# DISRUPTIONS IN THE HOMEOSTASIS OF NORMAL SKIN FIBROBLASTS AND MELANOCYTES INDUCED BY DOXYCYCLINE PHOTOTOXICITY – MODEL STUDIES USING A SUNLIGHT SIMULATOR

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## INTRODUCTION

Drug-induced phototoxicity constitutes a significant medical issue, taking into account epidemiological aspects, therapy safety, and the consequences of skin cell damage. Typically, the initial risk assessment for the occurrence of phototoxic reactions is carried out using the NRU test and mouse fibroblasts 3T3.

## AIM OF THE STUDY

The aim of this study was to assess changes in the homeostasis of human skin fibroblasts (HDF) and melanocytes with light (HEMn-LP) and dark (HEMn-DP) pigmentation caused by phototoxic action. Doxycycline (DOX) was selected as a model drug due to exhibiting phototoxic potential and forming complexes with melanin biopolymers.

## METHODS

### CELL CULTURE AND TREATMENT

*In vitro* studies were performed on human dermal fibroblasts (HDF) obtained from Sigma-Aldrich and human epidermal melanocytes (HEMn-LP and HEMn-DP) which were purchased from Cascade Biologics. HDF cells were cultured in Fibroblasts Growth Medium. Melanocytes were cultured in the growth medium M-254 supplemented with HMGS-2 and antibiotics.

The cells were preincubated in the appropriate growth medium at 5% $CO_2$ humidity and 37°C. Subsequently, one of the following procedures was applied:

**Model 1:** the medium was replaced by DOX solutions in PBS with calcium and magnesium (PBS++) and incubated for 1h. Then the cells were irradiated with the sunlight simulator SXL-3000V4 (UVA dose: 5 J/cm$^2$). Then, the cells were incubated for 24h in the appropriate medium until analysis.

**Model 2:** the medium was replaced by DOX solutions in medium and the cells were incubated for 23h. Subsequently, DOX solutions in PBS++ was added 1h before the irradiation with the sunlight simulator SXL-3000V4 (UVA dose: 5 J/cm$^2$). Then, the cells were incubated for 24 h in the appropriate medium prior the analysis.

Sunlight simulator SXL-3000V4.

### NRU ASSAY

The general assessment of cytotoxic and phototoxic potential were made using neutral red based In Vitro Toxicology Assay Kit (TOX4). Tested cells were seeded in 96-well plates and cultured for 48 hours. Then, they were treated according to experimental model 1 and 2. After that, 10 µL of 0.33% neutral red solution was added to each well. After 3-h incubation, the medium was removed and cells were rinsed with Neutral Red Assay Fixative. In the next step 100 µL of Neutral Red Assay Solubilization Solution was added to each well and the plates were stirred for 10 min at RT. The absorbance of the samples was measured at 540 nm with a reference wavelength of 690 nm using the microplate reader Infinite 200 PRO controlled by the Magellan software.

### INTRACELLULAR LEVEL OF REDUCED THIOLS

The measurement of the intracellular level of thiols was made using a fluorescence imaging cytometer NucleoCounter® NC-3000. The assay is based on VitaBright-48 - a highly specific dye staining cells with a high level of reduced thiols, e.g. GSH. Cell suspension was stained with Solution 5 (containing VitaBright-48, propidium iodide, and acridine orange) and analyzed with the image cytometer. The obtained histograms are used to differentiate the subpopulation of cells with high and low levels of reduced thiols.

### CELL CYCLE AND DNA FRAGMENTATION ASSAY

Cell cycle phase distribution and DNA fragmentation was assessed using the image cytometer. The analysis is based on differences in DNA content between the pre-replicative phase (G1 phase) cells, the cells that actually replicate DNA (S phase), the post-replicative plus mitotic (G2-M phase) cells and the late apoptotic cells. Following treatment, cells were counted, and fixed with ice-cold 70% ethanol. After washing, cell pellets were stained with solution containing DAPI and Triton X-100 and analyzed using the NC-3000 system.

### STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism 7. Data are presented as mean values ± SD of three independent experiments in at least three repetitions The results were analyzed statistically using one-way ANOVA or two-way ANOVA and Dunnett's or Tukey's test. $p < 0.05$ was considered to indicate a statistically significant difference.

