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

Tanja Prunk Zdravković[1,2], Bogdan Zdravković[3], Mojca Lunder[4], Polonca Ferk[5*]

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

ABSTRACT

Titanium dioxide (TiO_2) 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 TiO_2 in different cell lines, including human skin cells, studies investigating the effects of micro-TiO_2 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-TiO_2 (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-TiO_2 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-TiO_2 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-TiO_2 the metabolic activity of melanoma cells decreased, especially at the two highest micro-TiO_2 concentrations. Comparably, the cytotoxicity of micro-TiO_2 on melanoma cells increased after 48 and 120 hours of exposure, in a time-dependent manner. The *ABCB5* mRNA expression in micro-TiO_2-treated melanoma cells also decreased significantly after 24 and 48 hours, in a time-dependent manner. Overall, our results suggest inhibitory effects of micro-TiO_2 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 (TiO_2) 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 Ferk, Faculty of Medicine, University of Ljubljana, Vrazov trg 2, 1000 Ljubljana, Slovenia. Phone: +386 (1) 543 77 00; Fax: +386 (1) 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 TiO_2, 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 TiO_2 as possibly carcinogenic to humans (Group 2B carcinogen) [14]. Furthermore, numerous *in vitro* studies showed that TiO_2 nanoparticles (nano-TiO_2) are able to induce cytotoxicity, reactive oxygen species (ROS), and genotoxicity in different cell lines [15]. For micro-sized TiO_2 (micro-TiO_2), 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-TiO_2 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-TiO_2 on human health.

A study investigating the effect of micronized TiO_2 (nanoparticles) on the barrier function of the human skin (epidermis) transplanted to immunodeficient mice showed that nano-TiO_2 does not penetrate through the intact epidermal barrier. On the other hand, when exposed directly, TiO_2 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-TiO_2 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-TiO_2 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-TiO_2 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% CO_2, ≥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

TiO_2 in the rutile form and with an average particle size of ≤5 μm (micro-TiO_2) was purchased from Sigma-Aldrich, USA (AL-224227-5G). A 10 mg/mL stock solution of micro-TiO_2 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 TiO_2, 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 1×10^4 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-TiO_2 (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-TiO_2. 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-TiO_2 in melanoma cells, WM-266-4 cells were exposed to five selected micro-TiO_2 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: ((Test Sample - Low Control)/(High Control - Low Control)) × 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-TiO_2 (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 $2^{-\Delta\Delta Ct}$ 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-TiO_2 on the metabolic activity of metastatic melanoma cells

The MTT assay showed that after 24 hours of exposure to micro-TiO_2, the metabolic activity of WM-266-4 cells significantly increased at all tested concentrations (the mean metabolic activity was 174.8% ± 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-TiO_2 concentrations; i.e., at 250 μg/ml micro-TiO_2 the cell metabolic activity was 51.5% ± 9.2% and at 100 μg/ml it was 64.5% ± 0.7% ($p = 0.004$). Dose- and time-dependent curves of the metabolic activity of micro-TiO_2-treated WM-266-4 cells in relation to untreated control cells are shown in Figure 1.

Cytotoxic effects of micro-TiO_2 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-TiO_2, was related to the cytotoxic effects of TiO_2. After 48 hours of micro-TiO_2 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% ± 0.4% at 250 μg/ml micro-TiO_2 and 15.2% ± 1.6% at 100 μg/

ml micro-TiO_2 (Table 1). After 120 hours of exposure, the rate of cytotoxicity significantly increased (p = 0.047), to 97.0% ± 2.8% at 250 μg/ml and 60.0% ± 2.8% at 100 μg/ml micro-TiO_2 (Table 1).

