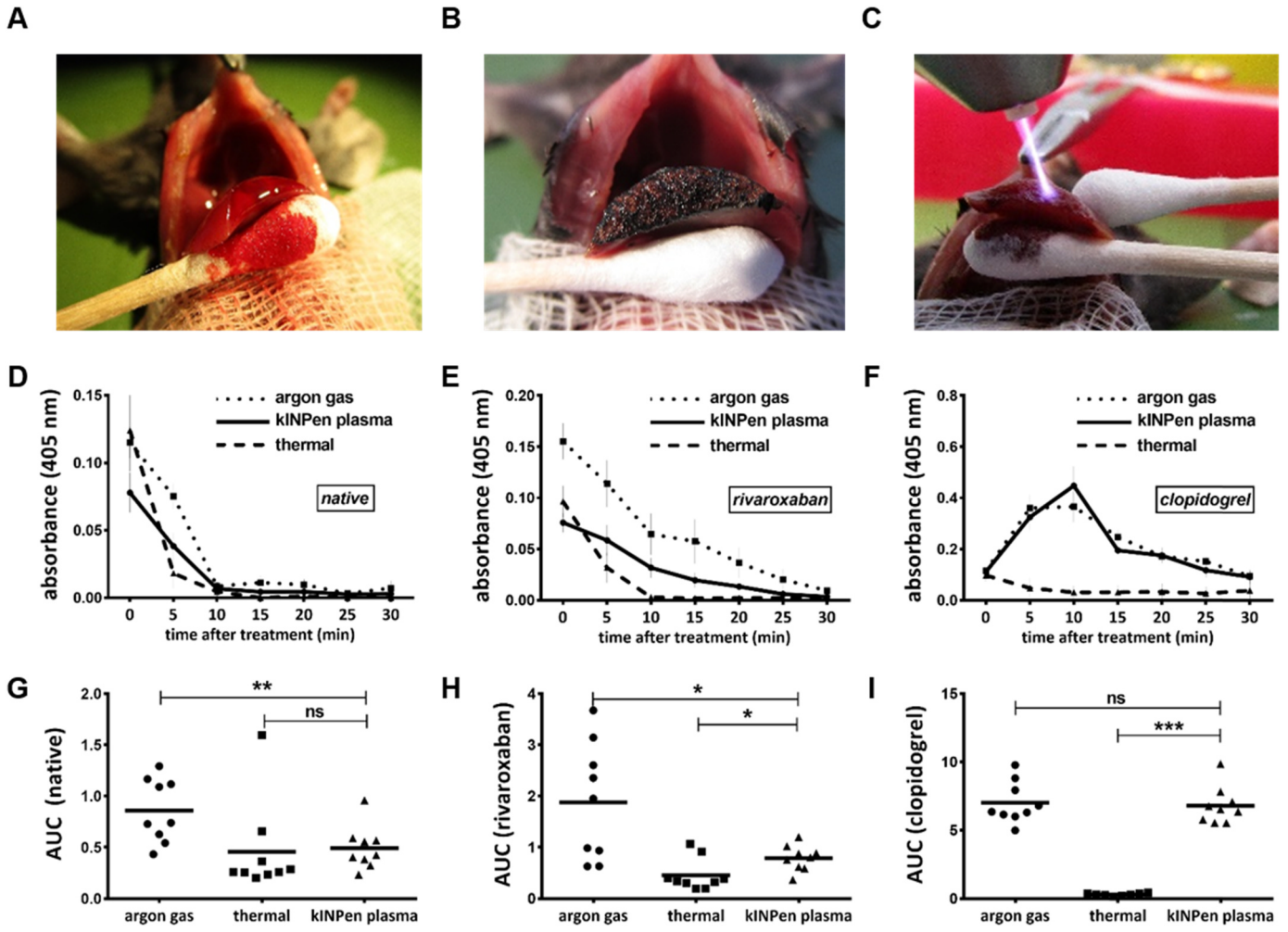

Fig. 4. kINPen MED plasma-aided hemostasis was dependent on platelets in a murine liver incision model Mice were pre-treated with clopidogrel, rivaroxaban, or vehicle, murine livers were incised, and blood loss was quantified spectroscopically from blood taken up by cotton buds (A). Livers were treated with argon gas alone (120 s), 120 s thermal cauterization (B, post treatment), or 120 s kINPen MED plasma (C, during treatment). Blood loss was quantified during operation and every 5 min over 30 min following incision in native (D), rivaroxaban treated (E), and clopidogrel treated (F) animals. Area under the curve (AUC) were calculated for each condition and treatment. kINPen MED plasma but not argon gas treatment performed similarly to cauterization in native (G) and rivaroxaban (I) but not clopidogrel treated mice (H). Data are presented as one representative (A–C), mean and S.E. (D–F), or mean and single data points (G–I) of nine to ten animals per group and treatment condition.

and ii) to decipher platelet and fibrin distribution in the different coagulation models used. Argon gas controls were assumed to closely resemble natural coagulation. Histological HE staining clearly indicated tissue destruction and necrosis at the surface exposed to electrocauterization (Fig. 5**I**). By contrast, natural (Fig. 5**I'**) and kINPen MED plasma-assisted (Fig. 5**I''**) coagulation did not disturb tissue integrity. Fluorescent staining against fibrinogen revealed its dispersed distribution in electrocauterized tissue (Fig. 5**II**) whereas in argon gas (Fig. 5**II'**) and kINPen MED plasma (Fig. 5**II''**) treated tissues fibrinogen was present rather at the coagulated margins. In cauterized tissues, platelets were randomly existent in bulk at different spots (Fig. 5**III**). In naturally (Fig. 5**III'**) and kINPen MED plasma (Fig. 5**III''**) coagulated tissues, platelets were finely aligned predominantly at the coagulated tissue margins. These results suggest that – in contrast to electrocauterization – the kINPen MED plasma coagulation closely resembles natural hemostasis processes, not interfering with tissue integrity.

