

The effect of micro-sized titanium dioxide on WM-266-4 metastatic melanoma cell line

Tanja Prunk Zdravković^{1,2}, Bogdan Zdravković³, Mojca Lunder⁴, Polonca Feršković^{5*}

¹Dermatovenerology Department, Celje General and Teaching Hospital, Celje, Slovenia, ²Institute of Anatomy, Histology and Embryology, Faculty of Medicine, University of Maribor, Maribor, Slovenia, ³Department of Anesthesiology, Intensive Care and Pain Management, University Medical Centre Maribor, Maribor, Slovenia, ⁴Department for Pharmaceutical Biology, Faculty of Pharmacy, University of Ljubljana, Ljubljana, Slovenia, ⁵Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia

ABSTRACT

Titanium dioxide (TiO₂) is widely used as an inorganic UV-filter in cosmetic products; however, it has been classified as possibly carcinogenic to humans. While numerous studies demonstrated cytotoxic and genotoxic effects of nano-sized TiO₂ in different cell lines, including human skin cells, studies investigating the effects of micro-TiO₂ on human keratinocytes and melanocytes, in healthy and cancer cells, are scarce. Adenosine triphosphate (ATP) binding cassette subfamily B member 5 (ABCB5) is a plasma membrane protein known for its role in the tumorigenicity, progression, and recurrence of melanoma. Here, we investigated the effect of micro-TiO₂ (average particle size ≤5 μm) on the metabolic activity, cytotoxicity and ABCB5 mRNA expression in metastatic melanoma cells. Metastatic melanoma cell line WM-266-4 was treated with different concentrations of micro-TiO₂ for different incubation times to obtain dose- and time-dependent responses. Untreated WM-266-4 cells, cultured under the same conditions, were used as control. The cell metabolic activity was determined by MTT assay. Cytotoxicity of micro-TiO₂ was analyzed by lactate dehydrogenase (LDH) cytotoxicity assay. The ABCB5 mRNA expression in melanoma cells was analyzed using quantitative reverse transcription polymerase chain reaction (RT-qPCR). After 120 hours of exposure to micro-TiO₂ the metabolic activity of melanoma cells decreased, especially at the two highest micro-TiO₂ concentrations. Comparably, the cytotoxicity of micro-TiO₂ on melanoma cells increased after 48 and 120 hours of exposure, in a time-dependent manner. The ABCB5 mRNA expression in micro-TiO₂-treated melanoma cells also decreased significantly after 24 and 48 hours, in a time-dependent manner. Overall, our results suggest inhibitory effects of micro-TiO₂ on the metabolic activity and ABCB5 mRNA expression in metastatic melanoma cells, indicating its potential use as an anticancer agent.

KEY WORDS: Titanium dioxide; UV filter; ABCB5 protein; melanoma

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INTRODUCTION

The use of topical sunscreens and other personal care products containing UV filters has been increasing, due to an increase in public awareness of the harmful effects of solar ultraviolet (UV) radiation [1-4]. Broad spectrum protection from UVA and UVB rays and regular application of sunscreens in sufficient amounts (e.g., 2 mg/cm² of skin surface) have proven to be useful in the prevention of actinic keratosis (AK), squamous cell carcinomas (SCCs) and skin ageing; however, a significant benefit of regular sunscreen use in the primary prevention of basal cell carcinoma (BCC) and melanoma has not yet been demonstrated [5,6]. The active ingredients (UV filters) used in sunscreens have different absorption spectra and

mechanism of action, and can be classified as organic (chemical) and inorganic (physical) filters. Inorganic sunscreens such as those based on zinc oxide (ZnO) and titanium dioxide (TiO₂) cover a wider spectral range compared to most of the organic sunscreens; however the cosmetic acceptability of inorganic UV filters is still inferior as they produce white coloration when applied to the skin [7]. To overcome the undesired effect (i.e. a white film on the skin) of the opaque sunscreen products, micro-sized inorganic UV filters have been increasingly replaced by the nano-sized filters [8] which, due to the very small size of particles, are transparent and thus provide improved aesthetic outcome [9]. Conventionally, particles that are less than 100 nm in size are classified as nanoparticles and those larger as microparticles [10]. Nanotechnology is considered to be the next logical step in science, nevertheless, the toxicological and environmental impact of nanoparticles is still the subject of considerable debate [11]. Thus, it is questionable whether the cosmetic acceptability of nano-sized UV