Microscopic observations of metastatic melanoma cells after exposure to micro-TiO_2

Micro-TiO_2 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-TiO_2, 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-TiO_2 (100 and 250 μg/ml), the number of WM-266-4 cells markedly decreased compared to cells treated with a lower concentration of micro-TiO_2 (i.e., 20 μg/ml) and untreated control cells. Also, after 120 hours of exposure more melanoma cells with granular cytoplasm were observed. At lower concentrations of micro-TiO_2, there was no significant change in the number of WM-266-4 cells compared to untreated control cells (Figure 3).

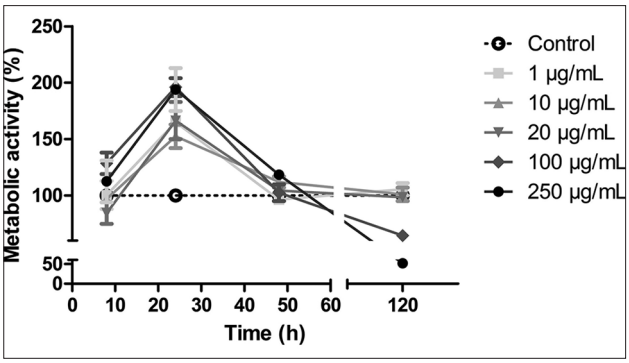

FIGURE 1. Metabolic activity of micro-TiO_2-treated WM-266-4 metastatic melanoma cells at selected micro-TiO_2 concentrations and exposure times in relation to untreated WM-266-4 cells (control), as evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. All values are expressed as mean ± standard error of the mean (SEM). After 24 hours of exposure to micro-TiO_2, the metabolic activity of WM-266-4 cells significantly increased at all tested concentrations (the mean metabolic activity was 174.8% ± 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-TiO_2 concentrations; i.e. at 250 μg/ml micro-TiO_2 the cell metabolic activity was 51.5% ± 9.2% and at 100 μg/ml it was 64.5% ± 0.7% (p = 0.004).

Micro-TiO_2 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-TiO_2 for 24 and 48 hours compared to the untreated control cells and compared to WM-266-4 cells exposed to micro-TiO_2 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-TiO_2, 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-TiO_2 and OCT [27,28]. However, nano-sized TiO_2 and micro-sized TiO_2 particles can differ in their effects and should not be treated in the same way. For example, nano-sized TiO_2 powders have

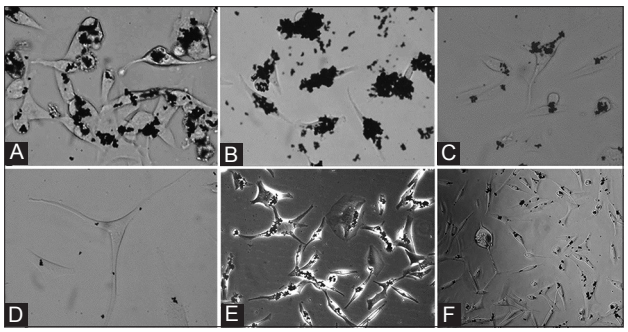

FIGURE 2. Representative phase-contrast images acquired at different magnifications, showing WM-266-4 metastatic melanoma cells treated with different micro-TiO_2 concentrations at different incubation times. A) Right after 50 μg/ml TiO_2 was added (0 hours; 630× magnification). B) After 48 hours of exposure to 250 μg/ml TiO_2 (400× magnification). C) After 48 hours of exposure to 10 μg/ml TiO_2 (200× magnification). D) After 24 hours of exposure to 20 μg/ml TiO_2 (400× magnification). E) After 24 hours of exposure to 20 μg/ml TiO_2 (200× magnification). F) After 48 hours of exposure to 10 μg/ml TiO_2 (200× magnification). Micro-TiO_2 tended to aggregate and arrange into spherical assemblies at the cell surface (A-C), and after 24 hours of exposure to micro-TiO_2, we observed altered size and shape in some WM-266-4 cells (D-E).