## RESULTS AND CONLUSIONS

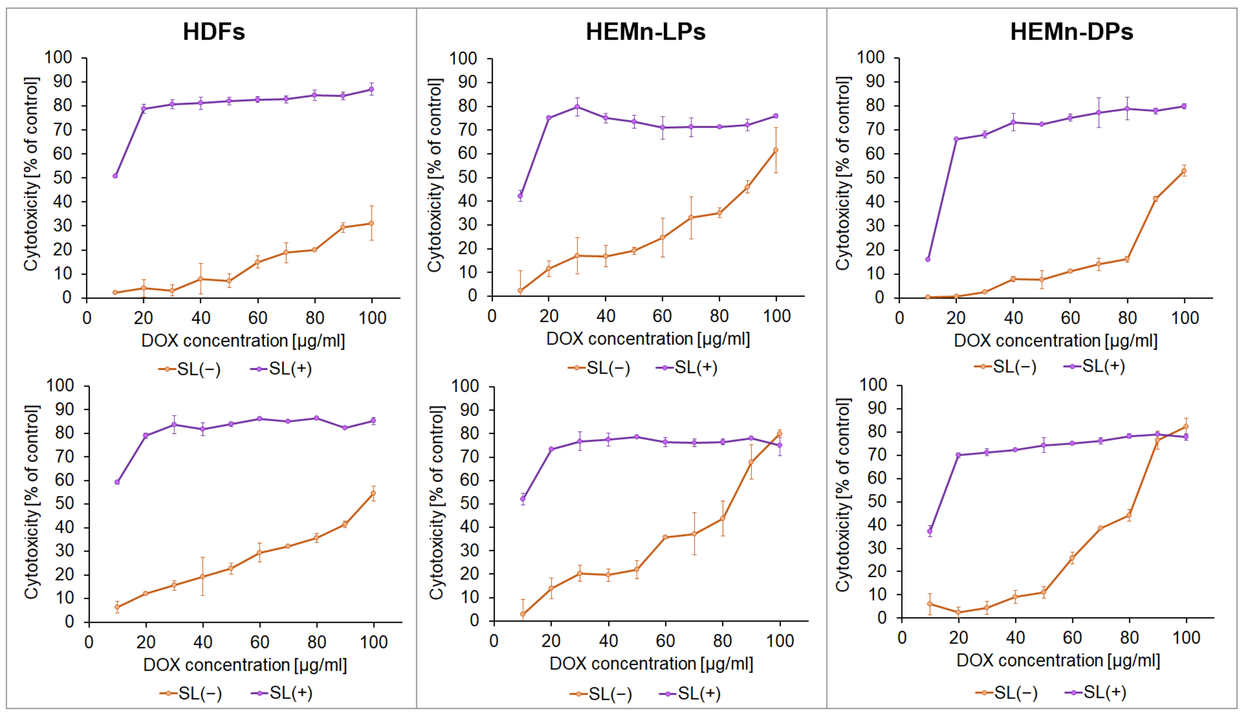


**Fig.1.** Effect of doxycycline (DOX) on the metabolical activity of human skin cells non-irradiated or exposed to sunlight (SL). Human dermal fibroblasts (HDFs), human epidermal melanocytes lightly pigmented (HEMn-LPs) and human epidermal melanocytes darkly pigmented (HEMn-DPs) were treated as described in Materials and Methods and tested using the WST-1 assay. The results are demonstrated as the percentage of control.