*Corresponding author: Polonca Feršković, Faculty of Medicine, University of Ljubljana, Vrazov trg 2, 1000 Ljubljana, Slovenia. Phone: +386 (0) 543 77 00; Fax: +386 (0) 543 7701. E-mail: polonca.ferk@guest.arnes.si

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filters can be justified without positive and improved effects on human health.

One of the most widely used physical UVA and UVB filters is TiO₂, which has three crystal structures, i.e., anatase, rutile and brookite. Its ability to block the UV radiation through scattering, reflecting and/or absorbing makes it a very effective active ingredient in sunscreen cosmetics, where it is used in concentrations up to 25% [12,13]. However, the International Agency for Research of Cancer has classified TiO₂ as possibly carcinogenic to humans (Group 2B carcinogen) [14]. Furthermore, numerous *in vitro* studies showed that TiO₂ nanoparticles (nano-TiO₂) are able to induce cytotoxicity, reactive oxygen species (ROS), and genotoxicity in different cell lines [15]. For micro-sized TiO₂ (micro-TiO₂), an *in vivo* study showed that it could induce DNA damage and micronuclei in bone marrow cells, increase the mitotic index in forestomach and colon epithelia and the frequency of spermatids with two and more nuclei, in mice [16]. Another, *in vitro*, study showed that micro-TiO₂ was able to induce ROS formation and single-strand DNA breaks in human Caco-2 cells [17]. Nevertheless, more data is needed to make a final conclusion on the effect of micro-TiO₂ on human health.

A study investigating the effect of micronized TiO₂ (nanoparticles) on the barrier function of the human skin (epidermis) transplanted to immunodeficient mice showed that nano-TiO₂ does not penetrate through the intact epidermal barrier. On the other hand, when exposed directly, TiO₂ nanoparticles were able to affect the functional properties of epidermal and dermal cells *in vitro* [18]. In contrast, there is a lack of studies on the effect of micro-TiO₂ on human keratinocytes and melanocytes, both in healthy and cancer cells. To the best of our knowledge, no published *in vitro* or *in vivo* study has investigated the molecular effects of micro-TiO₂ on melanoma cells.

Adenosine triphosphate (ATP) binding cassette subfamily B member 5 (ABCB5) is a plasma membrane protein and the member of ABC transporter superfamily (subfamily B or MDR/TAP [multidrug resistance/transporter associated with antigen processing]), encoded by the *ABCB5* gene (chromosome 7p21.1) [19]. Tumor cells expressing ABCB5 may have properties of stem cells and a survival advantage compared to other cell (sub)populations in tumor microenvironment [20,21]. The ABCB5 transmembrane protein plays an important role in the tumorigenic potential and metastatic disease progression of diverse human malignancies, including melanoma [22]. This leads to a relapse in patients with supposedly cured melanoma, even several years after the treatment with chemotherapy, radiotherapy or immunotherapy [21,23,24]. Generally, the treatment of metastatic melanoma represents a great clinical challenge, as the metastatic form is highly aggressive and resistant to conventional therapies, with an average survival time

of 6–10 months [25,26].

This study aims to investigate the effect of micro-TiO₂ on the metabolic activity, cytotoxicity, and *ABCB5* mRNA expression in WM-266-4 human metastatic melanoma cell line.

MATERIALS AND METHODS

Cell culture

Human metastatic melanoma cell line WM-266-4 (ATCC® CRL1676™) was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were grown in complete medium containing Eagle's Minimum Essential Medium (EMEM, ATCC® 30-2003™) with 1% fetal bovine serum [FBS] (ATCC® 30-2021™) and 0.02% MycoZap Plus-CL (Lonza, Portsmouth, NH, USA), and incubated at 37 °C, 5% CO₂, ≥90% relative humidity (RH). The complete medium was replaced every 48 hours. When the culture became confluent, the cells were subcultivated by trypsinization using Detach kit (Catalog number C-41220, PromoCell, Heidelberg, Germany, EU) and replated.