4. Discussion

The increased medication of new oral anticoagulants (NOAC) [4,35] and the absence of specific reversal agents cause problems in case of emergency [36]. In addition, there is a potential risk of blood clot detachment and re-bleeding from thermally coagulated tissues [10], substantiating the need for alternative hemostatic techniques during surgery. Especially gastrointestinal bleeding are even enhanced under treatment with NOACs (e.g. rivaroxaban) [37]. Cold physical plasma may be an alternative as it does not require necrotizing tissue. Its hemostatic efficacy was demonstrated in the present work in mice *in vivo* and to some extend *ex vivo*.

Blood clotting worked well in native and rivaroxaban-anticoagulated animals with electrocauterization or kINPen MED plasma treatment. The cauterizing, however, gave visible necrosis which can under special circumstances be associated with pain, re-bleeding, perforation, strictures, disturbances in swallowing, and the development of gas emphysema of the mucosa in patients [38]. As an alternative and similar to our approach, cold physical plasma-assisted hemostasis was found to be effective before, e.g. in human spleen [19], in saphenous veins cut in SKH1 hairless mice [39], on live pig skin [40], and in blood of hemophilic patients [41] using a floating electrode surface barrier discharge.

The results of our model suggested, that platelets were key regulators in kINPen MED plasma mediated hemostasis *ex vivo* and *in vivo*. A previous report stressed the role of blood plasma proteins in cold physical plasma mediated hemostasis [19]; however, this study was done in frozen and thawed human blood plasma lacking blood cells whereas our study was done in freshly recovered murine whole blood.

