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Non-thermal plasma treatment induces MAPK signaling in human monocytes

Abstract: The application of non-thermal atmospheric pressure plasma raises a hope for the new wound healing strategies. Next to its antibacterial effect it is known to stimulate skin cells. However, monocytes are also needed for the complex process of a wound healing. This study investigates the impact of plasma on the intracellular signaling events in the primary human monocytes. The proliferative MEK-ERK (MAPK/ERK kinase-extracellular signal-regulated kinase) pathway was activated by short plasma treatment times. In contrast, an induction of the apoptotic JNK (c-Jun N-terminal kinase) cascade as well as activation of caspase 3 were observed after long plasma exposure. These findings indicate that monocytes can be differentially stimulated by plasma treatment and may contribute to the proper wound recovery.

Keywords: human monocytes, MAPK signaling, non-thermal plasma, plasma medicine, wound healing

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1 Introduction

Medical applications of non-thermal atmospheric pressure plasma have attracted increased attention over the last

decades. It exhibits temperatures below thermal cell damage and has a unique composition of excited atoms, ions, electrons, neutral molecules, free radicals, electric fields, ultraviolet (UV), thermal and infrared radiation [1,2]. Its antibacterial effects are well documented [3,4] and there have been various medical applications such as decontamination of heat sensitive endoscopes and ablation of dental biofilms [5,6]. Preliminary trials showed that plasma application significantly reduced the bacterial load in chronic wounds [7] and potentially stimulated epithelial cells [8,9]. Thus, it seems promising in wound recovery since effective therapies are urgently needed.

Acute wound healing requires a complex interplay of various cell types, cytokines, growth factors and extracellular matrix (ECM) components [10]. Next to the skin, the immune system plays a leading part in wound regeneration [11]. Immediately after skin injury, blood vessels constrict and platelets aggregate to stop the bleeding. Pro-inflammatory cytokines are released by the platelets to attract phagocytic cells. First neutrophils and subsequently monocytes are recruited to the wound bed to remove foreign particles and microbes. Monocytes differentiate into macrophages in response to bacterial products like lipopolysaccharide (LPS) or cytokines secreted by neutrophils. Macrophages display antimicrobial activities, e.g. secretion of the inflammatory mediators nitric oxide (NO) and interleukin (IL)-6, and engulf pathogens and cell debris [12]. Following phagocytosis, granulation tissue forms, the vascular network is restored and the wound closes. Subsequently, granulation stops and a scar forms [13]. If a wound fails to heal, a chronic wound is formed. Since wound healing is a complex process, defects at different stages are not uncommon. Impaired healing can be caused by alterations of cytokines, growth factors or proteases, which play important roles in wound regeneration [14].

The mitogen-activated protein kinases (MAPK) extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK are implicated in monocyte differentiation to macrophages [15-17]. These three MAPK cascades are serine/threonine kinases,

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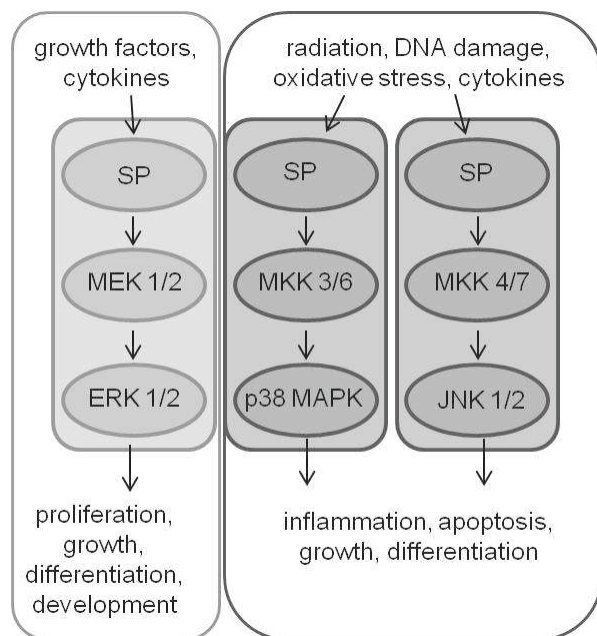