UV filter

TiO₂ in the rutile form and with an average particle size of ≤5 μm (micro-TiO₂) was purchased from Sigma-Aldrich, USA (AL-224227-5G). A 10 mg/mL stock solution of micro-TiO₂ was prepared and diluted with the EMEM (ATCC® 30-2003™) based complete medium to make 250 μg/mL, 125 μg/mL, 100 μg/mL, 50 μg/mL, 20 μg/mL, 10 μg/mL, and 1 μg/mL concentrations of TiO₂, which were then applied to WM-266-4 cells grown in 6-well, 24-well or 96-well culture plates.

MTT cell metabolic activity assay

The cells were plated at a density of 1 × 10⁴ viable cells per well in 24-well culture plates and cultured for 24 hours in the EMEM-based complete medium to allow cell attachment.

To measure the cell metabolic activity, WM-266-4 cells were exposed to five selected concentrations of micro-TiO₂ (250, 100, 20, 10, and 1 μg/mL) and cultured for 8, 24, 48 and 120 hours. Control cells were cultured under the same conditions but without the addition of micro-TiO₂. Afterwards, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed using the Colorimetric Cell Viability Kit IV (PromoKine, PromoCell, Heidelberg, Germany, EU), following the manufacturer's instructions. The absorbance was measured at 570 nm and then the background absorbance (at 630 nm) was subtracted (OD₅₇₀-OD₆₃₀). All tests were performed in triplicates. The percentage of metabolically active cells was calculated with the following equation: ((OD₅₇₀-OD₆₃₀) test sample value/

(OD₅₇₀-OD₆₃₀) control value) × 100.

LDH cell cytotoxicity assay

The cells were plated at a density of 9×10^2 viable cells per well in 96-well culture plates and cultured for 24 hours in the EMEM-based complete medium to allow cell attachment. To evaluate the cytotoxicity of micro-TiO₂ in melanoma cells, WM-266-4 cells were exposed to five selected micro-TiO₂ concentrations (250, 100, 20, 10, and 1 µg/mL) and incubated for 48 and 120 hours. Then, the lactate dehydrogenase (LDH) assay using the LDH Cytotoxicity Kit II (PromoKine, PromoCell, Heidelberg, Germany, EU) was carried out, following the manufacturer's instructions. All tests were performed in triplicates. The percentage of cytotoxicity was calculated with the following equation: $((\text{Test Sample} - \text{Low Control}) / (\text{High Control} - \text{Low Control})) \times 100$, where the cells grown in EMEM plus cell lysis solution represented high control and the cells grown in EMEM alone represented the low control.

RNA isolation and cDNA synthesis

The WM-266-4 cells were seeded at a density of 2×10^4 into 6-well culture plates and incubated overnight in the EMEM-based complete medium. Afterwards, the cells were treated with six different concentrations of micro-TiO₂ (250 µg/mL, 125 µg/mL, 50 µg/mL, 20 µg/mL, 10 µg/mL, and 1 µg/mL) and incubated for 2, 24 and 48 hours. The total RNA was isolated from the treated and control cells using High Pure RNA Isolation Kit (Cat. No. 11 828 665 001, Roche, Basel, Switzerland), following the manufacturer's protocol. The concentration and purity of RNA were determined using the Thermo Scientific NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, MA, USA). The purified RNA was stored at -80°C. Reverse transcription (RT) into complementary DNA (cDNA) was performed using the Transcriptor Universal cDNA Master Kit (Cat. No. 05 893 151 001, Roche, Basel, Switzerland), following the manufacturer's instructions.

