Blue light inhibits proliferation of melanoma cells

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ABSTRACT

Photobiomodulation with blue light is used for several treatment paradigms such as neonatal jaundice, psoriasis and back pain. However, little is known about possible side effects concerning melanoma cells in the skin.

The aim of this study was to assess the safety of blue LED irradiation with respect to proliferation of melanoma cells. For that purpose we used the human malignant melanoma cell line SK-MEL28.

Cell proliferation was decreased in blue light irradiated cells where the effect size depended on light irradiation dosage. Furthermore, with a repeated irradiation of the melanoma cells on two consecutive days the effect could be intensified.

Fluorescence-activated cell sorting with Annexin V and Propidium iodide labeling did not show a higher number of dead cells after blue light irradiation compared to non-irradiated cells.

Gene expression analysis revealed down-regulated genes in pathways connected to anti-inflammatory response, like B cell signaling and phagosome. Most prominent pathways with up-regulation of genes were cytochrome P450, steroid hormone biosynthesis. Furthermore, even though cells showed a decrease in proliferation, genes connected to the cell cycle were up-regulated after 24h. This result is concordant with XTT test 48h after irradiation, where irradiated cells showed the same proliferation as the no light negative control.

In summary, proliferation of melanoma cells can be decreased using blue light irradiation. Nevertheless, the gene expression analysis has to be further evaluated and more studies, such as in-vivo experiments, are warranted to further assess the safety of blue light treatment.

Keywords: SK-MEL28, melanoma cells, blue light, in vitro, cell proliferation, gene expression, photobiomodulation, low level light therapy, LLLT

1. INTRODUCTION

Statistics suggest that in the year 2014 76,000 people were affected by melanoma in the United States of America [1] and, according to the world health organization (WHO), the number of melanoma cases worldwide is increasing faster than any other type of cancer [2]. The current problem is that melanoma is very aggressive and does not respond to conventional treatments like immunotherapy, radiation or chemotherapy. Most patients relapse after chemotherapy, even though targeted molecular therapies have been designed [3]. New, safe therapeutic methods are needed to reduce the mortality of this type of skin cancer. Furthermore, it is important not to induce melanoma growth or even melanoma formation with any other therapeutic method.

Photobiomodulation is used for different medical treatments like neonatal jaundice [4], psoriasis [5] and back pain [6]). Likewise it is known to have anti-microbial [7], anti-inflammatory [8] and anti-proliferative effects [9, 10].

In a previous study the effect of blue light on the HaCaT cell line was tested with proliferation tests and gene expression analysis [11]. Cell growth was slowed down and 2192 genes were deregulated 24h after 30 minutes of blue light irradiation. Gene Set Enrichment Analysis using Kyoto Encyclopedia of Genes and Genomes (KEGG)-database and Gene Ontology (GOBP) presented pathways/biological functions containing deregulated genes due to blue light irradiation. Steroid hormone biosynthesis, metabolism of xenobiotics by cytochrome P450 and electron transport were the most prominent pathways containing up-regulated genes, whereas pathways containing down-regulated genes were mainly connected to inflammatory responses and diseases.
Interestingly also genes connected to the pathway associated with melanoma were down-regulated.

A previous study by Lewis and colleagues investigated the effect of blue light on oral tumor cells (OSC2) and found enhanced ROS production. Moreover, the succinate dehydrogenase activity was decreased and hence the respiratory chain was inhibited and the cells showed signs of apoptosis [12]. Results pointing in the same direction were obtained by Ohara and colleagues (2002), Oh and colleagues (2015), Sato and colleagues (2013) and Sparsa and colleagues (2010) [13-16]. The results of all these studies lead to the conclusion that blue light irradiation had anti-proliferative and/or apoptotic effects on the utilized cancer cells. Unfortunately, these research groups did not use human melanomas.

Therefore, the aim of this study was to assess the safety of blue LED irradiation with respect to proliferation and gene expression of the human melanoma cell line SK-MEL28.