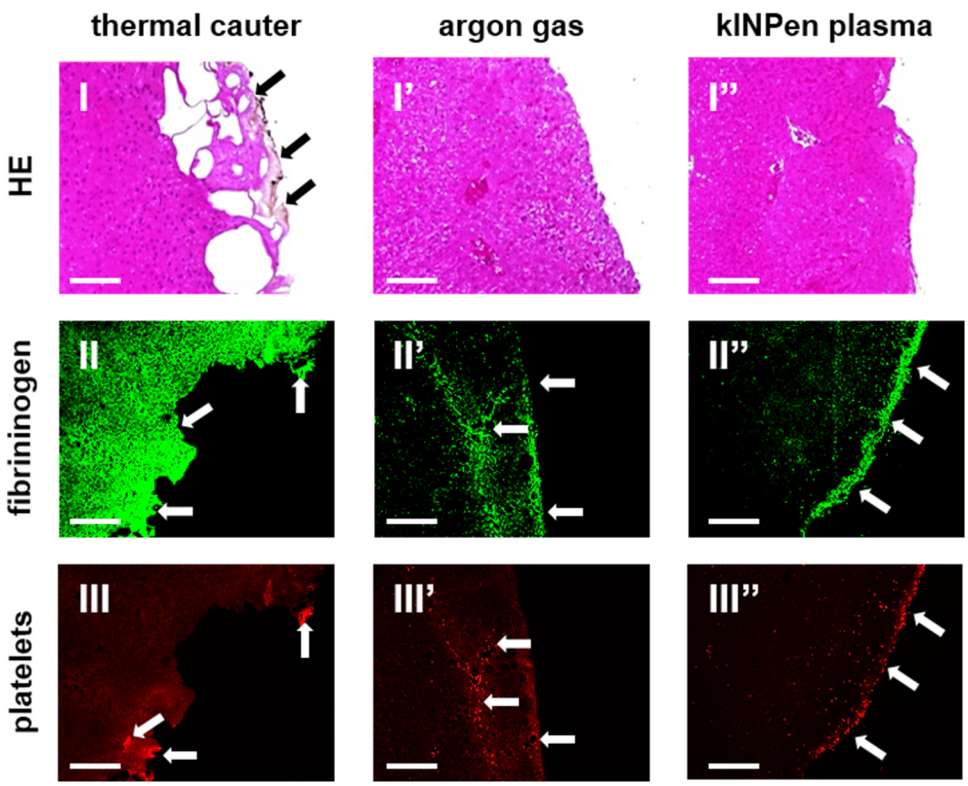

Fig. 5. kINPen MED plasma but not thermal cauterization showed similar hemostasis to natural coagulation Murine livers were incised *in vivo* and coagulated with thermal electrocauterization, argon gas treatment (natural coagulation), or cold physical plasma. Tissue sections were stained with HE, and thermal coagulation showed extensive tissue destruction (black arrows) (I). By contrast, natural (I') and kINPen MED plasma-assisted (I'') coagulation did not disturb tissue integrity. Fluorescent staining against fibrinogen revealed its dispersed distribution in electrocauterization (II) whereas in argon (II') and kINPen MED plasma (II'') treated tissues fibrin was present at the coagulated margins. In cauterized tissues, platelets were only randomly seen in bulk at different spots (III). In control (III') and kINPen MED plasma (III'') coagulated tissues, platelets were finely aligned predominantly at the coagulated tissue margins. Representative images from 3 to 4 animals per group are shown. Scale bars are 5 mm.

Corroborating a previous study [42], we have also found that calcium was dispensablesince i) its concentration was not significantly changed, and ii) results were similar in heparin and citrate anticoagulated murine blood. Another report using the kINPen 09 and bovine blood agrees with this finding [18]; yet, they had only treated 50 µl and found strong effects for longer treatment times up to 240 s, suggesting a significant contribution of the drying effect of feed gas alone. One study had used human blood anticoagulated with either heparin, citrate, or EDTA and – regardless of the anticoagulant used – found the pH to be markedly decreased [42] which was also reproduced with our findings.