Quantitative RT polymerase chain reaction (RT-qPCR)

RT-qPCR was used to determine the expression of the *ABCB5* gene in control and treated WM-266-4 cells. The PCR reaction included 100 ng of cDNA, LC480 Probes Master (Roche) and RT Ready Designer/Catalog assays (Roche, Basel, Switzerland) and was carried out on the LightCycler480 system II real-time PCR device (Roche, Basel, Switzerland). The *LDHA* gene was used as an endogenous control. PCR amplification efficiency was determined from a standard curve using template dilutions of 1:1, 1:10, 1:100 and 1:1000 at the same PCR conditions. The PCR protocol included an initial pre-incubation step at 95 °C for 10 minutes, followed by 45 cycles

of denaturation at 95 °C for 10 seconds, annealing at 60 °C for 30 seconds and elongation at 72 °C for 1 second; the final step was cooling at 40 °C for 30 seconds. The PCR efficiencies for the *ABCB5* and *LDHA* genes were verified by generating standard curves. All reactions were run in triplicates. Relative changes in *ABCB5* mRNA expression were determined by the $2^{-\Delta\Delta C_t}$ method, normalized to the reference *LDHA* gene.

Cell morphology

Changes in cell morphology were recorded with a digital camera (DFC365 FX Leica, Buffalo Grove, IL, USA) attached to an inverted microscope (DMI6000B, Leica).

Statistical analysis

Student's t-test, one-way analysis of variance (ANOVA) and post-hoc Bonferroni tests were conducted using IBM SPSS Statistics for Windows, Version 20.0. (IBM Corp., Armonk, NY). The level of significance was set at $p < 0.05$. All values are expressed as mean ± standard error of the mean (SEM). Graphs were created with GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, CA, USA).

RESULTS

The effect of micro-TiO₂ on the metabolic activity of metastatic melanoma cells

The MTT assay showed that after 24 hours of exposure to micro-TiO₂, the metabolic activity of WM-266-4 cells significantly increased at all tested concentrations (the mean metabolic activity was $174.8\% \pm 25.0\%$), compared to untreated control cells at the same incubation time ($p = 0.030$). However, after 120 hours of exposure, a marked decrease in the cell metabolic activity and viability was observed, especially at the two highest micro-TiO₂ concentrations; i.e., at 250 µg/ml micro-TiO₂ the cell metabolic activity was $51.5\% \pm 9.2\%$ and at 100 µg/ml it was $64.5\% \pm 0.7\%$ ($p = 0.004$). Dose- and time-dependent curves of the metabolic activity of micro-TiO₂-treated WM-266-4 cells in relation to untreated control cells are shown in Figure 1.

Cytotoxic effects of micro-TiO₂ on metastatic melanoma cells

Using the LDH assay, we investigated whether the decrease in metabolic activity of WM-266-4 cells, observed after longer exposure to micro-TiO₂, was related to the cytotoxic effects of TiO₂. After 48 hours of micro-TiO₂ exposure, we observed a significant cytotoxicity on WM-266-4 cells compared to control ($p = 0.012$). The percentage of cytotoxicity in WM-266-4 cells after 48 hours of exposure was $22.1\% \pm 0.4\%$ at 250 µg/ml micro-TiO₂ and $15.2\% \pm 1.6\%$ at 100 µg/

ml micro-TiO₂ (Table 1). After 120 hours of exposure, the rate of cytotoxicity significantly increased ($p = 0.047$), to $97.0\% \pm 2.8\%$ at $250 \mu\text{g/ml}$ and $60.0\% \pm 2.8\%$ at $100 \mu\text{g/ml}$ micro-TiO₂ (Table 1).

Microscopic observations of metastatic melanoma cells after exposure to micro-TiO₂

Micro-TiO₂ tended to aggregate close to WM-266-4 cells in every well and arrange into spherical assemblies at the cell surface (Figure 2A-C). After 24 hours of exposure of metastatic melanoma cells to micro-TiO₂, we observed altered size and shape in some cells (Figure 2D-E). After 48 hours, a few spindle cells and numerous melanospheres were observed, and some of these cells were with granular cytoplasm. Following 120 hours of exposure to the two highest concentrations of micro-TiO₂ (100 and $250 \mu\text{g/ml}$), the number of WM-266-4 cells markedly decreased compared to cells treated with a lower concentration of micro-TiO₂ (i.e., $20 \mu\text{g/ml}$) and untreated control cells. Also, after 120 hours of exposure more melanoma cells with granular cytoplasm were observed. At lower concentrations of micro-TiO₂, there was no significant change

