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Identification of the Molecular Basis of Non-thermal Plasma–Induced Changes in Human Keratinocytes

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ABSTRACT: A comprehensive gene expression profiling was conducted to explore cellular effects after non-thermal plasma atmospheric pressure plasma treatment. We performed a high-content microarray comparison by assessing several categories of target probes in identical conditions of labeling, hybridization, and data analysis to compare specific gene expression profiles of human epithelial skin cells with their nontreated counterparts. For assessment of transcriptome changes, cell culture medium was plasma treated and applied to the HaCaT keratinocyte cell culture. We show that even this indirect argon plasma treatment performs as well as incubation time–dependent effects on gene expression. These effects range from cell proliferation and growth to the induction of cell death pathways. It is hypothesized that these effects are evoked through plasma-based formation of reactive oxygen and nitrogen species. Several significant biological pathways, such as oxidative stress, repair, and inflammation signaling, as well as >300 transcription factors (e.g., zinc finger or homebox) were identified. Our results contribute to a better understanding of plasma-mediated changes in cells at the transcriptional level. In addition, plasma may serve as a promising biomedical tool for stimulation of skin cells with regard to wound healing.

KEY WORDS: non-thermal plasma, plasma medicine, reactive oxygen species, reactive nitrogen species, gene expression profiling, HaCaT keratinocytes

I. INTRODUCTION

Specific properties of non-thermal atmospheric pressure plasma lead to various biomedical applications in mammalian cells or tissues. Thus far, several sources have been used as agents for decontamination of biological surfaces and implants.[1–4] To date, little work has been done to investigate the effects of plasma in skin-healing processes or treatment of skin diseases such as dermatitis or inflammation.[5,6] Our research thus focuses on the improvement of plasma effects on treated cells and on active regulation of the interaction between plasma and surrounding tissue during wound healing.

Biological processes and cell interactions during wound healing are complex.[7] Wound healing progresses via four phases: hemostasis, inflammation, granulation, and tissue remodeling. During the granulation phase, keratinocytes proliferate and migrate to close the wound.[8,9] The human epithelial keratinocyte cell line (HaCaT) is an often applied model system for wound healing.[10] In this regard, HaCaT cells are extensively characterized for their properties in cell culture.[11] We thus utilized

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HaCaT cells in this study to assess qualitative and quantitative molecular changes after plasma application.

Plasma was generated by a jet (kINPen) containing reactive oxygen species (ROS; e.g., $HO^{\cdot}$, O_2^{-}, O_3, and H_2O_2) and reactive nitrogen species (RNS; e.g., $NO^{\cdot}$ and $ONOO^{-}$), photons (ultraviolet radiation), charged particles, and ions.[12–15] Changes in the metabolism of ROS are important for mammalian cells. Depending on the amounts present, ROS can feature either beneficial or damaging effects to cells.[16] Studies show that at lower concentrations in cells, ROS may serve as secondary messengers by activating signal transduction pathways mediated by several protein kinases and phosphatases as well as nuclear factor-κB.[17] By contrast, overproduction of ROS and deficiency in antioxidant enzymes lead to cytotoxicity by damaging cell components.[18]

In recent years, microarrays have been widely used to study complex molecular mechanisms in a broad range of diseases and cancer. Thus, this study aimed to profile differences in gene expression following indirect plasma treatment of keratinocytes using a high-content oligonucleotide microarray to understand fundamental molecular processes involved in plasma–cell interactions. Our data demonstrate that plasma has dose-dependent as well as incubation time–dependent effects ranging from cell proliferation and growth to induction of cell death pathways. Our results further indicate that networks of genes related to cellular growth, proliferation, function, and maintenance are affected at short-term and middle-term treatment, which likely may contribute to the beneficial effects of plasma during wound healing.