2. METHODS

Experiments were performed as previously described in Becker et al. 2015 with some adjustments to the new cell line [11].

2.1 Culturing cells

SK-MEL28 cells (human melanoma cells) from Cell Line Service (CLS) GmbH (Heidelberg/Germany) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) high glucose containing 10% fetal bovine serum (FBS), 1mM sodium pyruvate and 100 U/mL penicillin/streptomycin (Gibco® by life technologies TM AG (Carlsbad/USA)). The cells were cultured under standard conditions at 37°C with 5% CO2. For detachment 0.25% Trypsin-EDTA (1x) phenol red from Gibco® was used 1:2.5 diluted with 10 minutes (min) incubation time. 1/10 to 2/10 of the cells were seeded into a new flask.

2.2 Light application

For light irradiation and following gene expression or proliferation tests 8500 (5000 for experiments >24h) SK-MEL28 cells/well were plated in black 96 well plates, with sterile clear flat bottom wells (Sigma Aldrich Co. LLC, St. Louis/USA), which are specifically designed for fluorescence application to reduce auto-fluorescence and background signal. After seeding, cells were incubated 24h at 37°C with 5% CO2. Medium was renewed and cells were illuminated with constant light for different exposure times. 30 min was finally selected as the optimal irradiation time for the following tests. The right half of the plate was taped with black foil for the no light negative control. After a defined time (1h, 3h, 6h, 12h, 24h or 48h) the cells of the upper part of the plate were harvested with TRIzol (Ambion® by life technologies TM AG (Carlsbad/USA)) and stored at -80°C for further use in RNA isolation and following gene expression analysis with microarrays. The lower part was used for a proliferation (XTT) test using the last row of the 96 well plate as a chemical blank.

As light source Lumileds LUXEON Rebel LXML-PR01-0275 was used (Koninklijke Philips N.V., Eindhoven/Netherlands). The intensity was 23mW/cm², with a beam divergence of ±15° and a peak wavelength at 453 nm (blue light).

2.3 Cell proliferation (XTT) test

Cell Proliferation Kit II from Roche Diagnostics GmbH (Mannheim/Germany) and the Colorimetric Cell Viability Kit III (XTT) from PromoKine (Heidelberg/Germany) were used. The assays are based on the formation of an orange formazan dye only by metabolic active and therefore viable cells. For quantification spectrophotometric absorption measurements were done with Infinite® 200 PRO microplate reader, (Tecan Group AG, Männedorf/Switzerland).

2.4 FACS

For marking of apoptotic cells with FITC labeled Annexin V (BioLegend, San Diego/USA) and Propidium iodide (PI) (InvitrogenTM by life technologies TM AG (Carlsbad/USA)) supernatant was harvested to collect possible apoptotic cells. After that, cells were washed with PBS, trypsinized and dissolved with the collected supernatant; 2x10⁶ cells were used. The cells were transferred to a 15ml Falcon tube and centrifuged 3min at RT and 2000×g. The supernatant was removed and the pellet was washed twice, first with PBS, secondly with Annexin-Binding Buffer (BioLegend(San Diego, USA)). Subsequently, the pellet was re-suspended in 100µl Annexin-Binding Buffer and cells were incubated with 5µl of Annexin V, and 2µl of PI 1mg/ml for 15min at RT in the dark. Finally, 100µl Annexin-Binding Buffer was added. For positive control Staurosporine (Sigma Aldrich Co. LLC, St. Louis/USA) was added to SK-MEL28 cells to...
induce apoptosis. The subsequent measurement was performed on a BD FACSCalibur (BD Biosciences, Heidelberg, Germany) while Flowing Software version 2.5.1 was used to perform a distribution analysis for statistical evaluation.

