BASIC RESEARCH IN PLASMA MEDICINE: SUPPORT FOR THE TRANSFER INTO CLINICS

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ABSTRACT

During the last decade Plasma Medicine has been established as a new field of research. Applying cold physical plasmas in the treatment of biological matter the inactivation of microorganisms was the focus at the beginning. In the meantime basic research realized the stimulation of eukaryotic cells by the same kind of plasmas. For further understanding the underlying molecular processes in plasma-cell interaction, a controlled and detailed diagnostic of the reactive plasma components is essential. On the biological side the disentanglement of the molecular mechanisms triggered by plasma in cell culture led to a deeper understanding of the signalling pathways and on gene regulation.

In this work we present first results of the molecular mechanisms triggered by non-thermal plasma in human skin biopsies. The biopsies have been analysed by specific immuno-histochemistry to identify markers for stimulation (e.g. Ki67) in the stratum basale - the layer of the skin responsible for growth and regeneration. No harmful side effects were detected in these skin biopsies. We could proof that short term plasma treatment (up to 1 minute) indeed is able to stimulate the proliferation of human skin cells without the induction of cell death.

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1. INTRODUCTION

The development of devices generating cold physical atmospheric pressure plasmas (partially ionized gases operating closely to body temperature) is a new field of research with a great potential in medical applications [1, 2]. During the past decade several studies in the field of plasma medicine showed that cold plasmas are able to influence cell fate [3]. In this period of time lot of effort was put into the understanding of plasma processes in order to generate well tolerated cold plasmas for medical application focusing on diverse health care subjects [4, 5]. Most of the articles are focussing on the inactivation of micro-organisms or decontamination processes [6-8]. This also applies to the first clinical trials which could proof that cold atmospheric argon plasma was able to decrease bacterial load in chronic wounds [9]. Latest developments indicate that there is a more specific way of plasma treatment showing a differential sensitivity [10, 11]. Even the activation status of specific immune cell sub-populations seems to be differentially influenced by cold atmospheric pressure plasmas [10].
However, there is still a big gap in the understanding of the molecular mechanisms triggered by cold plasmas. Several efforts on a transcriptomic level revealed a treatment time and incubation time dependent manner of plasma mediated in-/ activation of gene activities [12]. Those alterations of the gene activation level of course are followed by a differential expression of proteins. Recently it was shown that plasma treatment is leading to the activation

or inactivation of certain signalling cascades [13], indicating that cold plasma specifically modulates cellular activities on the level of cell communication.
Although the understanding of the plasma mediated effects rose over the past decade, most of the experiments were performed either in cell culture or on isolated cells. There is still the need of translating those results into the next dimension of 3D tissue samples consisting of several different cell types, all of them interacting with each other. Here we present the next step in which the molecular mechanisms described above, are investigated on skin biopsies. This will help to understand the various possibilities on how cold plasma is interacting with the cellular networks. Most importantly this study shows that the results from cell culture of cold plasma application also confers to the real situation found in skin.

2. METHODS

- Plasma source

The kinpenMED manufactured and distributed by neoplas tools GmbH (Greifswald, Germany) was employed for generating plasma. In order to keep the appropriate distance of 10 mm to the skin surface a spacer was integrated. The kinpenMED is a certified medical device of class II, type BF. Plasma was ignited by applying a voltage of 2-3 kV at a high frequency of 1MHZ. The carrier gas was argon flowing at 5slm. By the high frequency discharge the argon is converted into plasma.

- Treatment of skin samples

Skin samples were obtained from patients undergoing surgery at the clinic of Oral and Maxillofacial Surgery / Plastic Surgery, University Medicine Greifswald, Germany. Although the location of skin samples varied between body sites, they were by the majority from the face and head/neck area. Immediately after excision biopsy samples were washed in William's E medium (Lonza, Verviers, Belgium) supplemented with 0,25 µg/ml amphotericin B (PromoCell GmbH, Heidelberg, Germany), 100 IU/ml penicillin/10µg/ml streptomycin, and transported to the laboratory within 10 min.
The skin specimen of 5 mm in diameter were directly exposed to plasma at the side of the stratum corneum for 1 min (n=5), and compared to untreated control skin (n=5) of the same patient. Immediately after plasma exposure skin biopsies were placed into 500 µl serum free William's E medium supplemented with 100 IU/ml penicillin/10µg/ml streptomycin, 10 µg/ml insulin, 10 ng/ml hydrocortisone and 2 mmol/l L-glutamine (all Lonza, Verviers, Belgium) and cultured for the next 24 hours. Samples were maintained at 37°C and 5% CO_2 making sure that the epidermis floated at the medium-air interface.

