THE DISTURBANCES OF REDOX HOMEOSTASIS IN NORMAL SKIN CELLS TRIGGERED BY THE PHOTOTOXIC ACTION OF MELOXICAM - IN VITRO STUDY ON MELANOCYTES AND FIBROBLASTS

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BACKGROUND

Adverse drug reactions (ADRs) are among the top 10 causes of diseases and death, increasing the frequency of hospitalizations and generating additional costs for the health care system. Cutaneous side effects, including phototoxicity, constitute the third most common ADR.

Meloxicam (MLX) belongs to the non-steroidal anti-inflammatory drug group, is a selective COX2 inhibitor widely used in the pharmacotherapy of musculoskeletal pain, mainly osteoarthritis and rheumatoid arthritis. The simultaneous skin exposition to the MLX and UVA irradiation (UVAR) result in the phototoxic reaction occurrence that contributes to disturbances of redox homeostasis.

AIM OF THE STUDY

The main aim of this study was to assess the content of reactive oxygen species (ROS) in melanocytes and fibroblasts exposed to MLX and UVAR and to determine changes in the activity and expression of antioxidant enzymes - superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in normal skin cells.

METHODS

Cell Culture and the Treatment

The studies were performed on human epidermal melanocytes, lightly pigmented (HEMn-LP), and human dermal fibroblasts (HDF). The cells used in the studies were from passages 5 to 10. Fibroblasts were maintained in ready-to-use Fibroblasts Growth Medium. Melanocytes were cultured in the M-254 growth medium, with the addition of HMGS-2 supplement, and antibiotics: penicillin (100 µg/mL), amphotericin B (0.25 µg/mL), and neomycin sulfate (10 µg/mL). The cells were incubated in the incubator CB 160 in humidified 5% CO_2 atmosphere at 37◦C. A filtered lamp BVL-8.LM was the source of UVA irradiation (λmax = 365 nm). The analyzed skin cells were irradiated at 5 J/cm^2 of UVAR at the intensity of 720 µW/cm^2. The irradiation was carried out after 24 h treatment with meloxicam. Simultaneously, the non-irradiated cell cultures were kept in dark conditions at 5% CO_2 and 37◦C. Before the UVA exposition, the medium was replaced by PBS. After irradiation the PBS was removed and skin cells were incubated in an appropriate growth medium for another 24 h.

Confocal microscopy imaging

The laser confocal microscope Nikon Eclipse Ti-E A1R-Si controlled by Nikon NIS-Elements AR software was used to perform oxidative stress analysis. Both fibroblasts and melanocytes were seeded in 4-well chamber slides. Cells after exposure to MLX and UVAR procedurę were incubated with CellROX® Green Reagent as well as Hoechst 33342 for 30 min, subsequently washer in PBS three times, and then imaged. CellROX® dye allowed to detect oxidative stress occurrence by demonstrating a bright green fluorescence in the oxidized state, and weak fluorescence in the reduced form. To estimate cells morphology transmitted light confocal imaging was applied.

The analysis of antioxidant enzymes activity

The assessment of antioxidant enzymes activity - catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx), was made with assay kits (from Cayman Chemical) following the manufacturer instructions. In the first stage, both melanocytes and fibroblasts after the treatment and UVA exposure were suspended in the lysis buffer which contains a protease inhibitor (1.4 mg/mL), and a phosphatase inhibitor (10 µL/mL).