2.5 RNA-Isolation

The cell lysates where stored in TRIzol at -80° C. For RNA isolation 200µl of chloroform was added for 1ml TRIzol. They were incubated for 2-3min at RT. Then the mixture was vortexed and centrifuged at 12000xg for 15min at 4° C to separate the organic from the aqueous phase. The aqueous upper phase was transferred into a new tube and precipitated with 500µl isopropanol. After incubation for 10min at RT, the mixture was centrifuged at 12000xg for 10min at 4° C. The supernatant was discarded. The pellet was washed with 1ml of ethanol and centrifuged at 12000xg for 5min at 4° C. After discarding the supernatant, the pellet was dried. Subsequently, the pellet was re-suspended in 20µl RNase-free water.

2.6 Microarrays

RNA was isolated as described above with TRIzol followed by purification using the RNA Clean-Up and Concentration Micro Kit. cDNA synthesis was performed using the SuperScript Choice System according to the recommendations of the manufacturer. Using ENZO BioArray HighYield RNA Transcript Labeling Kit biotin-labeled cRNA was produced. Standard protocol from Affymetrix was used for the in vitro transcription (IVT). Quantification of cRNA was performed by spectrophotometric analysis with acceptable A260/A280 ratio of 1.9 to 2.1. Fragmentation of the cRNA was achieved using Affymetrix defined protocol. For gene expression profiling, labeled and fragmented cRNA was hybridized to Affymetrix Hugene-2_0st microarrays with an incubation time of 16h at 45° C. The Affymetrix fluidics station 450 was used to wash the microarrays, scanning was performed with Affymetrix Genechip scanner 3000.

2.7 Bioinformatic evaluation

For annotation a Custom CDF Version 18 with Entrez based gene definitions was used [17]. Applying quantile normalization, the raw fluorescence intensity values were normalized. Based on OneWay-ANOVA, differential gene expression was analyzed using a commercial software package: SAS JMP10 Genomics, version 7, from SAS. A false positive rate of a=0.05 with FDR correction was taken as the level of significance.

Gene Set Enrichment Analysis (GSEA) was used to determine whether defined lists (or sets) of genes exhibit a statistically significant bias in their distribution within a ranked gene list (see http://www.broadinstitute.org/gsea/ for details (Subramanian et al, 2005)). Pathways belonging to various cell functions, such as cell cycle or apoptosis were obtained from public external database (KEGG).

3. RESULTS

3.1 XTT test (proliferation test)

To test the influence of blue light irradiation on cell proliferation of the human melanoma cell line SK-MEL28 spectrophotometric measurements were performed determining absorption of a formazan dye, which is produced by metabolization of XTT reagent by viable cells. Therefore, higher absorption values are connected to an accelerated cell metabolism.

The XTT test was performed 24h after irradiation with different exposure times (0, 2.5, 5, 7.5, 15, 30, 45, 60, 90 and 120min) of blue light (Figure 1). To exclude a possible batch/plate effect, the absorption values of each plate were normalized to no light negative control.

After 15min cell proliferation was significantly decreased. The maximum effect was reached after 30min blue light irradiation and seemed to be constant after that; there was no further decrease in cell proliferation with irradiation times higher than 30min. To ensure a maximum effect of blue light irradiation and, in addition, to keep experiment time short, 30min was chosen in the subsequent experiments for testing blue light effect on cells.
Figure 1: XTT test results 24h after exposure times of 0, 2.5, 5, 7.5, 15, 30, 45, 60, 90 and 120min blue light irradiation. Decrease of cell proliferation was first seen with 15min whereas maximum effect was reached after 30min. Batch normalization was done with the no light negative control. The box-and-whisker plots represent the distribution of XTT data values. These are ranked into quartiles, which divide the data set into a box of four equal groups; the band inside the box reflects the median. The whiskers extend from the ends of the box to the outermost data point that falls within the distances calculated as follows: 3rd quartile+1.5*(interquartile range) and 1st quartile-1.5*(interquartile range).

Moreover, the different time points (=harvesting time) 1h, 3h, 6h, 12h and 24h after 30min of blue light irradiation were tested with XTT (Figure 2). For batch normalization the no light negative control of each plate was used. Cell proliferation started to decrease already after 3h and continuously progressed until 24h. As the biggest difference in proliferation was seen after 24h this time point was chosen for further experiments enclosing analysis of gene expression level.