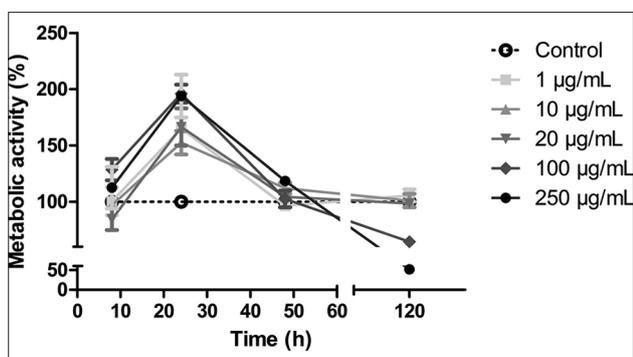


FIGURE 1. Metabolic activity of micro-TiO₂-treated WM-266-4 metastatic melanoma cells at selected micro-TiO₂ concentrations and exposure times in relation to untreated WM-266-4 cells (control), as evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. All values are expressed as mean \pm standard error of the mean (SEM). After 24 hours of exposure to micro-TiO₂, the metabolic activity of WM-266-4 cells significantly increased at all tested concentrations (the mean metabolic activity was $174.8\% \pm 25.0\%$), compared to untreated control cells at the same incubation time ($p = 0.030$). After 120 hours of exposure, a marked decrease in the cell metabolic activity and viability was observed, especially at the two highest micro-TiO₂ concentrations; i.e. at $250 \mu\text{g/ml}$ micro-TiO₂ the cell metabolic activity was $51.5\% \pm 9.2\%$ and at $100 \mu\text{g/ml}$ it was $64.5\% \pm 0.7\%$ ($p = 0.004$).

in the number of WM-266-4 cells compared to untreated control cells (Figure 3).

Micro-TiO₂ progressively decreased the ABCB5 mRNA expression in metastatic melanoma cells

The expression of ABCB5 gene was significantly lower in WM-266-4 cells exposed to micro-TiO₂ for 24 and 48 hours compared to the untreated control cells and compared to WM-266-4 cells exposed to micro-TiO₂ for 2 hours [$p = 0.002$] (Figure 4).

DISCUSSION

Previously, we showed that ABCB5 mRNA expression significantly increases in metastatic melanoma cells exposed to the UV filters octocrylene (OCT) and nano-TiO₂, in a time-dependent manner. This result indicates that the two UV filters may promote tumor invasion, progression and recurrence; therefore, patients diagnosed with metastatic melanoma should avoid products containing nano-TiO₂ and OCT [27,28]. However, nano-sized TiO₂ and micro-sized TiO₂ particles can differ in their effects and should not be treated in the same way. For example, nano-sized TiO₂ powders have