Western blotting analysis

Melanocytes HEMn-LP and fibroblasts HDF after 24 h incubation with meloxicam in concentrations of 0.5 mM and 1.0 mM and UVA exposure were lysed with RIPA buffer that includes phosphatase and protease inhibitors and incubated for 30 min on ice. Subsequently, the protein concentration in obtained lysates was quantitated using a microvolume spectrophotometer DS-11 and Pierce™ BCA Protein Assay Kit. Protein extracts were fractionated on 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. After 1 h of incubation with a blocking buffer containing 5% dry milk in TBS supplemented with Tween 20, the assayed membranes were washed and incubated overnight with the primary antibodies: rabbit anti-CAT (1:500), rabbit anti-SOD1 (1:1000), rabbit anti-SOD2 (1:1000), rabbit anti-GPx (1:1000) and rabbit anti-GAPDH (1:1000). Subsequently the membranes were carefully rinsed and incubated with horseradish peroxidase conjugated secondary antibody at room temperature for 1.5 h. The protein signals were detected with the ECL chemiluminescence reagent. Immunoreactive proteins were visualized via Syngene G:Box Chemi-XT4 Imaging System. Densitometry measurements were made using GeneTools Software. GAPDH was used to normalize for loading variations.

Statistical analysis

The obtained data were analyzed with GraphPad Prism 8. The mean values and standard deviations (SD) of three separate experiments were calculated. Statistical differences have been evaluated with a two-way ANOVA and Tukey's test. The correlation between the level and activity of the tested enzymes was calculated using the Pearson's correlation test (r). Statistical significance was found with a $p < 0.05$.

RESULTS

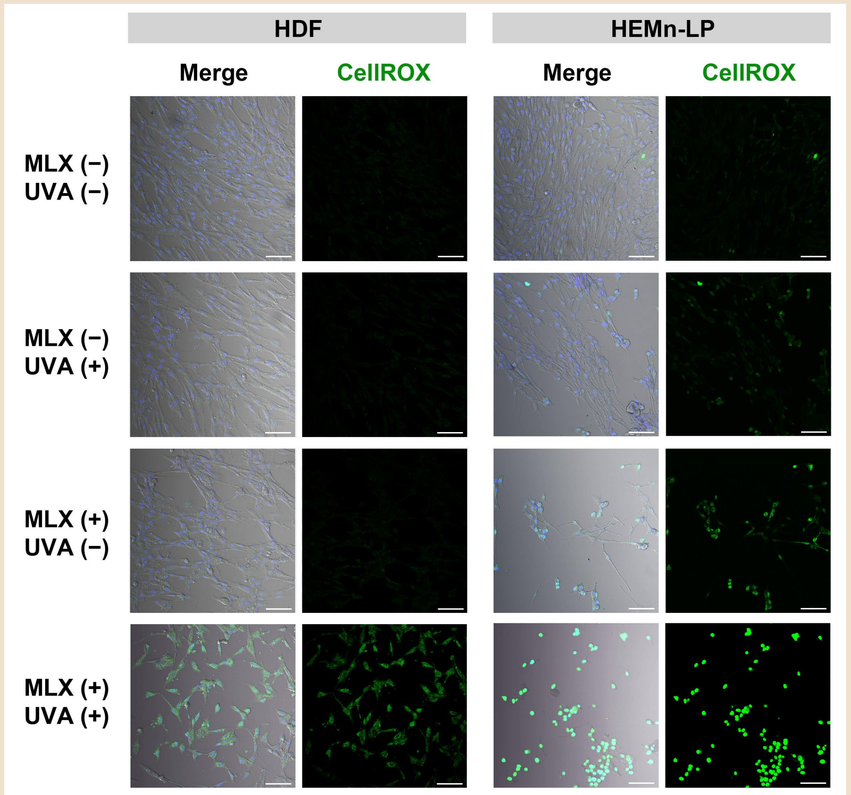

Fig. 1. UVAR enhances meloxicam-induced oxidative stress in melanocytes and fibroblasts. Confocal imaging of melanocytes and fibroblasts intracellular oxidative stress induced by treatment with 1.0 mM of MLX and/or UVAR stained with CellROX® Green Reagent and Hoechst 33342 that allow for nuclei visualization. To assess morphological changes in fibroblasts and melanocytes the transmitted light detector has been used. Scale bar = 100 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