Figure 2: XTT test results after 30min of blue light irradiation at different time points of cell harvesting: 1, 3, 6, 12 and 24h. Cell proliferation already decreased after 1h, the effect progressed continuously up to 24h. Batch normalization was done with no light negative control. (For legend see Figure 1).

Furthermore proliferation tests were extended to two days containing a consecutive irradiation of 30min of blue light, with 24h between the two irradiations and harvesting time 24h after the last irradiation (Figure 3), and one irradiation of 30min of blue light with harvesting time of 48h (Figure 4).
Two consecutive irradiations with 30min of blue light showed a higher decrease in cell proliferation compared to 24h after one-time irradiation.

![Figure 3: XTT test results of consecutive irradiation with 30min of blue light and harvesting time point of 24h after the last irradiation. With two consecutive irradiations the decrease in proliferation is more pronounced compared to a one-time irradiation (For legend see Figure 1).](image1)

However, 48h after 30min of blue light the irradiated melanoma cells showed about the same proliferation as the no light negative control cells.

![Figure 4: XTT test results with 30min of blue light irradiation and the different harvesting time points of 24h and 48h after irradiation. Proliferation of melanoma cells 48h after irradiation is comparable to proliferation of no light negative control cells (For legend see Figure 1).](image2)

3.2 FACS

FACS analysis with Annexin-V and Propidium iodide-stained cells was performed 24h after blue light irradiation of 30min. For a positive control cells were treated with Staurosporine. The percentage of the live and dead cell populations were reported in each graph with 50,000 total events analyzed for each of the three conditions (Figure 5). Results represent the mean of three independent experiments (Figure 6), with living cells counted in the lower left quadrant and dead cells in the three other quadrants. Therefore the analysis will not distinguish between early apoptosis, late apoptosis, or necrosis.
Cells irradiated with 30min of blue light do not show any difference in dead and live cell numbers compared to the no light negative control. Both, no light negative control and irradiated cells, show a significant difference with \( p<0.0001 \) to the Staurosporine treated positive control.

![FACS analysis 24h after 30min of blue light irradiation](image)

**Figure 5:** FACS analysis 24h after 30min of blue light irradiation. The four quadrants can be distinguished as follows: lower left quadrant=intact cells, lower right quadrant=early apoptosis, upper right quadrant=late apoptotic or secondary necrotic apoptotic cells and upper left quadrant=primary necrotic cells.

![FACS analysis 24h after 30min of blue light irradiation](image)

**Figure 6:** FACS analysis 24h after 30min of blue light irradiation. For comparison between live and dead cells the lower left quadrant was used for the numbers of intact cells and the other three quadrants were taken together to show the dead cells. In this graph there is no distinction between early or late apoptosis or necrosis. 30min of blue light did not induce apoptosis in SK-MEL28 melanoma cells.
3.3 Gene expression profiling

For measuring gene expression Affymetrix Hugene-2.0st microarrays were used, containing sequences for more than 26,000 genes or around 40,000 transcripts. Exposure time was 30min with the time point (=harvesting time) 24h after blue light irradiation. Gene expression was analyzed using JMP10 Genomics applying one-way analysis of variance (ANOVA) and a false discovery rate of \( \alpha = 0.5 \) (FDR correction).

3.3.1 24h after blue light irradiation

Cluster analysis grouped the set of microarrays 24h after blue light irradiation in such a way, that microarrays with irradiated samples (blue) were in the same group, whereas microarrays with no light negative control samples (control=red) were in a second group. Thus clustering revealed a difference between blue light irradiated samples and no light negative control (Figure 7).

![Figure 7: Clustering of microarrays with samples 24h after blue light irradiation demonstrated a grouping of blue light irradiated (constant=blue) and no light negative control (ctrl=red) samples.](http://proceedings.spiedigitallibrary.org/)

1486 genes were differentially expressed 24h after blue light irradiation. 777 genes were significantly up-regulated and 709 genes significantly down-regulated. In Figure 8 the volcano plot 24h after 30min blue light irradiation is shown.