Figure 1: MAPK pathways. Scaffold proteins (SP) are kinases upstream of the MAP kinases, which are activated through mediators such as cytokines or growth factors. MEK 1/2 and MKKs (mitogen-activated protein kinase kinases) are among the SPs. The kinases ERK 1/2, p38 MAPK or JNK 1/2 are subsequently activated. These enzymes induce different cellular processes e.g. proliferation or apoptosis [19]. Image adapted from [18].

which are expressed ubiquitously and play major roles in cell fate decisions (Fig. 1). ERK is activated mainly through its upstream activator MEK (MAPK/ERK kinase). This MEK-ERK pathway can be induced by growth factors and cytokines and leads to proliferation, growth, differentiation and development. The JNK and p38 MAPK cascades can be activated by radiation, DNA damage, oxidative stress or cytokines. Induction of these pathways results mainly in inflammation and apoptosis including caspase 3 activation, but they are also known to induce growth and differentiation [18-20].

Plasma treatment has recently been shown to support human and animal wound healing [21,22] but little is known about its intracellular effects on primary human monocytes, which are the key players in the process of wound recovery. Thus, we investigated the modulation of intracellular signaling effects of primary monocytes by the non-thermal atmospheric pressure plasma jet kINPen 09, which was previously done for the THP-1 monocyte cell line [23]. To do so, MAPK signaling cascades and caspase 3 activation were examined.

2 Experimental Procedure

Unless indicated otherwise, all cell culture ingredients were purchased from Lonza (PAA Laboratories GmbH, Pasching, Austria) and all chemicals from Sigma-Aldrich (Deisenhofen, Germany).

2.1 Monocyte isolation

Human donor blood was kindly provided by the Institute of Transfusion Medicine from the University of Greifswald. Peripheral mononuclear cells (PBMC) were isolated by density gradient centrifugation on lymphocyte separation medium 1077 (PAA Laboratories GmbH). Remaining erythrocytes were lysed with $1 \times$ red blood cell lysis buffer (Biolegend, San Diego, USA). Cell counting was performed with a Neubauer chamber (Carl Roth, Karlsruhe, Germany). Subsequently, untouched CD14 positive monocytes were isolated with the monocyte isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) by negative selection following the manufacturer's instructions. Monocyte purity was analyzed by flow cytometry (Gallios, Beckman Coulter, Brea, USA); cells were stained with FITC-conjugated CD14 antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocyte purity (CD14 positive cells) was 80% or higher (data not shown).

2.2 Plasma source and plasma treatment

Non-thermal plasma treatment was performed with the atmospheric pressure jet kINPen 09 (Neoplas GmbH, Greifswald, Germany), based on the setup of Weltmann *et al.* in 2009 [24] with the quartz capillary replaced by ceramic. Plasma was generated at atmospheric pressure and approximately 1 MHz. The kINPen 09 was operated with argon (99.999%) at 3 standard liters per minute controlled by a mass flow controller (MKS Instruments, Munich, Germany). To reduce the strong effect of feed gas humidity on the cells [25], stainless steel tubing was used and the apparatus was first flushed for 60 minutes.

Immediately after monocyte isolation, plasma treatment was performed indirectly; This means that 5 mL of monocyte culture medium (RPMI 1640 containing 10% FCS, 2 mM L-glutamine and 1% penicillin/streptomycin) was plasma treated for distinct times (15, 30, 60, 180 or 360 s) and then added to 1×10^6 monocytes. Appropriate volumes of sterile distilled water were immediately added to the culture medium to compensate molarity changes

Table 1: Primary antibodies used for Western blotting. Table adapted from [23].