Heparin was used as an anticoagulant for this experiment. It inhibits thrombin that degrades fibrinogen to fibrin in the physiological coagulation cascade [43]. The kINPen MED plasma did not circumvent this inhibition to induce plasmatic hemostasis because fibrinogen levels remained constant following treatment. Platelets store and release fibrinogen upon activation [44], capable of replenishing fibrinogen levels in our study. Yet, this is unlikely, as we did not observe significant platelet activation upon kINPen MED plasma treatment of whole blood *ex vivo*. A central role was attributed to a plasma-induced fibrin clot over ten years ago [42] and recently [45]. Yet, both studies derive their speculation from fibrinogen-enriched PBS and not experiments in whole bloods, solidifying our findings of fibrinogen to be of less importance with regard to being a primary mechanism of cold physical plasma mediated hemostasis. Moreover, a recent study suggested white blood cells to be of relevance [41]. Our results are not in agreement with this hypothesis, as *ex vivo* platelet activation was present in the absence of white blood cells but vice versa not in whole blood. An argon plasma jet was shown to foster hemostasis in rear leg incisions in mice but no mechanism was suggested in that work [46]. The present work clearly suggests platelets being the key effectors in cold physical plasma-induced hemostasis.

Depending on type, concentration, and site of generation, ROS/RNS can have harmful or beneficial effects [47]. The generation of various reactive oxygen and nitrogen species (ROS/RNS) is a key peculiarity of exposure to cold physical plasmas [48], leading to effective redox signaling in many cell types [49–51]. Importantly, platelet physiology and function are highly redox controlled as well [52]. For example, nitric oxide induces anti-thrombotic effects which inhibit platelet adhesion [53]. The effluent of a plasma jet similar to that used in the present study shows significant concentrations of nitric oxide [54]. Exogenous nitric oxide is relevant in supporting wound healing [55] but could be possibly targeted to increase the plasma's hemostatic activity. Atomic oxygen, related to the generation of RNS in the plasma source utilized [56], was previously speculated to be involved in plasma-assisted blood coagulation [41]. Peroxynitrite has also been shown to be formed in liquids following exposure to the kINPen MED [57], and this oxidant is important in activated platelets [58]. Ozone is another oxidant that can induce platelet aggregation [59], and is readily present within our plasma source [60]. However, this ozone-mediated platelet activation seems to be calcium-dependent and therefore present in heparin but not citrate anticoagulated blood [61] which would not corroborate our findings. Nonetheless, ozone treatment seems to involve the generation of hydrogen peroxide in blood plasma [62], which was identified to be a central mediator with this plasma source *in vitro* [63].

We hypothesized that kINPen MED plasma-derived ROS/RNS oxidize platelets, thereby increasing their activation, finally mediating hemostasis. Yet, there may be other or related explanations to our findings. First, blood is buffered well and has a strong antioxidant capacity, granting less investigated processes such as lipid peroxidation [64] a possible role in pro-oxidant coagulation. Second, following excessive stimulation platelets undergo apoptosis [65], and it may be feasible that our observations are secondary to such a process. Third, our results may be a byproduct of aggregation of other cell types, as for example erythrocytes have been shown before to readily aggregate upon exposure to cold physical plasma [66].

5. Conclusion

Cold physical plasma may be of clinical relevance in the management of hemostasis. We demonstrated a profound hemostatic activity of the kINPen MED plasma jet during liver resection in a murine model. The final result was comparable to benchmark electrocauterization.

Platelets were identified to be important in kINPen MED plasma-induced hemostasis. Importantly, this process resembled naturally occurring hemostasis along with a lack of tissue destruction. Future experiments will identify the role of oxidation and redox regulation to further advance this methods for future clinical use.

Acknowledgements

The authors acknowledge the contribution of Julia Klauke and Antje Janetzko during animal handling and experiments.

Financial disclosure

The authors have no financial interest to declare.

Ethical statement

The local ethics committee (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern, Rostock, Germany) approved blood collection and animal experiments (approval number: LALLF M-V/TSD/7221.3-1-056/14).

Funding

The German Federal Ministry of Education and Research (grant number 03Z22DN11) supported this work.

Conflict of interest

The authors declare no conflict of interest.