![Figure 8: Volcano plot of gene expression 24h after blue light irradiation.](http://proceedings.spiedigitallibrary.org/)

Pathway analysis was performed using KEGG pathways (download version) revealing 258 deregulated pathways with 177 pathways containing up regulated genes and 81 pathways comprising down regulated genes. In Table 1 selected pathways containing deregulated genes in the irradiated group 24h after blue light irradiation are presented.
Table 1: Selected deregulated pathways from gene expression analysis 24h after 30min blue light irradiation. The last four columns present the numbers of deregulated genes, normalized enrichment score (NES), probability values without adjustment (NOM p-val) and false discovery rate with adjustment of multiple testing (FDR q-val).

<table>
<thead>
<tr>
<th>Name</th>
<th>No. of genes</th>
<th>NES</th>
<th>NOM p-val</th>
<th>FDR q-val</th>
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<tr>
<td>Chemical carcinogenesis</td>
<td>69</td>
<td>1.97</td>
<td>&lt;.001</td>
<td>0.033</td>
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<tr>
<td>Metabolism of xenobiotics by cytochrome P450</td>
<td>65</td>
<td>1.94</td>
<td>&lt;.001</td>
<td>0.020</td>
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<td>Steroid hormone biosynthesis</td>
<td>51</td>
<td>1.91</td>
<td>&lt;.001</td>
<td>0.019</td>
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<tr>
<td>Pentose and glucuronate interconversions</td>
<td>31</td>
<td>1.90</td>
<td>&lt;.001</td>
<td>0.018</td>
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<tr>
<td>Fanconi anemia pathway</td>
<td>50</td>
<td>1.84</td>
<td>0.002</td>
<td>0.030</td>
</tr>
<tr>
<td>Dilated cardiomyopathy (DCM)</td>
<td>90</td>
<td>1.77</td>
<td>&lt;.001</td>
<td>0.053</td>
</tr>
<tr>
<td>Drug metabolism - cytochrome P450</td>
<td>60</td>
<td>1.76</td>
<td>0.002</td>
<td>0.048</td>
</tr>
<tr>
<td>Glutathione metabolism</td>
<td>46</td>
<td>1.66</td>
<td>0.008</td>
<td>0.113</td>
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<tr>
<td>Ovarian steroidogenesis</td>
<td>47</td>
<td>1.65</td>
<td>0.006</td>
<td>0.113</td>
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<tr>
<td>Cell cycle</td>
<td>124</td>
<td>1.44</td>
<td>&lt;.001</td>
<td>0.209</td>
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<tr>
<td>Glycosaminoglycan degradation</td>
<td>19</td>
<td>-1.40</td>
<td>0.083</td>
<td>1.000</td>
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<tr>
<td>Taste transduction</td>
<td>51</td>
<td>-1.38</td>
<td>0.060</td>
<td>1.000</td>
</tr>
<tr>
<td>Collecting duct acid secretion</td>
<td>27</td>
<td>-1.31</td>
<td>0.125</td>
<td>1.000</td>
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<tr>
<td>Glycosaminoglycan biosynthesis - KS</td>
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<tr>
<td>Phagosome</td>
<td>146</td>
<td>-1.29</td>
<td>0.051</td>
<td>1.000</td>
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<tr>
<td>Renin-angiotensin system</td>
<td>17</td>
<td>-1.29</td>
<td>0.147</td>
<td>1.000</td>
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<tr>
<td>Non-small cell lung cancer</td>
<td>55</td>
<td>-1.28</td>
<td>0.118</td>
<td>1.000</td>
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<tr>
<td>Adipocytokine signaling pathway</td>
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<td>1.000</td>
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<td>Melanoma pathway</td>
<td>70</td>
<td>-1.08</td>
<td>0.324</td>
<td>0.905</td>
</tr>
</tbody>
</table>