target	target molecular mass [kDa]	phosphorylation site	host	product number
β -Actin	45	-	rabbit	4970
ERK 1/2	42/44	-	rabbit	9102
JNK 1/2	46/54	-	rabbit	9258
MEK 1/2	45	-	rabbit	9126
phospho-ERK 1/2	42/44	T202/Y204	rabbit	4370
phospho-JNK 1/2	46/54	T183/Y185	rabbit	4668
phospho-MEK 1/2	45	S217/S221	rabbit	9154

caused by evaporation during plasma exposure. Next to the plasma treatment, monocytes were incubated in medium, which was left untreated (0 s). To induce proliferation, cells were treated with medium containing $1 \mu\text{g mL}^{-1}$ LPS. In contrast, cells were incubated with medium including $100 \mu\text{M H}_2\text{O}_2$ to activate apoptotic pathways. Subsequently, samples were incubated at 37°C , 95% relative humidity and 5% CO_2 for different time periods depending on the further investigation.

2.3 Western blotting procedure

To examine the proliferative MEK-ERK signaling pathway monocytes were lysed 15 min after plasma treatment, while the apoptotic cascade JNK 1/2 was studied 3 h after plasma exposure, similarly to the investigations of the THP-1 cell line [23]. Suspended cells were collected in Falcon tubes, while adherent monocytes were washed once with $1 \times$ PBS and trypsinized for 5 min. Suspended and adherent cells were recombined and rinsed once with $1 \times$ PBS at $230 \times g$ for 5 min. Cell lysis was performed with ice-cold RIPA lysis buffer ($1 \times$ PBS with 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS and 20 mM EDTA) containing protease and phosphatase inhibitors (cOmplete Mini, phosSTOP; both ROCHE, Mannheim, Germany) and freshly added 2 mM phenylmethanesulfonyl fluoride (Carl Roth, Karlsruhe, Germany). To complete cell lysis, samples were incubated on ice for 30 min, vortexing every 10 min, followed by sonication (Labsonic M, Satorius AG, Göttingen, Germany). Samples were then centrifuged at $10,000 \times g$ for 5 min at 4°C .

Supernatants were collected and their protein concentrations determined by the DCTM Protein Assay (Bio-Rad Laboratories GmbH, Munich, Germany). The protein concentrations of all samples were adjusted and proteins were subsequently denatured in $1 \times$ sample buffer

(0.25 M tris, 2% SDS, 10% glycerol, 2% β -mercaptoethanol, 0.004% bromphenol blue) at 95°C for 5 min.

Samples and marker (PageRulerTM Prestained Protein Ladder, Fermentas, St. Leon-Rot, Germany) were subjected to SDS-PAGE on precast 10% PAGE gels (Anamed Gelelektrophorese GmbH, Rodau, Germany). Blotting onto Roti-PVDF membranes (Carl Roth, Karlsruhe, Germany) was done on a Trans-Blot TurboTM (Bio-Rad Laboratories GmbH, Munich, Germany) according to the manufacturer's instructions (30 min at 25 V). This was followed by nonspecific binding site blocking using 5% nonfat milk powder (Premier Foods Marvel, St. Albans, UK) in tris-buffered saline (20 mM tris, 13.7 mM NaCl) containing 0.1% Tween 20 (TBS-T) for 30 min. Afterwards, the membrane was incubated with one of the phospho-specific primary antibodies (Cell Signaling, Beverly, USA) listed in Table 1 (adapted from [23]) 1:1,000 in TBS-T at 4°C overnight. The next day the membrane was washed three times with TBS-T and incubated with horseradish peroxidase-coupled secondary antibodies (Jackson ImmunoResearch, West Grove, USA; rabbit IgG, product 211-032-171) 1:10,000 for 1 h at room temperature. The membrane was washed three times followed by incubation with Serva Light Polaris chemiluminescence detection reagent (Serva Electrophoresis GmbH, Heidelberg, Germany) and imaged using the ImageQuantLAS4000 (GE Healthcare, St. Louis, USA). Target protein identification was assisted by the molecular mass marker. The membranes were stripped twice in stripping buffer (62.5 mM tris with 1% SDS and 111 mM β -mercaptoethanol; pH 6.7) for 25 min at 70°C and reprobed with the equivalent total protein antibody for the respective target as well as β -Actin antibody (all from Cell Signaling, Beverly, USA). Band intensities were quantified with ImageQuant TL Software (GE Healthcare, St. Louis, USA). Relative phosphorylation levels were given by the ratios of phospho-protein to total protein intensities.