II. MATERIALS AND METHODS

Non-thermal plasma was generated using a kINPen atmospheric pressure plasma jet (neoplas GmbH) that ionized a flow of argon gas.[19] A voltage of 2–6 kV_{pp} was applied with a frequency of approximately 1 MHz. Immediately after plasma treatment with 5 mL Roswell Park Memorial Institute (RPMI) medium supplemented with 8% fetal calf serum, 0.1 mg/mL penicillin/streptomycin, and 2 mM L-glutamine, the medium was transferred to 1×10^6 HaCaT keratinocytes. The treatment time equivalents of 100, 300, and 900 ms-µL/cell corresponded to 20-s, 60-s, and 180-s treatment times of 5 mL RPMI medium, respectively. To assess qualitative and quantitative changes over time, cells were incubated with fresh media for an additional 2 h or 23 h and were collected 3 h or 24 h after plasma exposure. To exclude effects of the carrier gas, cells incubated with argon gas–treated medium were used as negative controls. Cells treated with 50 µM H_2O_2 served as positive controls for oxidative stress.

Total RNAs from plasma-treated and untreated HaCaTs were purified according to RNA kit instructions (Bio & Sell). RNA integrity was confirmed using the Bioanalyzer 2010 (Agilent). cDNA was synthesized from 10 µg of total RNA using the cDNA Synthesis Kit (Invitrogen) in the presence of oligo-dT (200 ng/mL) and random hexamer primer (100 ng/mL). Double-strand cDNAs were end-labeled with fluorescent Cy3-dye (One-Color DNA Labeling Kit; Roche). A global gene expression study was conducted using NimbleGen multiplex arrays containing a 4-plex format (4 × 72K) with 24,000

different human-specific probes per array ($n \geq 3$). Hybridization and washing of gene chips was performed according to the supplier's instructions (Roche). Microarrays were analyzed by a Microarray Laser Scanner MS 200 as previously described.[12]

Image generation and quality analysis were performed using NimbleScan 2.6 software (Roche). Background-corrected signal intensities were determined and processed using a robust multichip averaging (RMA) analysis.[20] Background adjustment, normalization of microarray data, statistical tests (false discovery rate [FDR] < 0.001; $P \leq 0.05$), and further filtering methods were accomplished by DEVA1.1 analysis software (Roche NimbleGen). Signal intensity values were translated into gene ID lists including expression values. Data handling and all calculations including cluster analysis were performed using the Partek Genomic Suite (PGS; Partek Inc.). To find the differentially expressed genes, expression data were grouped according to treatment conditions and were statistically analyzed using multiple testing corrections.[21] Gene ontology (GO) analysis was performed using the Protein Analysis Through Evolutionary Relationships (Panther) classification system. The Panther classification system was designed to classify proteins and their genes in order to facilitate high-throughput analysis.[22] Ingenuity Pathway Analysis (IPA; Ingenuity Systems Inc.) was applied to summarize the effect of gene expression changes and to obtain the top biological functions, pathways, and networks associated with non-thermal plasma treatment.

To quantify mRNAs by quantitative real-time PCR (qPCR) on a LightCycler 480 qPCR system (Roche Diagnostics Ltd), 1 μg of total RNA was reverse transcribed using Omniscript reverse transcriptase, oligo-dT primer, and 10 mM dNTP (Qiagen). Fifty nanograms of the cDNA sample was mixed with SYBR Green I Master (Roche Diagnostics Ltd) as well as the specific primer pairs for oxidative stress responsive 1 (OXSR1), glutathione peroxidase 1 (GPX1), NADH dehydrogenase FS4 (NDUFS4), methionine sulfoxide reductase A (MSRA), and the housekeeping gene ribosomal protien L13A (RPL13A) (Roche Diagnostics Ltd, Germany). The protocol included pre-incubation at 95°C for 3 min, 45 cycles of 95°C for 10 s, annealing for 20 s at 55°C, and an amplification step at 72°C for 1 s. The expression of the single gene was analyzed using the ΔΔCT method. The final value for gene expression in each plasma-treated sample was determined as the ratio of the gene expression in the respective sample related to the untreated control.