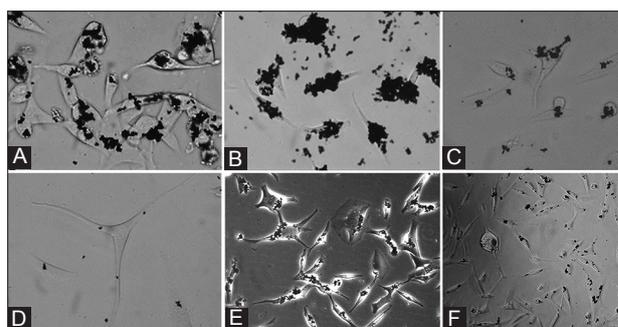


FIGURE 2. Representative phase-contrast images acquired at different magnifications, showing WM-266-4 metastatic melanoma cells treated with different micro-TiO₂ concentrations at different incubation times. A) Right after $50 \mu\text{g/ml}$ TiO₂ was added (0 hours; $630\times$ magnification). B) After 48 hours of exposure to $250 \mu\text{g/ml}$ TiO₂ ($400\times$ magnification). C) After 48 hours of exposure to $10 \mu\text{g/ml}$ TiO₂ ($200\times$ magnification). D) After 24 hours of exposure to $20 \mu\text{g/ml}$ TiO₂ ($400\times$ magnification). E) After 24 hours of exposure to $20 \mu\text{g/ml}$ TiO₂ ($200\times$ magnification). F) After 48 hours of exposure to $10 \mu\text{g/ml}$ TiO₂ ($200\times$ magnification). Micro-TiO₂ tended to aggregate and arrange into spherical assemblies at the cell surface (A-C), and after 24 hours of exposure to micro-TiO₂, we observed altered size and shape in some WM-266-4 cells (D-E).

TABLE 1. Cytotoxic effects of micro-TiO₂ on WM-266-4 metastatic melanoma cell line, evaluated by the lactate dehydrogenase (LDH) cytotoxicity assay after 48 and 120 hours of exposure. Increased cytotoxicity was observed with longer times of exposure to micro-TiO₂

WM-266-4 cell line	Incubation time	Micro-TiO ₂ concentrations ($\mu\text{g/ml}$)				
		10	10	20	100	250
	0 hours	0	0	0	0	0
Cytotoxicity (%)	48 hours	0.2 ± 0.1	0.5 ± 0.1	1.2 ± 0.2	15.2 ± 1.6	22.1 ± 0.4
	120 hours	0.6 ± 0.4	0.9 ± 0.7	1.6 ± 0.5	60.0 ± 2.8	97.0 ± 2.8

