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Letter to Editor Open Access

Preliminary Evaluation of the Effects of Cold Atmospheric Plasma Application Rate on the Proliferation Behavior of Keratinocytes *In Vitro* Measured Using CK-5, CK-10, CK-14, Ki-67 and p53 Expression

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Introduction

Tissue tolerable plasma has been used in preclinical and more recently in clinical settings for the debridement of dead tissue and the removal of bacterial biofilms. Cold plasma therapy is an emerging field in medical sciences; it is mainly due to the beneficial effects that low temperature plasma has demonstrated: anti-inflammatory, anti-tumorigenic and anti-microbial effects [1-4]. The advantage of cold plasma therapy over conventional thermal plasma treatments, arc coagulators and desiccators, is that it allows for more precise application and therefore more controllable effects on the tissue. Additionally, cold plasma treatment showed stimulatory effects on wound healing and tissue regeneration [5-8]. Experiments show that cold atmospheric plasma treatment allows for efficient, non-contact, painless, and antiseptic effects without damaging healthy tissue [9-11]. As a result of the better understanding of complex plasma phenomena and the development of new plasma sources in the past few years, plasma medicine has developed into an innovative and promising field of research.

Keratinocytes have an important role in wound healing. They secrete humoral factors that regulate extracellular matrix production. Keratinocytes physically fill gaps in the skin and have an important role in scar formation and the remodelling phase. Therefore, it is imperative to understand the effects of therapeutics used in dermatology on keratinocytes, their proliferation and maturation.

After obtaining Institutional Review Board at the Schlosspark Clinic in Berlin approval for human tissue collection, we conducted *in vitro* studies in German Institute for Cell and Tissue Repair and the Department of Pathology of the Unfallkrankenhaus Berlin. Split skin biopsies (27 cm × 3 cm) were taken from tissue samples which had been obtained during the course of aesthetic procedures. The split skin biopsy was subsequently divided in 3 (9 cm × 3 cm) pieces and designated to receive cold atmospheric plasma (CAP) treatment with low or high plasma level or left untreated. Each skin sample designated to receive CAP treatment was positioned and spread uniformly on the sterile aluminium foil using sterile needles. Marks were made in the split skin biopsy at 1 cm intervals for the purpose of the velocity of the plasma jet point over the skin surface. CAP therapy was delivered using "kINPen 09" device. For more details about the principle of cold plasma generation and “kINPen” read Weltmann et al. [12]. The split-skin fragments were then exposed to the beam; plasma spectrum ranged from 200-600 nm and was applied at a voltage of 60 volts and a gas pressure of 40 kPa. The distance between the pen tip and the skin (length of the plasma beam jet) was about 10 (+/-2) mm (Figure 1). Because of the design of the plasma pen, a paintbrush motion was used to ensure a uniform distribution of the plasma over the skin surface. Low plasma level was achieved by progression of the plasma jet at speed of 8-10 mm/s while the High plasma level was achieved by speed of 2 mm/s. After the cold atmospheric plasma application, the skin sample was immediately cut to form fragments of 3 cm × 3 cm as preparation for the keratinocyte isolation and cultivation. The split-skin fragments that served as a control were not exposed to plasma and were immediately processed for keratinocyte isolation and cultivation. Keratinocytes were isolated and cultivated according to our previously published protocols in triplicates [13,14]. Dead and living cells were counted in parallel in the haemocytometer and cell viability was calculated as the percentage of all cells. During the maturation process, keratinocytes express several proteins that serve as markers of not only their maturation status (i.e., CK5 and CK14 are exclusively expressed on the dividing cells [15]), but also their position in the layers of epidermis (i.e., CK10 is expressed by keratinocytes closer to the surface of the skin). Immunohistochemistry was used to determine expression of Ki-67, CK5, CK14 and CK10 were used to evaluate the proliferation and the differentiation of keratinocytes in the control and cold plasma treated cells according to previously described methodology [13,14]. The percentage of positively stained cells was then determined and compared with that measured for the control group using light microscopy at 200X magnification. A one way ANOVA with post-hoc t-tests were performed on the triplicates of samples receiving High plasma level vs Low plasma level vs Controls.

Treatment with Low and High level of cold plasma had no negative effects on cell viability and the cell number in general. All flasks had a comparable number of cells between the groups (data not shown). Proliferation marker Ki-67 and transcription factor p53 were expressed more in High level plasma group (62% ± 11.8 and 49% ± 3.9) than in the cells of both the Low plasma group (41% ± 3.98 and 34% ± 7.5) and the Control group (49% ± 10.28 and 32% ± 4.7) (Figure 1). Immunohistochemical evaluation of CK5 expression showed no difference in the number of CK5+ cells among the three groups (90% of cells). In the keratinocytes that were exposed to Low plasma level we have seen an increase in expression of CK10 and a decrease in expression of CK14 when compared to the Control group and the keratinocytes exposed to High plasma level (CK10: 37%± 2.7 vs. 29% ±

7.9 and 25% ± 7.6; CK14: 68 ± 5.7 vs. 85 ± 7.9 and 83 ± 7.9, respectively). High plasma level group had comparable results to the Control group (p>0.05). Combined, our results suggest that High level plasma treatment induced proliferation of the keratinocytes, showing an increase in Ki-67 and suppression of the CK5/14 phenotype corresponding to basal keratinocytes, indicating that keratinocytes have more proliferating capacity. Additionally, our results suggest that Cold plasma therapy does not induce keratinocyte transformation.