References

[1] F.J. van der Meer, F.R. Rosendaal, J.P. Vandenbroucke, E. Briet, Bleeding complications in oral anticoagulant therapy. An analysis of risk factors, Arch. Intern. Med. 153 (1993) 1557–1562.
[2] J.J. You, D.E. Singer, P.A. Howard, D.A. Lane, M.H. Eckman, M.C. Fang et al., Antithrombotic therapy for atrial fibrillation: Antithrombotic therapy and prevention of thrombosis, 9th ed: American college of chest physicians evidence-based clinical practice guidelines. Chest 2012,141, pp. e531S–575S.
[3] C.E. Chiang, L. Naditch-Brule, J. Murin, M. Goethals, H. Inoue, J. O'Neill, et al., Distribution and risk profile of paroxysmal, persistent, and permanent atrial fibrillation in routine clinical practice: insight from the real-life global survey evaluating patients with atrial fibrillation international registry, Circ. Arrhythm. Electrophysiol. 5 (2012) 632–639.
[4] G.V. Naccarelli, H. Varker, J. Lin, K.L. Schulman, Increasing prevalence of atrial fibrillation and flutter in the united states, Am. J. Cardiol. 104 (2009) 1534–1539.
[5] J. Menke, L. Luthje, A. Kastrup, J. Larsen, Thromboembolism in atrial fibrillation, Am. J. Cardiol. 105 (2010) 502–510.
[6] N.J. Carter, G.L. Plosker, Rivaroxaban: a review of its use in the prevention of stroke and systemic embolism in patients with atrial fibrillation, Drugs 73 (2013) 715–739.
[7] T.R. Riley, M.L. Gauthier-Lewis, C.K. Sanchez, J.S. Douglas, Role of agents for reversing the effects of target-specific oral anticoagulants, Am. J. Health Syst. Pharm. 74 (2017) 54–61.
[8] B. Weiner, T. Fischer, S. Waxman, Hemostasis in the era of the chronic anticoagulated patient, J. Invasive Cardiol. 15 (2003) 669–673 (quiz 674).
[9] D.O. Castell, Consensus statement on therapeutic endoscopy and bleeding ulcers, Gastrointest. Endosc. 36 (1990) S62–S65.
[10] Y. Jung, I.K. Chung, Y.S. Cho, T.H. Lee, S.H. Park, J.S. Lee, et al., Do we perform a perfect endoscopic hemostasis prophylactically with argon plasma coagulation in colonic endoscopic mucosal resection? Dig. Dis. Sci. 60 (2015) 3100–3107.
[11] G.C. Gurtner, S. Werner, Y. Barrandon, M.T. Longaker, Wound repair and regeneration, Nature 453 (2008) 314–321.
[12] T.M. Dunlevy, T.P. O'Malley, G.N. Postma, Optimal concentration of epinephrine for vasoconstriction in neck surgery, Laryngoscope 106 (1996) 1412–1414.
[13] K. Broos, H.B. Feys, S.F. De Meyer, K. Vanhoorelbeke, H. Deckmyn, Platelets at work in primary hemostasis, Blood Rev. 25 (2011) 155–167.
[14] P. Savi, P. Nurden, A.T. Nurden, S. Levy-Toledano, J.M. Herbert, Clopidogrel: a review of its mechanism of action, Platelets 9 (1998) 251–255.
[15] F. Muller, T. Renne, Platelet polyphosphates: the nexus of primary and secondary hemostasis, Scand. J. Clin. Lab. Investig. 71 (2011) 82–86.
[16] E. Investigators, R. Bauersachs, S.D. Berkowitz, B. Brenner, H.R. Buller, H. Decousus, et al., Oral rivaroxaban for symptomatic venous thromboembolism, N. Engl. J. Med. 363 (2010) 2499–2510.