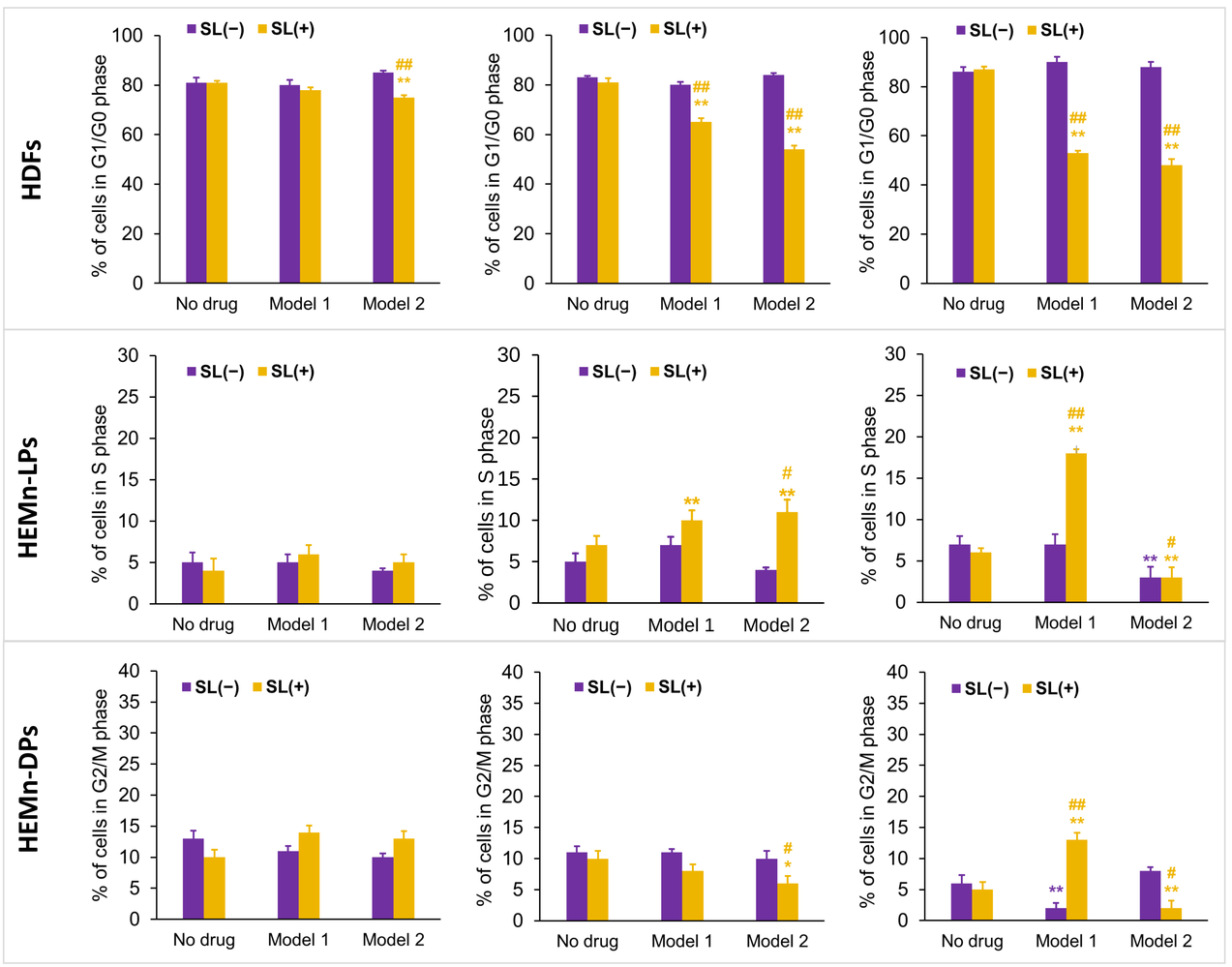


**Fig.2.** Effect of doxycycline on cell cycle of non-irradiated or exposed to sunlight (SL) human skin cells. Human dermal fibroblasts (HDFs), human epidermal melanocytes lightly pigmented (HEMn-LPs) and human epidermal melanocytes darkly pigmented (HEMn-DPs) were treated as described in Materials and Methods. Cell cycle was analyzed using the image cytometry. (**A**) The results expressed as mean values ± SD (*p<0.05, **$p$<0.01 *vs* untreated control, # $p < 0.05$, ## $p < 0.01$ *vs* cells exposed only to sunlight).