TABLE 1. Cytotoxic effects of micro-TiO_2 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-TiO_2

WM-266-4 cell line	Incubation time	Micro-TiO_2 concentrations (μg/mL)				
		10	10	20	100	250
Cytotoxicity (%)	0 hours	0	0	0	0	0
	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

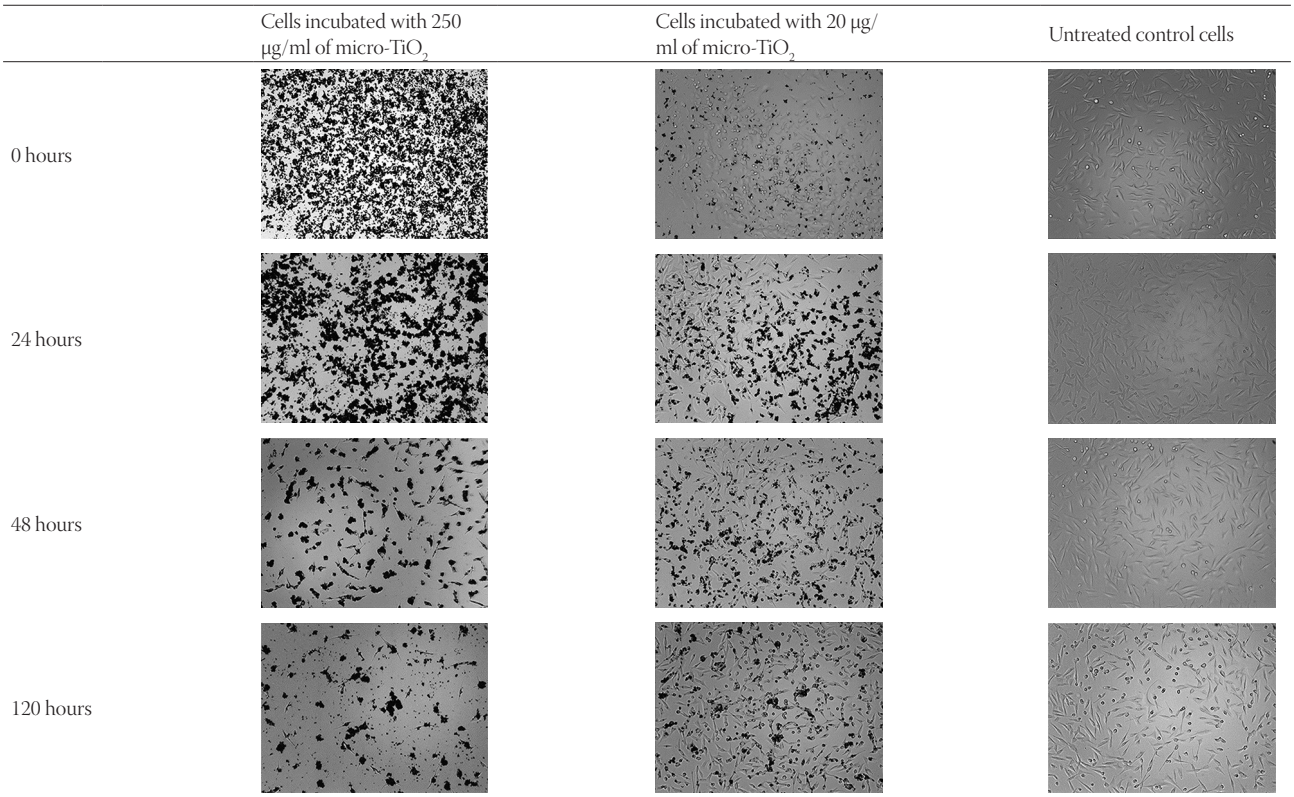

FIGURE 3. Microscopy images of WM-266-4 metastatic melanoma cells treated with different micro-TiO_2 concentrations right after the filter was added (0 hours) and after 24, 48 and 120 hours of incubation, compared with the corresponding control cells. All images are at 100× magnification. After 120 hours of exposure to the highest concentration of micro-TiO_2 (250 µg/ml), the number of WM-266-4 cells markedly decreased. At lower concentration (20 µg/ml) of micro-TiO_2, 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-TiO_2 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-TiO_2 on the metabolic activity, cytotoxicity, and *ABCB5* mRNA expression in metastatic melanoma cells. The tested concentrations of micro-TiO_2 were selected based on our previous studies on nano-TiO_2 and OCT [27,28], as specific studies on the effects of micro-TiO_2 on human skin cells are still scarce. Similar TiO_2 concentrations were also used in nano-TiO_2 penetration and toxicity studies on various cells, including keratinocytes [18,30,31], and in a study investigating the effect of micro-TiO_2 on human leukocytes and fibroblasts [32]. In the current study, we were interested only in the basic function of micro-TiO_2 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-TiO_2, 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-TiO_2 [100 and 250 µg/ml] (Figure 1). These results were consistent with our LDH results

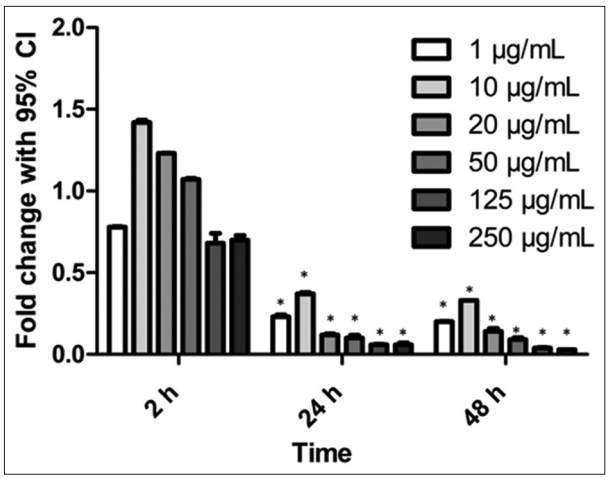

FIGURE 4. Fold change in adenosine triphosphate (ATP) binding cassette subfamily B member 5 (ABCB5) mRNA expression in WM-266-4 metastatic melanoma cells treated with different micro-TiO_2 concentrations as compared to untreated controls, after 2, 24 and 48 hours of incubation. All values are expressed as mean ± standard error of the mean (SEM). The expression of ABCB5 gene was significantly lower in WM-266-4 cells exposed to micro-TiO_2 for 24 and 48 hours compared to the untreated control cells and compared to WM-266-4 cells exposed to micro-TiO_2 for 2 hours ($p = 0.002$).