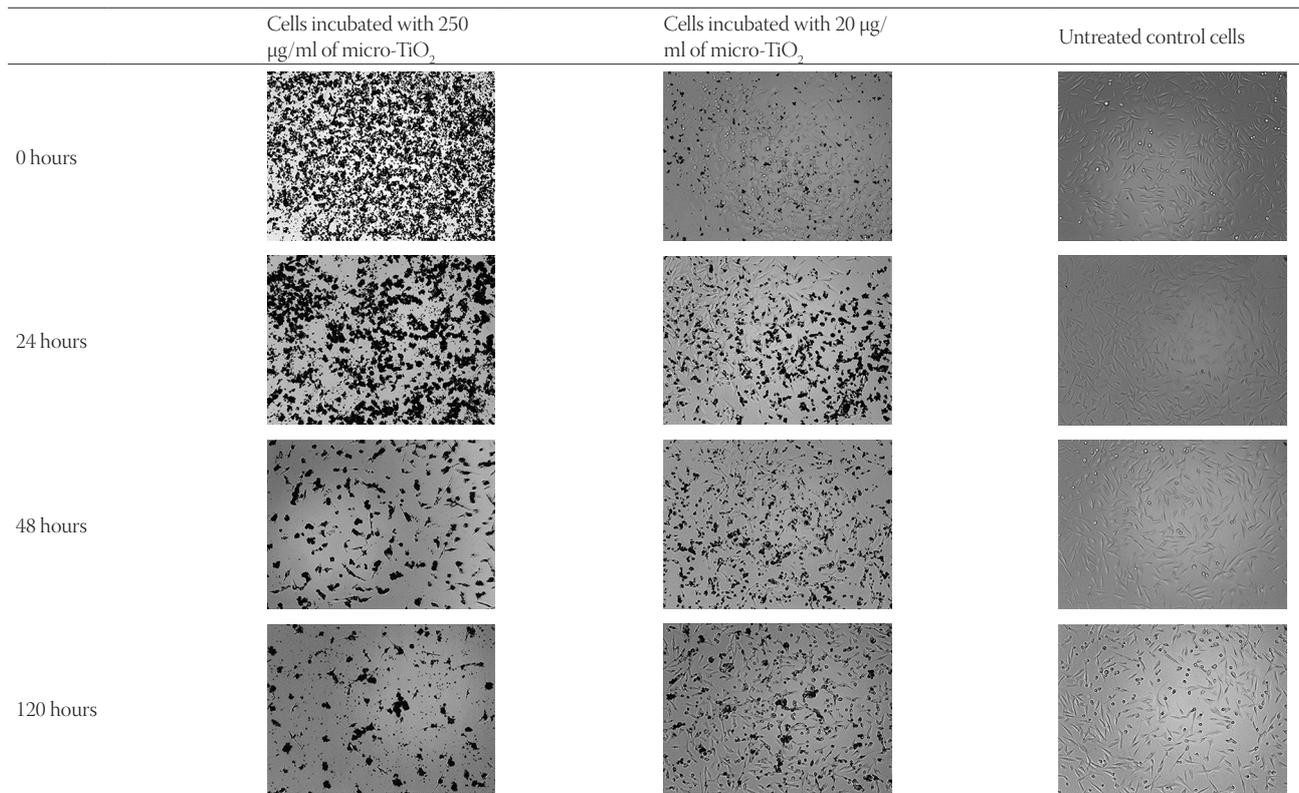


FIGURE 3. Microscopy images of WM-266-4 metastatic melanoma cells treated with different micro-TiO₂ concentrations right after the filter was added (0 hours) and after 24, 48 and 120 hours of incubation, compared with the corresponding control cells. All images are at 100x magnification. After 120 hours of exposure to the highest concentration of micro-TiO₂ 250 µg/ml, the number of WM-266-4 cells markedly decreased. At lower concentration (20 µg/ml) of micro-TiO₂, there was no significant change in the number of WM-266-4 cells compared to untreated control cells. Also, after 120 hours of exposure to micro-TiO₂ numerous melanoma cells with granular cytoplasm were observed.

significantly higher specific surface area and may exhibit physical and chemical properties different from those of microparticles [29].

In the present study, we investigated the effect of micro-TiO₂ on the metabolic activity, cytotoxicity, and *ABCB5* mRNA expression in metastatic melanoma cells. The tested concentrations of micro-TiO₂ were selected based on our previous studies on nano-TiO₂ and OCT [27,28], as specific studies on the effects of micro-TiO₂ on human skin cells are still scarce. Similar TiO₂ concentrations were also used in nano-TiO₂ penetration and toxicity studies on various cells, including keratinocytes [18,30,31], and in a study investigating the effect of micro-TiO₂ on human leukocytes and fibroblasts [32]. In the current study, we were interested only in the basic function of micro-TiO₂ in WM-266-4 cells and hence we did not expose the treated melanoma cells to UV light.

Our MTT results showed that, until 24 hours of incubation, the metabolic activity of metastatic melanoma cells increased with increased time of exposure to micro-TiO₂, in a concentration-independent manner. After 120 hours of incubation, a marked decrease in the cell metabolic activity and viability was observed, which was especially obvious at the two highest concentrations of micro-TiO₂ [100 and 250 µg/ml] (Figure 1). These results were consistent with our LDH results

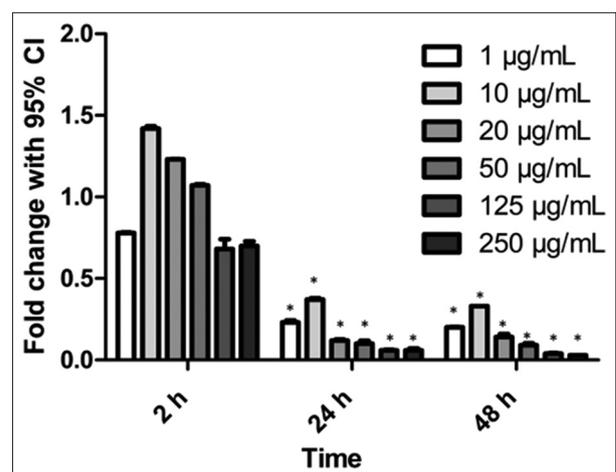


FIGURE 4. Fold change in adenosine triphosphate (ATP) binding cassette subfamily B member 5 (*ABCB5*) mRNA expression in WM-266-4 metastatic melanoma cells treated with different micro-TiO₂ concentrations as compared to untreated controls, after 2, 24 and 48 hours of incubation. All values are expressed as mean \pm standard error of the mean (SEM). The expression of *ABCB5* gene was significantly lower in WM-266-4 cells exposed to micro-TiO₂ for 24 and 48 hours compared to the untreated control cells and compared to WM-266-4 cells exposed to micro-TiO₂ for 2 hours ($p = 0.002$).