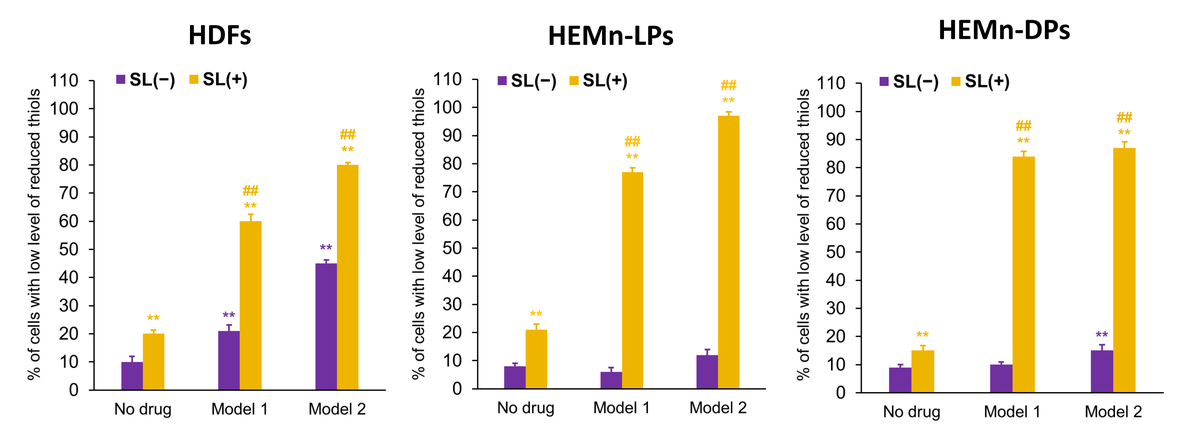


**Fig.3.** Effect of doxycycline on intracellular thiols status of non-irradiated or exposed to sunlight (SL) human skin cells. Human dermal fibroblasts (HDFs), human epidermal melanocytes lightly pigmented (HEMn-LPs) and human epidermal melanocytes darkly pigmented (HEMn-DPs) were treated as described in Materials and Methods. The status of cellular thiols was analyzed using the image cytometry. The results expressed as mean values ± SD (** $p < 0.01$ *vs* untreated control, ## $p < 0.01$ *vs* cells exposed only to sunlight).

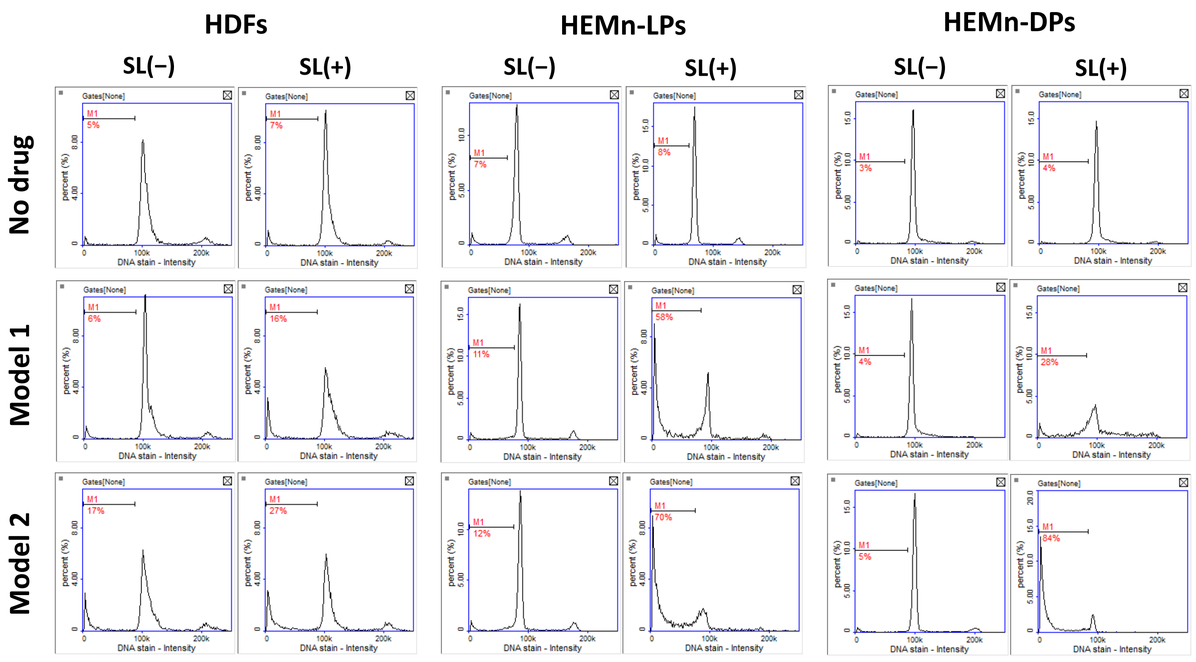


**Fig.4.** Effect of doxycycline on DNA fragmentation of non-irradiated or exposed to sunlight (SL) human skin cells. Human dermal fibroblasts (HDFs), human epidermal melanocytes lightly pigmented (HEMn-LPs) and human epidermal melanocytes darkly pigmented (HEMn-DPs) were treated as described in Materials and Methods. The status of cellular thiols was analyzed using the image cytometry. Representative histograms were shown (M1 – percent of cells with fragmented DNA).

## CONCLUSIONS

- **It was demonstrated that doxycycline had cyto- and phototoxic potential towards all examined cells, proportionally to the drug concentration. As a result, intracellular reduced glutathione levels decreased, and cell cycle disturbances occurred. The observed disturbances were more pronounced in the case of longer cell incubation with the drug.**
- **Analysis of DNA fragmentation showed greater damage to genetic material in melanocytes compared to fibroblasts. The noted effect may be associated with the formation of drug-melanin complexes and the accumulation of doxycycline in pigmented cells.**
- **The conducted studies indicate the need to assess the phototoxicity of drugs using cells with varying degrees of pigmentation.**

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