III. RESULTS AND DISCUSSION

This study aimed to evaluate the association between the differential gene expression profiles of non-thermal plasma–treated keratinocytes and their nontreated counterparts. Thus, we focused on the biological effects of plasma exposure after a short (1 h) incubation time and compared gene activity with nontreated HaCaT cells. Keeping in mind that plasma produces ROS and RNS in liquids,[23] we investigated the effects of different treatment time equivalents (100, 300, and 900 ms-μl/cell). We included multiple incubation times ranging from 3 h to 24 h to monitor the effects of plasma after short or long time periods. Total RNA samples were transcribed into Cy3-labeled cDNA probes and were hybridized onto a microarray containing 24,000 human DNA target sequences.

The Cy3-monocolor images from four experimental features were quantified, normalized, and translated into gene lists containing expression values using the RMA algorithm of the DEVA1.1 platform-specific analysis software (Fig. 1).

The resulting microarray data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus (accession number GSE46343). Evaluation of the data together with Venn diagrams allowed us to identify shared and exclusively modulated genes, according to plasma treatment condition. Gene expression data of increased and repressed gene groups were determined by PGS to identify regulated genes represented by several samples. After 1 h of plasma-cell incubation time, we obtained data on >3700 differentially expressed genes for all treatment times and experimental groups in contrast with the control. The number of modulated genes was identified as statistically significant (FDR < 0.001; $P \leq 0.05$). The heat map illustrates the sorting of genes in respect to their relative expression value between the experimental plasma-treated and control groups. Genes with similar expression levels were placed into corresponding subgroups in clusters of approximately 1800 upregulated genes or 1900 downregulated genes (Fig. 2, red and green, respectively).

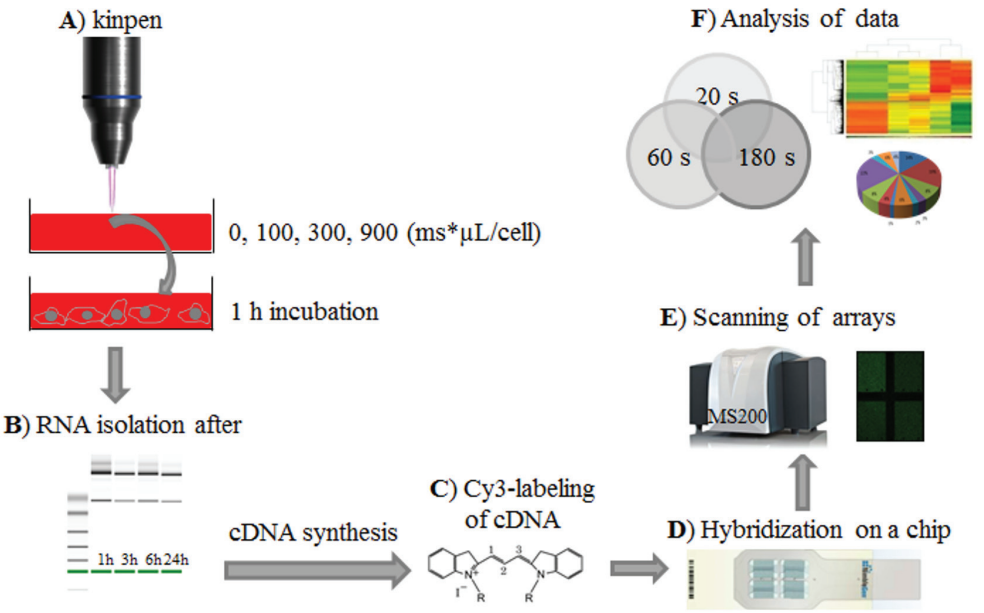

FIG. 1: Schematic illustration of gene expression profiling of the HaCaT cell line after non-thermal plasma treatment. (A) Cells were indirectly treated using a plasma jet (kINPen). (B and C) After RNA isolation and cDNA synthesis, double-strand cDNA was labeled with Cy3. (D) Gene expression of samples was analyzed with a 4 × 72K microarray chip containing 24,000 genes with three probes per target gene. (E and F) Scanned images were analyzed by PGS, Panther, and IPA, which provides insight into the causes of observed gene expression changes as well as into the predicted downstream biological effects of those changes.