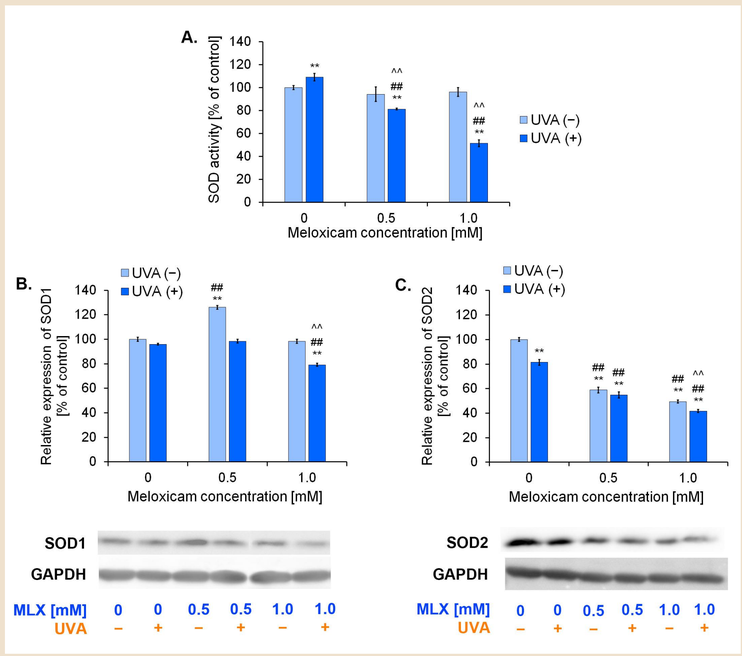

Fig. 2. Meloxicam (MLX) decreased superoxide dismutase (SOD) activity in fibroblasts through down-regulation of SOD2 expression. UVA irradiation (5 J/cm2) enhanced the suppressive effect on MLX-induced SOD expression and activity. The activity of SOD and expression of SOD1 and SOD2 isoenzyme was assessed by the usage of meloxicam in concentrations of 0.5 mM and 1.0 mM and/or UVAR on fibroblasts. ** $p < 0.01$ vs. untreated cells (control; -MLX; -UVAR), ## $p < 0.01$ vs. irradiated cells (-MLX; +UVAR), ^^ $p < 0.01$ vs. corresponding sample not treated with UVAR (+MLX; -UVAR).

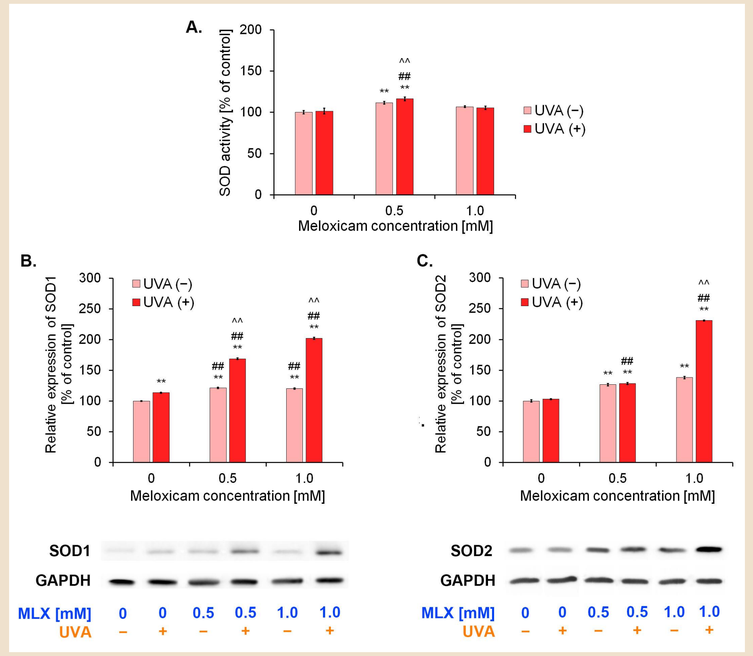

Fig. 3. Meloxicam (MLX) in concentrations of 0.5 mM and 1.0 mM increased superoxide dismutase (SOD) isoenzyme SOD1 and SOD2 expression in melanocytes. UVA irradiation (5 J/cm2) contributes to an additional increase in meloxicam-induced SOD1 and SOD2 expression. The activity of the SOD enzyme in melanocytes is only slightly affected by MLX and/or MLX and UVAR. ** $p < 0.01$ vs. untreated cells (control; -MLX; -UVAR), ## $p < 0.01$ vs. irradiated cells (-MLX; +UVAR), ^^ $p < 0.01$ vs. corresponding sample not treated with UVAR (+MLX; -UVAR).