[17] A. Amritkar, B. Cunningham, B. Hawkins, B. Batalla, D. Moore, E. Thompson, et al., Nonthermal plasma-assisted trauma management: hemostasis of noncompressible profuse hemorrhage, Plasma Med. 3 (2013) 291–314.
[18] C. Heslin, D. Boehm, V. Milosavljevic, M. Laycock, P.J. Cullen, P. Bourke, Quantitative assessment of blood coagulation by cold atmospheric plasma, Plasma Med. 4 (2014) 153–163.
[19] G. Fridman, M. Peddinghaus, M. Balasubramanian, H. Ayan, A. Fridman, A. Gutsol, et al., Blood coagulation and living tissue sterilization by floating-electrode dielectric barrier discharge in air, Plasma Chem. Plasma Process 26 (2006) 425–442.
[20] R. Bussiahn, N. Lembke, R. Gesche, T. von Woedtke, K.-D. Weltmann, Plasma sources for biomedical applications, Hyg. Med. 38 (2013) 212–216.
[21] H. Jablonowski, T. von Woedtke, Research on plasma medicine-relevant plasma–-liquid interaction: what happened in the past five years? Clin. Plasma Med. 3 (2015) 42–52.
[22] D.B. Graves, The emerging role of reactive oxygen and nitrogen species in redox biology and some implications for plasma applications to medicine and biology, J. Phys. D: Appl. Phys. 45 (2012) 263001.
[23] K.D. Weltmann, T. von Woedtke, Plasma medicine-current state of research and medical application, Plasma Phys. Control. Fusion 59 (2017) 014031.
[24] M. Klebes, C. Ulrich, F. Kluschke, A. Patzelt, S. Vandersee, H. Richter, et al., Combined antibacterial effects of tissue-tolerable plasma and a modern conventional liquid antiseptic on chronic wound treatment, J. Biophotonics 8 (2015) 382–391.
[25] A. Schmidt, S. Bekeschus, K. Wende, B. Vollmar, T. von Woedtke, A cold plasma jet accelerates wound healing in a murine model of full-thickness skin wounds, Exp. Dermatol. 26 (2017) 156–162.
[26] H.R. Metelmann, T. von Woedtke, R. Bussiahn, K.D. Weltmann, M. Rieck, R. Khalili, et al., Experimental recovery of CO_2-laser skin lesions by plasma stimulation, Am. J. Cosmet. Surg. 29 (2012) 52–56.
[27] A.S. Wolberg, Z.H. Meng, D.M. Monroe 3rd, M. Hoffman, A systematic evaluation of the effect of temperature on coagulation enzyme activity and platelet function, J. Trauma 56 (2004) 1221–1228.
[28] S. Bekeschus, A. Schmidt, K.-D. Weltmann, T. von Woedtke, The plasma jet kinpen – a powerful tool for wound healing, Clin. Plasma Med. 4 (2016) 19–28.
[29] S. Kluge, S. Bekeschus, C. Bender, H. Benkhai, A. Sckell, H. Below, et al., Investigating the mutagenicity of a cold argon-plasma jet in an het-mn model, PLoS One 11 (2016) e0160667.
[30] A. Schmidt, T.V. Woedtke, J. Stenzel, T. Lindner, S. Polei, B. Vollmar, et al., One year follow-up risk assessment in skh-1 mice and wounds treated with an argon plasma jet, Int. J. Mol. Sci. 18 (2017) 868.