(Table 1), i.e., after 48 hours of micro-TiO_2 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-TiO_2 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-TiO_2, and some of these cells were with granular cytoplasm. After 120 hours of exposure to micro-TiO_2 even more melanoma cells with granular cytoplasm were observed (Figure 3). Micro-TiO_2 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-TiO_2, some cells became larger than others and their shape changed (Figure 2D-F). This phenomenon was observed after 24 and 48 hours of micro-TiO_2 exposure when the metabolic activity of treated melanoma cells was higher than that of untreated control cells. These TiO_2-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-TiO_2. Figure 4 provides an overview of the results, which indicate that different concentrations of micro-TiO_2 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-TiO_2 for 24 hours and especially in cells exposed for 48 hours, compared to the cells exposed to micro-TiO_2 for 2 hours. Because the LDH assay showed very high cytotoxicity of micro-TiO_2 on melanoma cells after 120 hours of exposure, especially at the two highest concentrations of micro-TiO_2, 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-TiO_2 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-TiO_2 are less invasive and less resistant, compared to other melanoma cells or control cells. As such, micro-TiO_2 may be an effective anticancer agent. Our results suggest that, in UV protection cosmetics, it might be safer to use micro-TiO_2 rather than nano-TiO_2. 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-TiO_2 is ethically undesirable [33].

Further studies are necessary to confirm our results. The safety profile of micro-TiO_2 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-TiO_2 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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