(Table 1), i.e., after 48 hours of micro-TiO₂ exposure, we observed a significant cytotoxicity on WM-266-4 cells. The limitation of the LDH and most other cytotoxicity assays is

that they are based on the premise that due to highly compromised cellular membranes in dying cells they release their cytoplasmic components, including LDH, into the culture medium. However, because the loss of membrane integrity often occurs quite late in apoptosis and necrosis, we suspect that the cytotoxic activity of micro-TiO₂ observed in our study is even underestimated.

Our microscopic observations were also consistent with the LDH results as we observed only a few spindle cells and numerous melanospheres after 48 hours of exposure of metastatic melanoma cells to micro-TiO₂, and some of these cells were with granular cytoplasm. After 120 hours of exposure to micro-TiO₂ even more melanoma cells with granular cytoplasm were observed (Figure 3). Micro-TiO₂ particles were evenly distributed over the area without cells, but tended to aggregate and arrange into spherical assemblies at the cell surface (Figure 2A-C). Following 24 hours of exposure to micro-TiO₂, some cells became larger than others and their shape changed (Figure 2D-F). This phenomenon was observed after 24 and 48 hours of micro-TiO₂ exposure when the metabolic activity of treated melanoma cells was higher than that of untreated control cells. These TiO₂-treated melanoma cells did not have the shape of melanospheres nor did they have microscopic characteristics of cellular cannibalism, such as those previously observed in metastatic melanoma cells exposed to OCT after a decrease in their metabolic activity [27].

Using qRT-PCR we analyzed *ABCB5* mRNA expression in metastatic melanoma cells treated with micro-TiO₂. Figure 4 provides an overview of the results, which indicate that different concentrations of micro-TiO₂ within our tested concentration range significantly decrease the expression of the *ABCB5* mRNA in metastatic melanoma cells, in a time-dependent manner. The *ABCB5* gene expression was lower in cells exposed to micro-TiO₂ for 24 hours and especially in cells exposed for 48 hours, compared to the cells exposed to micro-TiO₂ for 2 hours. Because the LDH assay showed very high cytotoxicity of micro-TiO₂ on melanoma cells after 120 hours of exposure, especially at the two highest concentrations of micro-TiO₂, we did not analyze the expression of *ABCB5* gene at longer incubation time points, as the insufficient number of cells could have affected the accuracy of the results.

Taken together, our results indicate inhibitory effects of micro-TiO₂ on the metabolic activity and *ABCB5* mRNA expression in metastatic melanoma cells. Due to the known cellular roles of the *ABCB5* protein, it can be assumed that metastatic melanoma cells which survive exposure to micro-TiO₂ are less invasive and less resistant, compared to other melanoma cells or control cells. As such, micro-TiO₂ may be an effective anticancer agent. Our results suggest that, in UV protection cosmetics, it might be safer to use micro-TiO₂ rather than nano-TiO₂. Some authors even consider the introduction

of innovative technologies such as nanotechnology to be a societal experiment, and argue that the marketing of sunscreens containing nano-TiO₂ is ethically undesirable [33].

Further studies are necessary to confirm our results. The safety profile of micro-TiO₂ should be first established with normal melanocytes (primary culture) and melanoma cells from the primary tumor site, before any definitive conclusions. In addition, it would be informative to investigate the effect of micro-TiO₂ on other potential markers of melanoma cells, i.e. nerve growth factor receptor (NGFR or CD271), aldehyde dehydrogenases (ALDH), receptor activator of nuclear factor κ B (RANK), melanoma-associated chondroitin sulfate proteoglycan (MCSP), and melanoma cell adhesion molecule (MCAM or CD146) [34-37]. One of the most important goals in melanoma research is to improve the prevention and treatment strategies for this aggressive form of cancer, and the results of our study may serve as a starting point for future studies in this promising area of research.

DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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