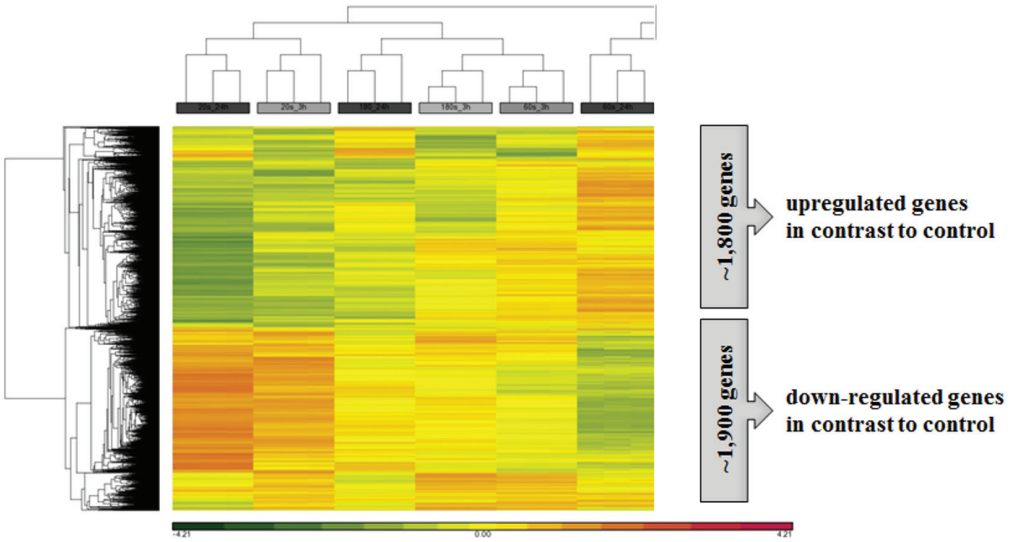

FIG. 2: Heat map of hierarchical clustering of differentially expressed genes identified by whole-genome gene chip analysis on plasma-treated HaCaTs using PGS. The transcripts were compared with each other and with the control 1 h and 24 h after plasma exposure. The genes are sorted in rows according to their expression level, and the different plasma treatments are aligned in columns. Genes are color coded according to the normalized expression values and the colors in the heat map represent expression levels. Of approximately 3700 differentially expressed genes, approximately 1800 were upregulated (red) and 1900 were downregulated (green) in comparison with the control.

Venn diagrams were constructed to distinguish the common and the exclusively modulated genes according to the above-mentioned features. Each of the circles depicts the number of different transcripts based on a binary comparison for each of the labeled sample groups from among the 5697 transcripts for 3 h or 7298 genes for 24 h (Fig. 3). Overlapping differences shared among more than one sample group comparison are represented in the areas of intersection between the two circles. The 757 or 112 transcripts in the center of the Venn diagram represent genes that are different among all group comparisons. Plasma-modulated genes were further divided into groups for upregulation (left) and downregulation (right) for each treatment time (Fig. 3A, B, lower panels).

Several biology-focused pathway analysis programs were then used to compare and interpret differential gene expression as well as to monitor involved signaling cascades. First, to better understand the significance of the differentially expressed genes in relation to each other, the response to biological function was analyzed by the Panther classification system.[22] GO, an expert-curated database that assigns genes to various functional categories, is a robust tool to summarize gene function and classify data into biological process, molecular function, and cellular component.[24] According to the major biological processes (Fig. 4A), modulated genes from each group were related to