2.4 Flow cytometry

18 h after plasma treatment the number of cells positive for the late apoptotic marker caspase 3 was determined by flow cytometry. 0.3×10^6 cells of each sample were stained with 1 μL FITC-DEVD-FMK (Green Caspase-3 Staining Kit, PromoCell, Heidelberg, Germany) in 300 μL culture medium and incubated for 30 min in the dark at 37°C, 95% relative humidity and 5% CO_2 . This was followed by two washings with 0.5 mL wash buffer (Green Caspase-3 Staining Kit, PromoCell, Heidelberg, Germany) at $1,000 \times g$. Cell pellets were resuspended in 300 μL wash buffer and the caspase 3 positive cell percentage was determined by flow cytometry.

2.5 H_2O_2 measurement

Hydrogen peroxide in the plasma-treated culture medium was determined with commercial H_2O_2 test strips (Merckoquant 110011, Merck, Darmstadt, Germany); 10 μL of treated medium was pipetted onto a test strip and incubated for 30 s. The color was recorded by a digital microscope camera (Conrad, Germany) and the H_2O_2 concentration calculated as described by Winter *et al.* [25].

2.6 Statistics

Western blot, results of the flow cytometry and H_2O_2 treatment were illustrated with Prism 6.0 (GraphPad software, La Jolla, USA). Means and standard deviation of three independent experiments were plotted as bar diagrams. One-way analysis of variances (ANOVA) and Dunnett's post hoc-test were used to calculate statistical significance of the samples referring to the untreated control (0 s). Statistical significance is displayed the following way: $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***)

3 Results and Discussion

3.1 Plasma-mediated regulation of MAPK signaling pathways

To analyze whether the pro-proliferative MEK-ERK pathway or the pro-apoptotic JNK cascade was induced by plasma treatment, the relative phosphorylation or activation levels of these kinases were determined

by Western blotting. Additionally, β -Actin expression was examined to ensure equal loading of the protein suspension onto the gels (Figs. 2 and 3).

Both short and long plasma treatments led to MEK-ERK activation (Fig. 2). As displayed in Fig. 2A, MEK 1/2 was activated in a plasma treatment time dependent way. The shorter plasma exposure times of 30 and 60 s already resulted in an induction of the relative phosphorylation level of MEK 1/2 (1.6- and 1.8-fold), while longer duration of plasma treatment led to significant activation ($p < 0.05$, *) up to 2.4-fold of untreated control after a treatment time of 360 s. A similar tendency could be demonstrated for the plasma-mediated activation of ERK 1/2 as depicted in Fig. 2B. Relative phosphorylation levels of ERK 1 and ERK 2 increased 2.7- and 6.1-fold after 360 s of plasma treatment. Both MEK 1/2 and ERK 1/2 of plasma-treated primary monocytes displayed the same trends as already described for plasma-treated THP-1 monocytes [23]. However, the levels were considerably higher for the primary cells. The activation of the MEK-ERK pathway is important for monocyte to macrophage transformation, essential for a proper wound healing process [15]. Furthermore, it has been reported that the MEK-ERK signaling pathway was weakened in diabetic rats, associated with the impaired wound regeneration [26]. Here, plasma treatment could be one option to reactivate this pathway and subsequently contribute to a proper wound healing process.

LPS up-regulated both MEK 1/2 (1.8-fold) and ERK 1/2 (2.7- and 7.2-fold) in agreement with the findings of Liu *et al.* for monocytes [27]. Additionally, H_2O_2 increased the relative phosphorylation of MEK 1/2 (1.8-fold) and ERK 1/2 (1.3- and 1.4-fold), consistent with Bhatt *et al.*, who showed that ERK 1/2 can be induced in human monocytes by exogenous reactive oxygen species such as H_2O_2 [28]. Since H_2O_2 is generated in plasma-treated liquids, it is hypothesized to be one of the species activating the MEK-ERK pathway [29].