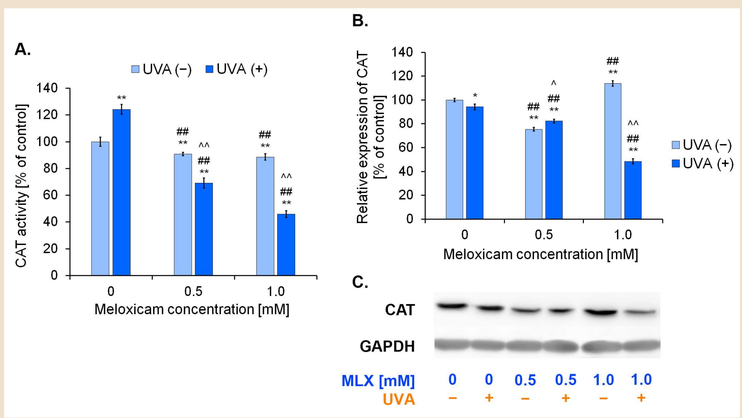

Fig. 4. The effect of meloxicam (MLX) and/or UVA radiation on catalase expression and activity. UVA radiation increase the CAT activity with a simultaneous decrease in its expression in fibroblasts. MLX reduces both catalase activity and expression, and the observed effect is further enhanced by UVA irradiation. * $p < 0.05$ vs. untreated cells (control; -MLX; -UVAR), ** $p < 0.01$ vs. untreated cells (control; -MLX; -UVAR), ## $p < 0.01$ vs. irradiated cells (-MLX; +UVAR), ^ $p < 0.05$ vs. corresponding sample not treated with UVAR (+MLX; -UVAR), ^^ $p < 0.01$ vs. corresponding sample not treated with UVAR (+MLX; -UVAR).

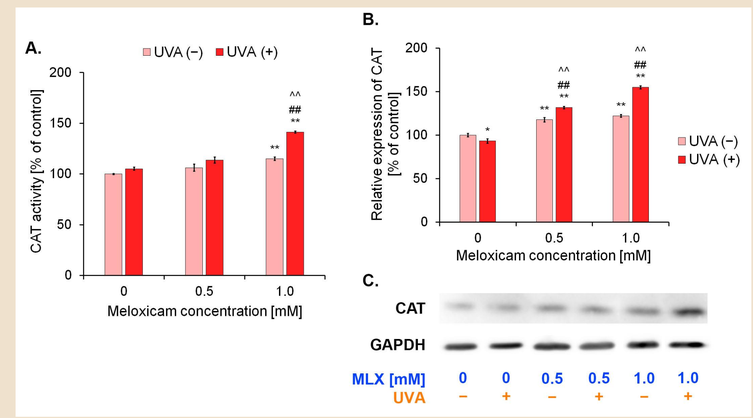

Fig. 5. Catalase activity and expression were increased in melanocytes by meloxicam (MLX) and/or UVAR treatment. The drug itself caused an increase in the activity and expression of the enzyme, and UVA radiation contributed to the intensification of the obtained effect. * $p < 0.05$ vs. untreated cells (control; -MLX; -UVAR), ** $p < 0.01$ vs. untreated cells (control; -MLX; -UVAR), ## $p < 0.01$ vs. irradiated cells (-MLX; +UVAR), ^^ $p < 0.01$ vs. corresponding sample not treated with UVAR (+MLX; -UVAR).