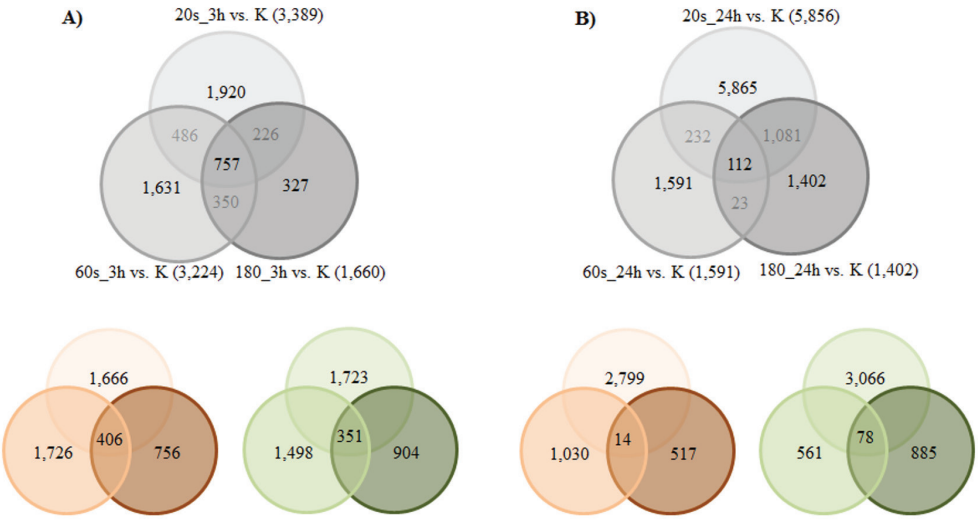

FIG. 3: Venn diagrams of microarray results representing the number of targets revealed similarities and differences in transcriptome profiles regulated by plasma. (A, B) Venn diagram visualizing the overlapping results between the differentially regulated genes found at 20 s, 60 s, and 180 s in non-thermal plasma–treated versus control groups (K) for 3 h (A) and 24 h (B). Single Venn diagrams (lower panels) for upregulation (left) and downregulation (right) for each cluster.

metabolic (37%–40%), cellular (29%–32%), developmental (11%–13%), and immune system processes (9%–10%), as well as cell communication (20%–23%), transport (13%), response to stimulus (6%–7%), cell adhesion (5%–7%), cell cycle (6%), cellular component organization (5%), and apoptosis (4%). Of approximately 3700 genes with an at least 2-fold change in the expression level, we found approximately 100 oxidoreductases including enzymes of the antioxidant defense system, which were further divided into different categories listed in Fig. 4B.

To validate microarray data, we measured the gene activity of OXSR1, GPX1, NDUFS4, and MSRA using qPCR. qPCR data are illustrated as the fold change of expression of an individual target related to the control. qPCR confirmed that OXSR1, GPX1, and NDUFS4 mRNA were increased in response to plasma, whereas MSRA mRNA was decreased (Fig. 4C). The product of OXSR1 belongs to the Ser/Thr protein kinase family and regulates downstream kinases in response to environmental stress. It may play a role in regulating the actin cytoskeleton.[25] GPX1, one of the most important antioxidant enzymes, functions in the detoxification of H_2O_2.[12] NDUFS4 encodes an accessory subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (complex I), which plays a vital role in cellular ATP production.[26] Gene expression of MSRA decreased in senescent cells, and this decline was associated with an alteration in catalytic activity.[27] The proposed function of these proteins is to repair oxidative damage of molecules (e.g., proteins, lipids) to restore biological activity. In conclusion,