[31] I.F.C.C.J. Guimaraes, Human platelets activation by convulxin is accompanied by tyrosyl-phosphorylation of plcgamma2 and occurs independently of integrin alphaiibbeta3, Platelets 9 (1998) 185–189.
[32] C. Hermans, C. Wittevrongel, C. Thys, P.A. Smethurst, C. Van Geet, K. Freson, A compound heterozygous mutation in glycoprotein vi in a patient with a bleeding disorder, J. Thromb. Haemost. 7 (2009) 1356–1363.
[33] A.D. Michelson, M.R. Barnard, H.B. Hechtman, H. MacGregor, R.J. Connolly, J. Loscalzo, et al., In vivo tracking of platelets: circulating degranulated platelets rapidly lose surface p-selectin but continue to circulate and function, Proc. Natl. Acad. Sci. USA 93 (1996) 11877–11882.
[34] P. Schubert, D.V. Devine, De novo protein synthesis in mature platelets: a consideration for transfusion medicine, Vox Sang. 99 (2010) 112–122.
[35] R. Thomas, R. Gonzalez, Which antidotes are available for the novel oral anticoagulants? JAAPA 29 (2016) 12–13.
[36] A.C. Pinho-Gomes, A. Hague, J. Ghosh, Management of novel oral anticoagulants in emergency and trauma surgery, Surgeon 14 (2016) 234–239.
[37] I.L. Holster, V.E. Valkhoff, E.J. Kuipers, E.T. Tjwa, New oral anticoagulants increase risk for gastrointestinal bleeding: a systematic review and meta-analysis, Gastroenterology 145 (2013) 105–112 (e115).
[38] H. Manner, M.D. Enderle, O. Pech, A. May, N. Plum, J.F. Riemann, et al., Second-generation argon plasma coagulation: two-center experience with 600 patients, J. Gastroenterol. Hepatol. 23 (2008) 872–878.
[39] A. Fridman, Plasma Chemistry, Cambridge University Press, Philadelphia, USA, 2008.
[40] D. Dobrynin, A. Wu, S. Kalghatgi, S. Park, N. Shainsky, K. Wasko, et al., Live pig skin tissue and wound toxicity of cold plasma treatment, Plasma Med. 1 (2011) 93–108.
[41] S.P. Kuo, O. Tarasenko, J. Chang, S. Popovic, C.Y. Chen, H.W. Fan, et al., Contribution of a portable air plasma torch to rapid blood coagulation as a method of preventing bleeding, New J. Phys. 11 (2009) 115016.
[42] S.U. Kalghatgi, G. Fridman, M. Cooper, G. Nagaraj, M. Peddinghaus, M. Balasubramanian, et al., Mechanism of blood coagulation by nonthermal atmospheric pressure dielectric barrier discharge plasma, IEEE Trans. Plasma Sci. 35 (2007) 1559–1566.
[43] A.S. Wolberg, Thrombin generation and fibrin clot structure, Blood Rev. 21 (2007) 131–142.
[44] S.W. Whiteheart, Platelet granules: surprise packages, Blood 118 (2011) 1190–1191.
[45] Z. Ke, Q. Huang, Haem-assisted dityrosine-cross-linking of fibrinogen under non-thermal plasma exposure: one important mechanism of facilitated blood coagulation, Sci. Rep. 6 (2016) 26982.
[46] E. Garcia-Alcantara, R. Lopez-Callejas, P.R. Morales-Ramirez, R. Pena-Eguiluz, R. Fajardo-Munoz, A. Mercado-Cabrera, et al., Accelerated mice skin acute wound healing in vivo by combined treatment of argon and helium plasma needle, Arch. Med. Res. 44 (2013) 169–177.
[47] L.A. Pham-Huy, H. He, C. Pham-Huy, Free radicals, antioxidants in disease and