In contrast to the pro-proliferative pathway, the pro-apoptotic JNK cascade was induced only after long plasma exposure times (Fig. 3). Short treatment times (30 and 60 s) did not activate JNK 1/2, while longer times (180 and 360 s) resulted in JNK 1/2 up-regulation. JNK 1 was induced up to 3.5-fold of control after 360 s ($p < 0.05$, *) of plasma exposure, while JNK 2 showed an up-regulation up to 4.5 for a plasma treatment of 360 s. JNK 1/2 cascade involvement in oxidative stress mediated-apoptosis induction has been reported [30]. Especially long treatments lead to reactive species accumulation in the medium [31]. This may explain why JNK 1/2 was only activated after the long 180 and 360 s treatments.

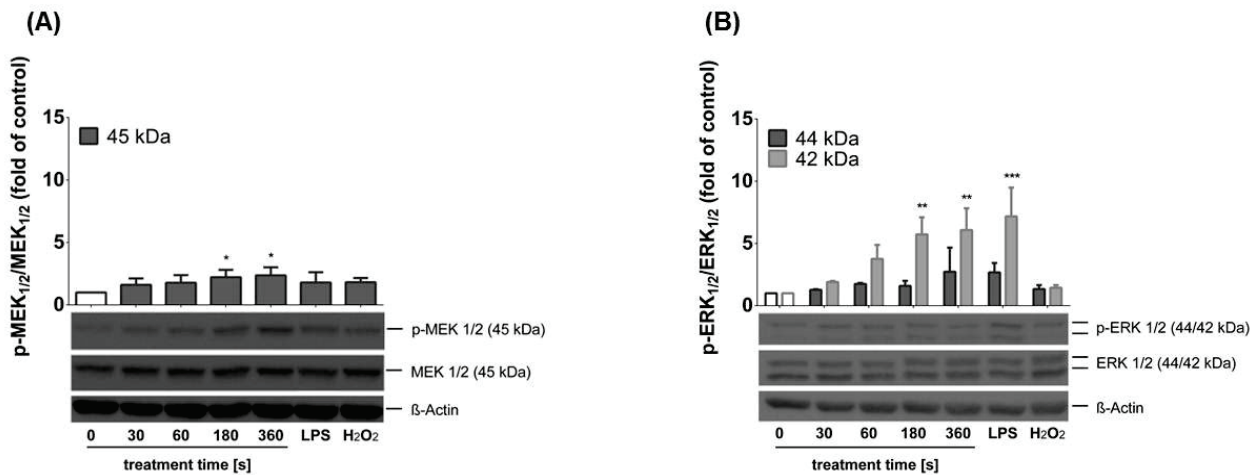


Figure 2: Western blot analysis of the MEK-ERK signaling pathway. Human monocytes were lysed after 15 min of plasma treatment. Subsequently, Western blot analysis was performed with the protein suspensions to determine the phosphorylated as well as the total level of the proteins MEK 1/2 at 45 kDa (A) and ERK 1/2 at 44 and 42 kDa (B). Relative phosphorylation level was calculated as a ratio of phosphorylated to total protein and plotted as bar diagrams. H₂O₂ (100 μ M) and LPS (1 μ g mL⁻¹) treated samples served as controls for apoptotic and proliferative pathways. All samples were normalized to the untreated control (0 s). β -Actin (45 kDa) was used as a loading control. Data from three independent experiments are shown. Statistical significance is depicted in the following way: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)