- Histochemistry, Immunofluorescence

After 24 hours skin biopsies were embedded in OCT (VWR chemicals, Leuven, Belgium) and snap frozen in liquid nitrogen. Using a Leica CM 199 cryomicrotome (Leica Microsystems, Nussloch, Germany) 6 µm sections were cut, placed on SuperFrost®Plus microscope slides (R. Langenbrinck, Teningen, Germany) and stored at -80°C until the staining procedure was performed. Fluorescence labeling was realized by incubation with secondary antibodies conjugated with Alexa488 (anti-rabbit) and Alexa546 (anti-mouse), respectively (both Life Technologies). The rabbit anti- phospho-histone γH2A.X antibody (Cell Signaling Technology, MA, USA) was used to detect possible DNA-damages. Further terminal dUTP nicked end labeling (TUNEL staining = in situ cell death detection kit, fluorescein, ROCHE Applied Science, Mannheim, Germany) was applied to analyze the amount of apoptotic cell death while proliferating cells were visualized by immune-reactivity of the Ki67 marker (DAKO, cloneMIB-1, Glostrup, Denmark). DAPI served as counter stain and was included in the mounting medium (Vectashield, Vector Laboratories, Burlingame, USA). Photographs were captured using an Axio Observer.Z1 microscope (Carl Zeiss Microscopy GmbH, Göttingen, Germany) and AxioVision software. Sections from one patient were treated at the same time in order to assure comparability.

3. RESULTS & DISCUSSION

Due to the nature of cold plasma containing reactive oxygen species (ROS), reactive nitrogen species (RNS) as well as various kinds of radiation, the likelihood to induce cellular damage rises with time. This fact is supported by several cell culture based studies [14, 15].
According to the literature we stained the biopsy samples for DNA damages applying a γH2A.X antibody [16] and counted positive cells (Fig 1). Comparing the untreated control to the samples treated for one minute with cold plasma we could not detect any significant increase of γH2AX

positive cells. Although in both groups relatively high amounts of positive cells were detected.

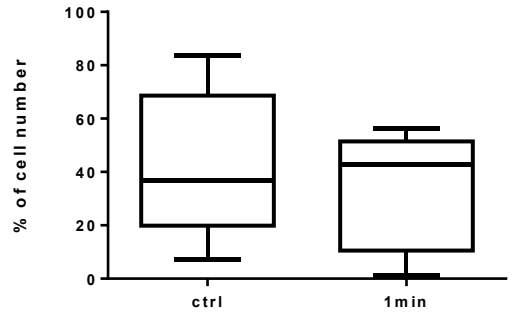

Figure 1 The ratio of γH2A.X -positive cells and all present cells counterstained with DAPI in human skin biopsy samples as a hint for DNA damages after application of plasma for 1 min. Depicted is the 10 to 90 percentile with median.

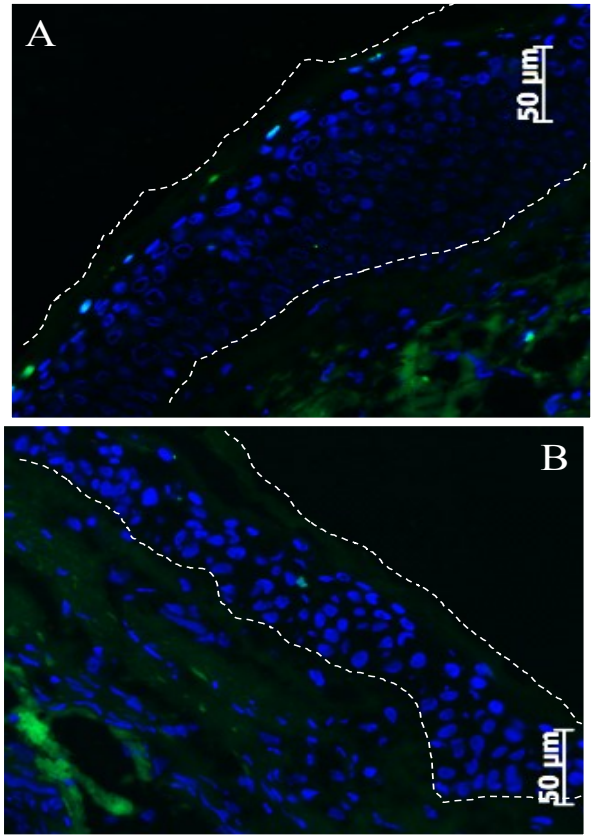

Figure 2 Human skin biopsy samples stained for late apoptotic cells (TUNEL staining, green) and counterstained with DAPI (blue fluorescence) in untreated (A) and plasma treated for 1 min (B).