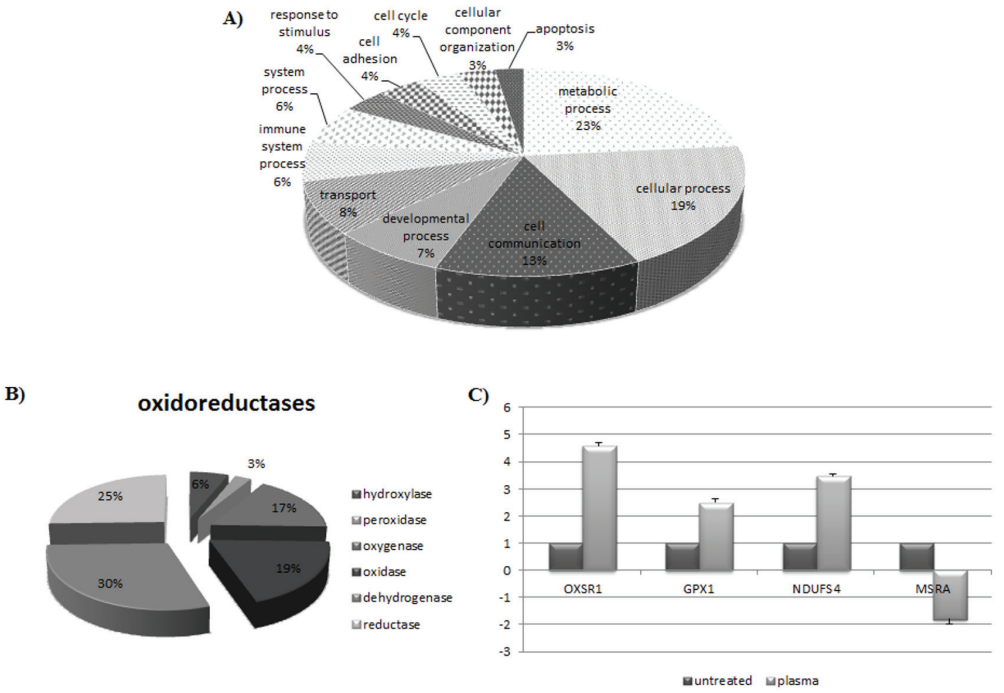

FIG. 4: Percentage distribution of significant GO biological process categories associated with non-thermal plasma. (A) The open access Panther program was used to analyze the gene lists for each experimental group (100, 300, and 900 ms-µl/cell versus control) to find all categories with the "biological process" functions domain of GO ($P \leq 0.05$). (B) Approximately 100 oxidoreductases were regulated and grouped into enzyme classes. (C) HaCaTs were incubated in plasma-treated media for 1 h. mRNA levels of OXSR1, GPX1, NDUFS4, and MSRA were measured by qPCR as described in the Materials and Methods. Results (gene expression values) are the means ± standard derivation of three measurements. A *p*-value of ≤ 0.05 was considered significant.

one major effect of plasma as observed is the activation of stress signals and the gene expression of enzymes of the antioxidative defense system.

Another more focused analysis beyond GO classification was subsequently performed on the molecular level utilizing the accumulated knowledge found in the literature. IPA predicted most significant biological signaling pathways and networks by analyzing gene expression differences between two features using the knowledge base created from previous findings. During the analysis, IPA finds the assigned function for given genes and further categorizes genes with related biological functions into several classes.[28] After short-term plasma treatment (100 ms-µL/cell), the microarray-evaluated mRNA expression ratio showed a significant increase in the expression of genes related to cell cycle, cellular growth, function, and maintenance as well as cellular assembly, morphology, and organization (Fig. 5A). However, a crucial shortcom-

ing is that biological processes usually involve more than one pathway; more precise pathways must be interconnected in a context-specific manner mainly resulting in a regulatory network for microarray results.[24] Therefore, based on the genes differentially expressed during plasma treatment, several networks were identified and summarized (Fig. 5A). The top-associated network functions mainly included "cell cycle, cellular assembly, and organization," "cellular growth and proliferation," and "cellular development, function, and maintenance." The ErbB4 growth factor signaling was induced after short plasma treatment. ErbB4 is a 180-kDa transmembrane receptor tyrosine kinase that regulates cell proliferation and differentiation.[29] Similarly, after