4. DISCUSSION

Studies have revealed that blue light irradiation can have an anti-proliferative effect on mouse melanoma cells [13-16]. In line with these findings the present proliferation tests on human melanoma cells revealed a decrease in cell proliferation up to 24h after irradiation. Nevertheless, FACS analysis did not result in an apoptotic effect of blue light with 30min irradiation. When the cells were irradiated twice, 24h and 48h after seeding, the anti-proliferative effect was even stronger. Moreover, it was found that the effect was dependent on the irradiation time with a maximum effect reached with 30min of blue light irradiation. The proliferation measured 48h after irradiation was equal to the no light control. The subsequently performed gene expression profiling revealed that 1486 genes were deregulated. To set this high number of genes into context with the pathways that are active, gene set enrichment analysis was performed. An up-regulation of genes was found in pathways connected to cytochrome P450, steroid hormone biosynthesis and cell cycle.
Many down-regulated genes were found in pathways connected to anti-inflammatory response, like B cell signaling and phagosome and the melanoma pathway.

These results are in many aspects comparable to the blue light effects seen in the human skin cell line HaCaT [11] and leads to the hypothesis that photoactive chromophores like flavins and cytochromes [9, 18], which are inter alia located in the mitochondrial respiratory chain [19, 20], are activated with the blue light. Also cytochrome c oxidase, which is thought to be the main chromophore for red light [21, 22], has an absorption peak in the blue [18, 21]; a fact that is often ignored for blue light therapy. This can lead to ROS production and activation of downstream processes [9, 18]. ROS production can result in a decreased cell proliferation [19] whereas the anti-inflammatory response can be induced by steroid hormone biosynthesis [23-25], which is a downstream process of cytochrome P450 [19, 25, 26].

On the other hand blue light irradiation led to an up-regulation of genes in the cell cycle for melanoma cells, this was not concordant with HaCaT results where genes connected to cell cycle were not significantly regulated, but showed a tendency to down-regulation. This leads to the conclusion that, after the ROS-induced anti-proliferative effect, cell metabolism of irradiated melanoma cells might increase again and that the cells have been able to either balance out the decrease in metabolism or even grow at a faster rate than the non-irradiated cells of the negative control. This is consistent with XTT results with harvesting time 48h after irradiation.

These effects are comparable with the results of Oh et al. in 2015 [14], who also saw an anti-proliferative effect in melanoma cells and an additional induction of apoptosis for higher power densities. In vivo the tumor growth rate was first decreased with blue light but similar in the control and blue LED groups after 9 days of blue LED irradiation.

5. FUTURE OUTLOOK

Among other effects, cell proliferation was reduced in human melanoma cells after blue light treatment up to at least 24h after irradiation. This might be due to ROS production, which occurs in such a manner that the cells do not have a higher apoptosis rate, but a slower growth rate. In further experiments ROS should be measured.

48h after irradiation the cells had the same metabolic rate measured by XTT as the no light negative control. This might be due to an activation of the cell cycle and as a consequence may result in a higher cell growth. However, these results have to be verified and the time course of cell proliferation and gene expression has to be tested in more detail. Possibly the metabolism of the cells is slowed down in the first hours after irradiation and the cells regain their fast metabolism rate after a while. This would lead to a treatment paradigm with consecutive irradiations at least every 24h.

Moreover, steroid hormone biosynthesis was activated, which creates an anti-inflammatory effect. Inflammation pathways like B cell signaling and phagosome show a non-significant down-regulation as well. The strength of the anti-inflammatory response could be tested by measuring the concentration of steroid hormones. In animal studies, the activity and number of B cells and phagosomes could be tested as well.

Furthermore the gene expression results will be analyzed more thoroughly with bioinformatical methods. The high number of deregulated genes may reveal further information about the complex processes induced by blue light irradiation. After the identification of individual important genes, they should be validated by real time PCR.

Finally, as melanoma cells in culture do not necessarily behave in the same way as melanoma cells in skin, the results cannot be transferred directly to the behavior of melanoma cells in human skin. It might therefore be necessary to analyze co-culture systems or animals with blue light and to test different markers for cell proliferation and inflammation in vivo.

REFERENCES