To answer the question if there are damages which could not be restored within a normal doubling period of 24 hours we performed a TUNEL staining, specific for late apoptotic cells. We could proof that for short term plasma treatment of one minute (Fig 2B) no increased level of apoptosis occurred when compared to untreated control skin (Fig 2A). Therefore the TUNEL staining revealed that there are no permanent destructive effects after the application of cold atmospheric pressure plasma.

Furthermore, we were interested if cold plasma would be able to stimulate the epidermis by increasing the amount of cells, which would be an advantage in wound healing processes. Therefore, a staining for Ki67 – a typical marker of proliferating cells - showed a significant elevation in the amount of proliferating cells found in the stratum basale – the skin layer responsible for skin regeneration and wound healing. Hence short-term plasma treatment leads to a stimulation of cell proliferation in the stratum basale (Fig 3).

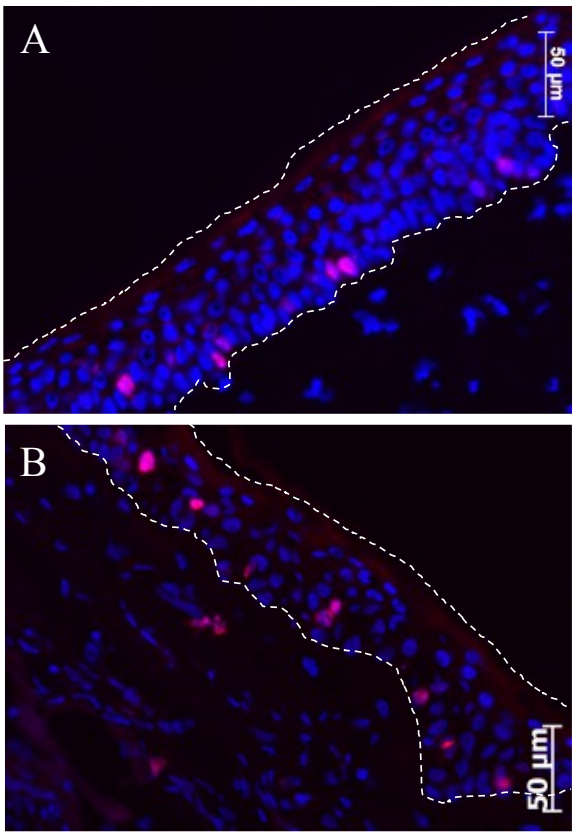

Figure 3 Proliferating cells within a human skin sample after plasma treatment (B) and in untreated skin (A) detected by applying the Ki67 marker (red) overlayed with all stained nuclei (DAPI, blue).

In order to quantify the amount of Ki67 positive cells the fluorescence signals for control samples and plasma treated samples were evaluated. The results are depicted in Fig 4 and clearly display a significant increase in proliferating cells found in the plasma treated skin samples with similar levels of apoptotic cells for both groups.

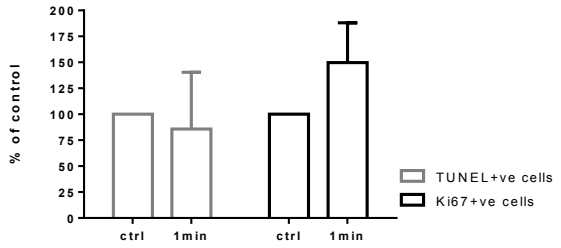

Figure 4 Percentage of apoptotic (grey bar = TUNEL staining) and proliferating cells (black bar = Ki67) of human epidermis after 1 min plasma application in comparison to their respective control (100%).

4. CONCLUSION

This study shows that in general the results for a plasma treatment of single cells from cell culture are transferable to three dimensional samples of human skin. Furthermore, the molecular markers applied in cell culture for both cell damages and proliferation also helped to identify the mechanisms triggered by cold plasma treatment within biopsy samples. Moreover, there are no adverse effects when biopsy samples were treated for one minute with cold plasma, since there was no difference in the content of early or late apoptotic events in both groups. Finally, we could proof a stimulation of cell proliferation, indicated by an elevated level of Ki67 positive cells. Since cells which stained positive for DNA damage (γH2A.X) were found close to the stratum corneum, and to less extent at the stratum basale, while the increase in proliferation was detected only in the stratum basale, the plasma mediated effects seemed to be transmitted through the skin, which further needs to be investigated. However, this study reveals that a short-term plasma treatment of human skin leads to a stimulation of proliferation without an incduction of cell death compared to control.