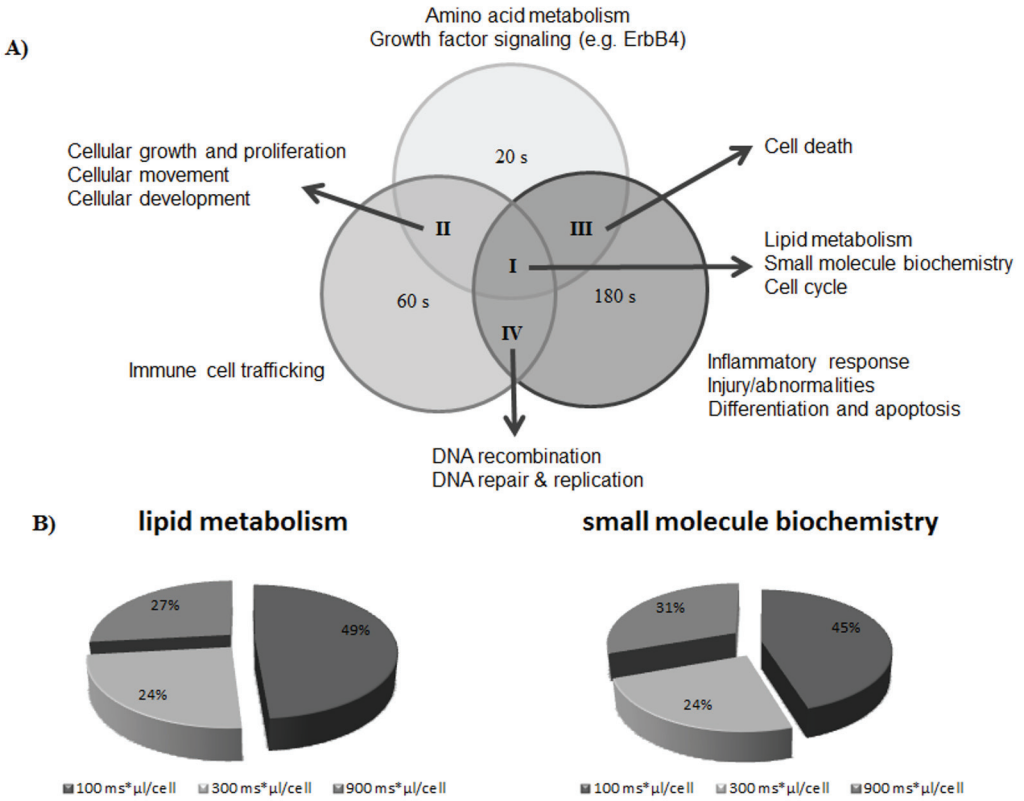

FIG. 5: Functional analysis and networks for plasma-treated keratinocytes. (A) Analysis of the top five molecular and cellular functions and their associated networks determined by IPA for all data sets demonstrates that "cell cycle," "small molecule signaling," and "lipid metabolism" are primary functions of the genes expressed after plasma treatment. "Cellular proliferation, growth, and differentiation" are important after short-term and middle-term plasma exposure. By contrast, inflammatory response is one of the primary functions of the genes expressed after long-term plasma treatment (see text). (B) The categories "lipid metabolism" and "small molecule biochemistry" were depicted in dependences on dose for each experimental group.

mid-term treatment (300 ms-µL/cell), a comparison of the cluster analysis confirmed pathways that are involved in the cell cylcle as well as in cellular movement, growth, proliferation. Some genes were associated with "DNA replication, recombination, and repair" and several were associated with "carbohydrate metabolism" and "cellular development." By contrast, long-term plasma treatment (900 ms-µL/cell) analysis revealed an expression pattern of genes similar to cell death, recombination, repair, and inflammatory response processes. Less altered genes were exclusively associated with a high plasma dose, whose major biological function was related to gene expression, cell signaling, and protein synthesis processes.

Interestingly, in all groups, we found genes strongly associated with small molecule biochemistry and lipid metabolism (Fig. 5B). Several small molecule species (O_2, NO, CO, or ROS/RNS) were previously shown to be endogenously generated signaling molecules.[30] They form an integrated signaling net that affects and regulates numerous physiological processes. The chemical interactions between these species and each other, as well as their influence on biological targets, is an important factor in their roles as signaling agents.[31] To note, plasma-generated reactive species also add to the cellular production level of endogenous ROS/RNS. A high level of oxidative stress is known to be detrimental to cell survival and in many cases leads to the induction of apoptosis.[32] Some of the networks identified are involved in several disorders, suggesting that the increased production of ROS/RNS by plasma enhanced apoptosis-dependent gene expression at the transcriptional level. Our study further indicates that oxidative stress induced by non-thermal plasma exposure leads to activation of several enzymes of the antioxidative defense system[12] whose number differs in dependence on treatment and incubation time (data not shown).

IV. SUMMARY

Although the activation of specific signaling pathways remains to be elucidated, our data suggest that gene expression by plasma is dramatically affected and present new insights on plasma effects in keratinocytes. In summary, our results indicate both that plasma can stimulate cells without causing significant cytotoxic bystander effects after moderate treatment times and that plasma may serve as a promising biomedical tool for stimulation of skin cells in wound healing.

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