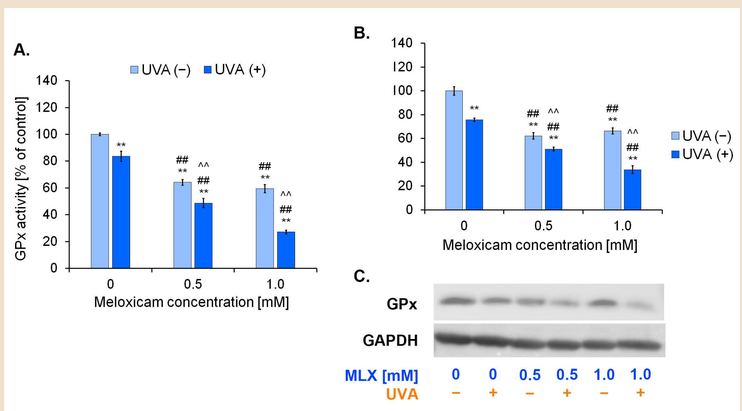

Fig. 6. Meloxicam (MLX) in concentrations of 0.05 mM and 1.0 mM decreased the expression and activity of glutathione peroxidase in fibroblasts. UVAR (5 J/cm2) caused a decrease in the expression and activity of GPx, and the simultaneous use of MLX and UVAR on fibroblasts deepens the obtained effect. ** $p < 0.01$ vs. untreated cells (control; -MLX; -UVAR), ## $p < 0.01$ vs. irradiated cells (-MLX; +UVAR), ^^ $p < 0.01$ vs. corresponding sample not treated with UVAR (+MLX; -UVAR).

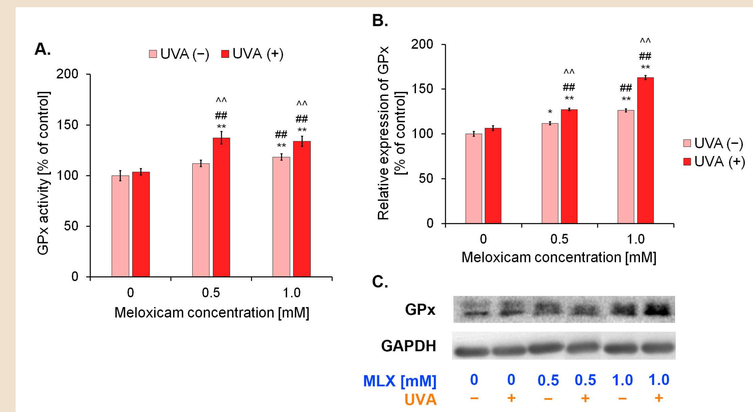

Fig. 7. The evaluation of changes in the activity and expression of glutathione peroxidase in melanocytes treated with meloxicam (MLX) and/or UVAR. MLX at a concentration of 1.0 mM causes an increase in enzyme activity and expression. UVA radiation does not affect cells but enhances the effect obtained with MLX. * $p < 0.05$ vs. untreated cells (control; -MLX; -UVAR), ** $p < 0.01$ vs. untreated cells (control; -MLX; -UVAR), ## $p < 0.01$ vs. irradiated cells (-MLX; +UVAR), ^^ $p < 0.01$ vs. corresponding sample not treated with UVAR (+MLX; -UVAR).

CONCLUSIONS:

- ✓ **The obtained results indicated that UVAR enhances meloxicam-induced oxidative stress in melanocytes and fibroblasts.**
- ✓ **It was found that MLX differently affects the expression and activity of SOD, CAT and GPx depending on the analyzed cell line.**
- ✓ **In the case of fibroblasts, a decrease in the expression and activity of SOD, CAT and GPx was observed. Conversely, an increase in the activity and expression of antioxidant enzymes was found in melanocytes.**
- ✓ **Due to the fact that MLX has the ability to bind to melanin biopolymers, creating its long-therm reservoir, the concentration of free drug in melanocytes is lower than in fibroblasts.**
- ✓ **The demonstrated differences indicate that melanin may have protective properties against the phototoxic effects of MLX.**

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