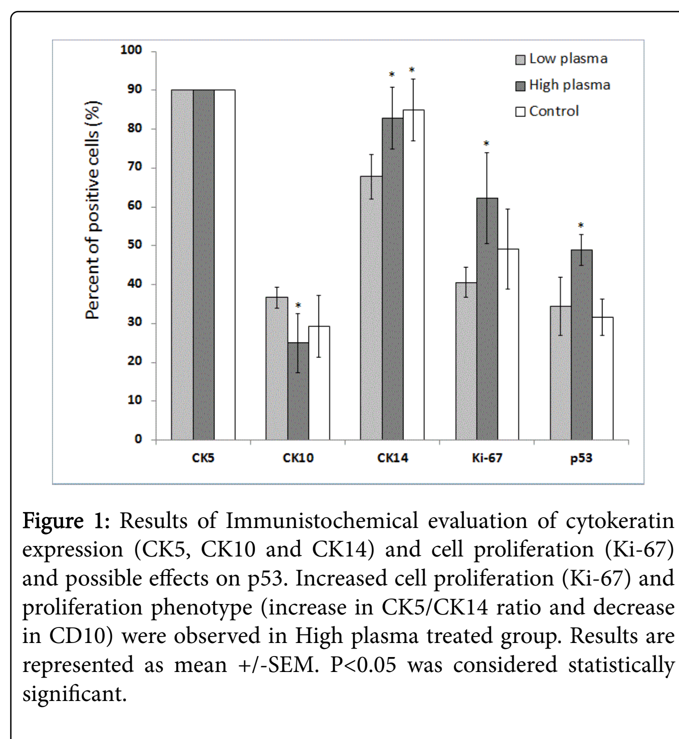

Figure 1: Results of Immunistochemical evaluation of cytokeratin expression (CK5, CK10 and CK14) and cell proliferation (Ki-67) and possible effects on p53. Increased cell proliferation (Ki-67) and proliferation phenotype (increase in CK5/CK14 ratio and decrease in CD10) were observed in High plasma treated group. Results are represented as mean +/-SEM. P<0.05 was considered statistically significant.

Our results confirm the results of Hasse et al. that showed the proliferating effects of CAP treatment in human skin keratinocytes [16]. Contrary to our results, HaCaT keratinocytes exposed to CAP had smaller viability [17]. Additionally, the same authors showed in a different study beneficial effects of CAP on expression of surface molecules. They showed that CAP treatment did not affect the expression of integrins in treated keratinocytes. CAP treatment induced suppression of EGFR, which is known to have important pro-migratory role in the wound healing [17,18]. CAP effects were evaluated in fibroblast, another cell type that is key for successful wound healing, showing increase in fibroblasts proliferation *in vitro* [19].

An important difference between our experimental set up and those by others has to be discussed. Contrary to those studies, we performed ex vivo treatment, on the split skin biopsy samples. After CAP treatment, we isolated keratinocytes. Therefore, our study is conducted over a much longer time period. Similarly to our experimental design, other researchers performed ex vivo experiments on the mucosal tissue isolated from pharynx and nose, showing a dose dependent cytotoxicity. Additionally, pig skin was exposed to CAP and dose dependent necrosis was reported after the treatment. Therefore, when comparing studies that are using cold atmospheric plasma, special attention has to be paid to the treatment parameters and the experimental set up, as effects can vary depending on the dose, energy and temperature [20]. CAP effects on wound healing have numerous mechanisms of action. These include cell proliferation effects, increased nitric oxide and oxygen free radical production, angiogenic properties, and reduced wound contamination [21]. CAP treatment increased the rate of wound healing in *in vivo* experiments and in clinical trials. In a murine animal model of wound healing, treatment with CAP increased the rate of wound healing and affected expression of wound healing (IL-6, IL-8, MCP-1) and angiogenesis relevant genes. Furthermore, there was an increase in intracellular concentration of oxygen radicals after CAP treatment [17]. Increase in oxygen free radicals concentration was utilized by Keratinocytes and melanoma cells grown in co-culture were exposed to CAP. The authors reported accumulation of reactive oxygen species in both cell types. However, melanoma cells are more sensitive to oxygen free radicals and CAP induced selective apoptosis in melanoma cells. Authors suggested that CAP could be used for targeted killing of melanoma cells [22]. In two different studies, CAP selectively targeted head and neck squamous cell carcinoma cells over normal epithelial cells [23,24]. These results may support our observation that CAP induces an increase in p53, which was significantly increased in the High plasma group.

Our study combined with the recent publications showing evidence for positive effects of cold atmospheric plasma on the healing of acute and chronic wounds [25,26] and aforementioned mechanistic studies, provide strong proof-of-principle evidence and warrants further investigations of cold plasmas on living tissues.

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