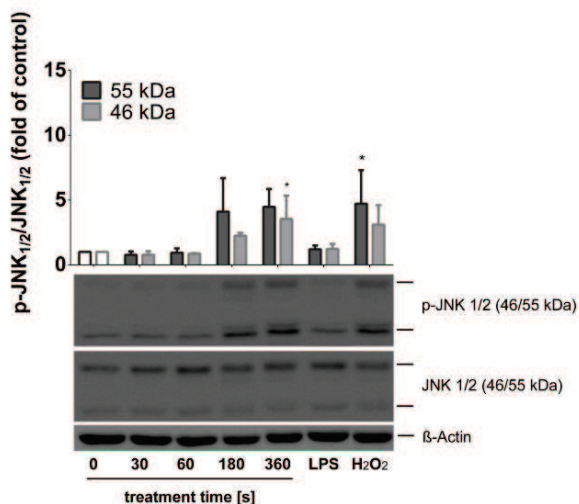


Figure 3: Western blot analysis of the JNK pathway. Human monocytes were lysed after 3 h of plasma treatment. Subsequently, Western blot analysis was performed with the protein solution to determine the phosphorylated as well as the total level of the protein JNK 1/2 at 46 and 55 kDa. Relative phosphorylation level was calculated as a ratio of phosphorylated to total protein and plotted as bar diagrams. H₂O₂ (100 μ M) and LPS (1 μ g mL⁻¹) treated samples served as controls for apoptotic and proliferative pathways. All samples were normalized to the untreated control (0 s). β -Actin (45 kDa) was used as a loading control. Data from three independent experiments are shown. Statistical significance is depicted in the following way: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)

Like the short treatments, incubation with LPS did not induce JNK 1/2 activation. This is consistent with the report of Heidenreich *et al.* that LPS activates monocytes by CD14

expression, leading to increased survival and apoptosis evasion [32]. Treatment with the apoptosis-inducing agent H₂O₂ [33] led to an up-regulation of JNK 1/2 to 3.1- and 4.7- ($p < 0.05$, *) fold of the control as described in the literature [34]. Next to JNK 1/2, p38 MAPK was examined regarding its activation after non-thermal plasma treatment. However, only a slight and insignificant induction of the relative phosphorylation level after the long plasma treatment times was found for this kinase (data not shown). Thus, the involvement of this pathway was considered negligible in plasma-treated primary monocytes.

Remarkably, short plasma treatment times of 30 and 60 s induced only the pro-proliferative MEK-ERK pathway, while the pro-apoptotic JNK cascade was only switched on after longer plasma durations of 180 and 360 s.

3.2 Apoptosis induction in response to plasma treatment

Since the pro-apoptotic JNK pathway was switched on only after long plasma exposure times, a caspase 3 assay was performed to investigate the percentage of cells undergoing apoptosis 18 h after plasma treatment.

Fig. 4 displays the percentage of late apoptotic monocytes (caspase 3 positive) after plasma treatment. As has been shown for the early apoptotic marker annexin V [35], the percentage of late apoptotic monocytes increased with plasma treatment time. The untreated

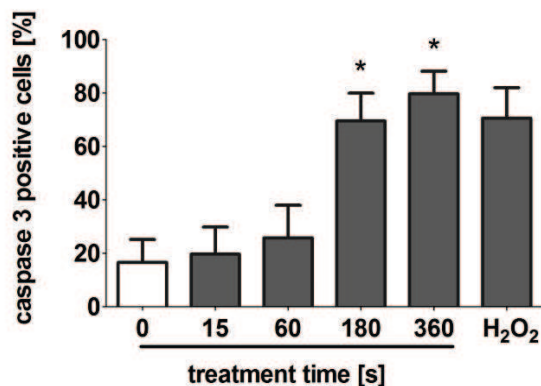


Figure 4: Flow cytometry analysis of caspase 3 positive cells after indirect plasma treatment. Percentages of caspase 3 positive monocytes were plotted according to plasma treatment time. 100 μM H_2O_2 was used as a positive control for apoptosis induction. Data are displayed with the mean and standard deviation of three independent experiments. Statistical significance is depicted in the following way: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)

control contained 17% apoptotic cells, as monocytes are relatively short living immune cells which undergo apoptosis on a daily basis [36]. Up to a plasma treatment time of 60 s no significant induction of the caspase 3 activation level was found in comparison to the untreated control. Plasma exposure times exceeding 60 s showed dose-dependent caspase 3 activation up to 80% (360 s). This is consistent with the findings of Barton *et al.*, who showed that cytotoxicity increases in THP-1 monocytes after long plasma exposure [37]. Recently, Avisetti *et al.* showed the connection of JNK and caspase 3 activation due to reactive oxygen species exposure, which may be responsible for the plasma-induced caspase 3 activation in the monocytes [20]. Additionally, incubation with 100 μM H_2O_2 up-regulated the number of caspase 3 positive cells to 71%, in good agreement with data of Tanigawa *et al.* [38].