health, Int. J. Biomed. Sci. 4 (2008) 89–96.
[48] A. Kramer, S. Bekeschus, R. Matthes, C. Bender, M.B. Stope, M. Napp, et al., Cold physical plasmas in the field of hygiene-relevance, significance, and future applications, Plasma Process Polym. 12 (2015) 1410–1422.
[49] S. Bekeschus, J. Moritz, A. Schmidt, K. Wende, Redox regulation of leukocyte-derived microparticle release and protein content in response to cold physical plasma-derived oxidants, Clin. Plasma Med. 7–8 (2017) 24–35.
[50] A. Schmidt, T. von Woedtke, S. Bekeschus, Periodic exposure of keratinocytes to cold physical plasma: an in vitro model for redox-related diseases of the skin, Oxid. Med. Cell. Longev. 2016 (2016) 9816072.
[51] S. Bekeschus, K. Wende, M.M. Hefny, K. Rodder, H. Jablonowski, A. Schmidt, et al., Oxygen atoms are critical in rendering thp-1 leukaemia cells susceptible to cold physical plasma-induced apoptosis, Sci. Rep. 7 (2017) 2791.
[52] D. Pietraforte, R. Vona, A. Marchesi, I.T. de Jacobis, A. Villani, D. Del Principe, et al., Redox control of platelet functions in physiology and pathophysiology, Antioxid. Redox Signal 21 (2014) 177–193.
[53] M.J. Ricciardi, B.P. Knight, F.J. Martinez, M. Rubenfire, Inhaled nitric oxide in primary pulmonary hypertension: a safe and effective agent for predicting response to nifedipine, J. Am. Coll. Cardiol. 32 (1998) 1068–1073.
[54] S. Iseni, S. Zhang, A.F.H. van Gessel, S. Hofmann, B.T.J. van Ham, S. Reuter, et al., Nitric oxide density distributions in the effluent of an rf argon appj: effect of gas flow rate and substrate, New J. Phys. 16 (2014) 123011.
[55] B.B. Childress, J.K. Stechmiller, Role of nitric oxide in wound healing, Biol. Res. Nurs. 4 (2002) 5–15.
[56] S. Bekeschus, S. Iseni, S. Reuter, K. Masur, K.-D. Weltmann, Nitrogen shielding of an argon plasma jet and its effects on human immune cells, IEEE Trans. Plasma Sci. 43 (2015) 776–781.
[57] S. Bekeschus, J. Kolata, C. Winterbourn, A. Kramer, R. Turner, K.D. Weltmann, et al., Hydrogen peroxide: a central player in physical plasma-induced oxidative stress in human blood cells, Free Radic. Res. 48 (2014) 542–549.
[58] J.E. Freedman, Oxidative stress and platelets, Arterioscler. Thromb. Vasc. Biol. 28 (2008) s11–s16.
[59] V. Bocci, G. Valacchi, R. Rossi, D. Giustarini, E. Paccagnini, A.M. Pucci, et al., Studies on the biological effects of ozone: 9. Effects of ozone on human platelets, Platelets 10 (2010) 110–116.
[60] W.V. Gaens, S. Iseni, A. Schmidt-Bleker, K.D. Weltmann, S. Reuter, A. Bogaerts, Numerical analysis of the effect of nitrogen and oxygen admixtures on the chemistry of an argon plasma jet operating at atmospheric pressure, New J. Phys. 17 (2015) 033003.
[61] G. Valacchi, V. Bocci, Studies on the biological effects of ozone: 10. Release of factors from ozonated human platelets, Mediat. Inflamm. 8 (1999) 205–209.
[62] V. Bocci, Biological and clinical effects of ozone. Has ozone therapy a future in medicine? Br. J. Biomed. Sci. 56 (1999) 270.
[63] S. Bekeschus, C.C. Winterbourn, J. Kolata, K. Masur, S. Hasse, B.M. Broker, et al., Neutrophil extracellular trap formation is elicited in response to cold physical plasma, J. Leukoc. Biol. 100 (2016) 791–799.
[64] V. Bocci, G. Valacchi, F. Corradeschi, C. Aldinucci, S. Silvestri, E. Paccagnini, et al., Studies on the biological effects of ozone: 7. generation of reactive oxygen species (ros) after exposure of human blood to ozone, J. Biol. Regul. Homeost. Agents 12 (1997) 67–75.
[65] S. Zharikov, S. Shiva, Platelet mitochondrial function: from regulation of thrombosis to biomarker of disease, Biochem. Soc. Trans. 41 (2013) 118–123.
[66] J. Kim, J.H. Kim, B. Chang, E.H. Choi, H.-K. Park, Hemorheological alterations of red blood cells induced by non-thermal dielectric barrier discharge plasma, Appl. Phys. Lett. 109 (2016) 193701.