5. REFERENCES

[1] T. von Woedtke, S. Reuter, K. Masur, K.D. Weltmann, Plasmas for medicine, Physics Reports, 530 (2013) 291-320.
[2] K.D. Weltmann, E. Kindel, T. von Woedtke, M. Hähnel, M. Stieber, R. Brandenburg, Atmospheric-pressure plasma sources: Prospective tools for plasma medicine, Pure Appl Chem, 82 (2010) 1223-1237.
[3] E. Stoffels, R.E.J. Sladek, I.E. Kieft, Gas Plasma Effects on Living Cells, Phys Scripta, 2004 (2004) 79.
[4] G.E. Morfill, M.G. Kong, J.L. Zimmermann, Focus on Plasma Medicine, New Journal of Physics, 11 (2009) 115011.
[5] K.D. Weltmann, M. Polak, K. Masur, T. von Woedtke, J. Winter, S. Reuter, Plasma Processes and Plasma Sources in Medicine, Contributions to Plasma Physics, 52 (2012) 644-654.
[6] G. Daeschlein, S. Scholz, T. von Woedtke, M. Niggemeier, E. Kindel, R. Brandenburg, K.-D. Weltmann, M. Junger, In Vitro Killing of Clinical Fungal Strains by Low-Temperature Atmospheric-Pressure Plasma Jet, Ieee Transactions on Plasma Science, 39 (2011) 815-821.
[7] R. Matthes, I. Koban, C. Bender, K. Masur, E. Kindel, K.D. Weltmann, T. Kocher, A. Kramer, N.O. Hubner, Antimicrobial Efficacy of an Atmospheric Pressure Plasma Jet Against Biofilms of Pseudomonas aeruginosa and Staphylococcus epidermidis, Plasma Processes and Polymers, 10 (2013) 161-166.
[8] T. von Woedtke, A. Kramer, K.D. Weltmann, Plasma sterilization: What are the conditions to meet this claim?, Plasma Processes and Polymers, 5 (2008) 534-539.
[9] G. Isbary, J.L. Zimmermann, T. Shimizu, Y.F. Li, G.E. Morfill, H.M. Thomas, B. Steffes, J. Heinlin, S. Karrer, W. Stolz, Non-thermal plasma—More than five years of clinical experience, Clinical Plasma Medicine, 1 (2013) 19-23.
[10] S. Bekeschus, K. Masur, J. Kolata, K. Wende, A. Schmidt, L. Bundscherer, A. Barton, A. Kramer, B. Bröker, K.-D. Weltmann, Human Mononuclear Cell Survival and Proliferation is Modulated by Cold Atmospheric Plasma Jet, Plasma Processes and Polymers, 10 (2013) 706-713.
[11] B. Haertel, F. Volkmann, T. von Woedtke, U. Lindequist, Differential sensitivity of lymphocyte subpopulations to non-thermal atmospheric-pressure plasma, Immunobiology, 217 (2012) 628-633.
[12] A. Schmidt, K. Wende, S. Bekeschus, L. Bundscherer, A. Barton, K. Ottmüller, K.D. Weltmann, K. Masur, Non-thermal plasma treatment is associated with changes in transcriptome of human epithelial skin cells, Free Radical Res, 47 (2013) 577-592.
[13] L. Bundscherer, K. Wende, K. Ottmüller, A. Barton, A. Schmidt, S. Bekeschus, S. Hasse, K.-D. Weltmann, K. Masur, U. Lindequist, Impact of non-thermal plasma treatment on MAPK signaling pathways of human immune cell lines, Immunobiology, 218 (2013) 1248-1255.
[14] S. Kalghatgi, J. Azizkhan-Clifford, DNA Damage in Mammalian Cells by Atmospheric Pressure Microsecond-Pulsed Dielectric Barrier Discharge Plasma Is Not Mediated Via Lipid Peroxidation, 1 (2011) 167-177.
[15] E.A. Sosnin, E. Stoffels, M.V. Erofeev, I.E. Kieft, S.E. Kunts, The effects of UV irradiation and gas plasma treatment on living mammalian cells and bacteria: A comparative approach, Ieee Transactions on Plasma Science, 32 (2004) 1544-1550.
[16] G. Isbary, J. Köritzer, A. Mitra, Y.F. Li, T. Shimizu, J. Schroeder, J. Schlegel, G.E. Morfill, W. Stolz, J.L. Zimmermann, Ex vivo human skin experiments for the evaluation of safety of new cold atmospheric plasma devices, Clinical Plasma Medicine, 1 (2013) 36-44.

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