In summary, apoptotic caspase 3 was mainly activated by long treatment times (180 and 360 s) as an expected downstream event of pro-apoptotic JNK 1/2 induction.

3.3 Hydrogen peroxide generation in plasma treated cell culture media

It is already known that H_2O_2 is one of the main products of cell culture medium treated with the kINPen 09 operated with argon [29]. Since hydrogen peroxide seems important in triggering cellular responses, its content in plasma-treated cell culture medium was investigated.

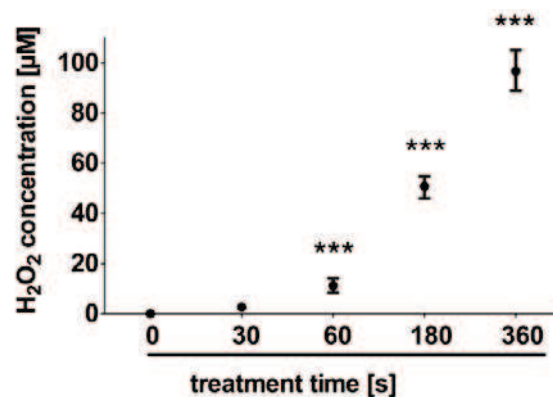


Figure 5: H_2O_2 concentration of plasma treated cell culture medium determined by H_2O_2 test strips after treatment. Data are displayed as mean with standard deviation of three independent experiments. Statistical significance is depicted in the following way: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)

Fig. 5 displays the H_2O_2 concentrations after plasma exposure. In untreated cell culture medium (0 s) none could be detected. After a short 30 s treatment only 3 μM H_2O_2 was present. Longer plasma treatment times resulted in a significant treatment time dependent induction of the H_2O_2 content ($p < 0.001$, ***) up to 97 μM after a 360 s of plasma exposure. Since induction of the relative phosphorylation level of JNK 1/2 after 360 s of plasma treatment resembled the value of a 100 μM H_2O_2 treatment, it can be assumed that JNK 1/2 is mainly activated by formation of hydrogen peroxide in the cell culture medium. However, incubation with 100 μM H_2O_2 led to activation levels of MEK 1/2 and caspase 3 similar to that from 180 s of plasma treatment and ERK 1/2 activation levels similar to 30 s of plasma exposure. We hypothesize that not only H_2O_2 but other reactive species like $\cdot\text{OH}$, $\text{O}_2^{\cdot-}$ and NO_2^- , known to be formed in the plasma treated medium [39,40], are able to induce these cellular responses.

In summary, hydrogen peroxide formed in plasma treated liquids plays an important role in eliciting cellular signaling responses. However, other reactive species are involved and should be further investigated.

4 Conclusions

Depending on the plasma exposure time, pro-proliferative or pro-apoptotic MAPK signaling cascades can be activated in human monocytes. While short plasma treatment (30 and 60 s) activates the pro-proliferative MEK-ERK cascade, the pro-apoptotic pathway JNK and the apoptotic protease caspase 3 are only induced after longer plasma

exposures (180 and 360 s). Plasma exposure up to 60 s stimulates the MEK-ERK cascade. Therefore, short plasma treatment might stimulate monocytes to contribute to the wound recovery. Plasma-generated hydrogen peroxide was shown to be an important signaling messenger. More studies of intra- and intercellular signaling in response to plasma treatment are needed for both immune and skin cells. This study improves our understanding of immune cell plasma modulation and its contribution to wound healing.

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