TITANIUM DIOXIDE NANOPARTICLES TOXICITY ON DNA METHYLATION: IN VITRO STUDY

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ABSTRACT

Background: It was shown by several studies that titanium dioxide nanoparticles (TiO2 NPs) can change the global and gene-specific patterns of DNA methylation. This may result in modifications to the DNA methylation pattern by affecting the expression of DNA methyltransferases (DNMTs). **Aim of the work:** Evaluation of the epigenetic changes of the genome (DNA methylation) resulting from exposure to TiO_2 NPs. **Material and Methods:** Human epithelial adenocarcinoma cell line (CaCo-2 cells) was divided into group 1: untreated cells, group 2: treated with 0.5 % dimethyl sulfoxide, group 3: treated with 100 µg/mL TiO2 NPs for 72 hours, group 4: treated with doxorubicin 30 microgram/ml. The cell line was subjected to cytotoxicity assessment, translocation of TiO2 NPs by transmission electron microscopy (TEM), global DNA methylation quantification, and quantitative measurement of DNMT1, 3A, and 3B gene expression by reverse transcription-PCR. **Results:** A dose-dependent cytotoxicity was found. TEM revealed the presence of TiO2 NPs within the cells in group 3. DNA methylation showed a significant decrease in groups 3 and 4 than group 1 and group 2. Expression levels of DNMT1 gene showed a significant increase in groups 3 and 4 when comparing them to group 1 and group 2. The expression levels of DNMT3A gene and DNMT3B gene showed a significant decrease in groups 3 and 4 in comparing them to groups 1 and 2. **Conclusion:** In Caco-2 cells, being exposed to TiO2 NPs can affect DNA methylation, indicating that the toxicity of TiO2 NPs may be related to this epigenetic pathway.

Keywords: *Titanium dioxide nanoparticles, Caco-2 cell line, DNA methylation, Methyl transferases, Epigenetic toxicity.*

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INTRODUCTION

One of the most often made nanoparticles in the world is titanium dioxide ***(Rashid et al., 2021)***. Electronics, optical, and pharmaceutical industries are among those that continue to report a rise in TiO2 NPs commercial applications. Because these nanoparticles are widely used in paints, sunscreen, cosmetics, and personal care products, a high risk to humans in contact with them, through the respiratory system, topically, or orally are different ways for TiO2 NPs entrance to the body ***(Shakeel et al., 2016)***.

Studies have demonstrated that the tissues and cells may undergo epigenetic modifications as a result of nanoparticle exposure. NPs can be cytotoxic and genotoxic even in sub-cytotoxic quantities. Research has demonstrated that TiO2 NPs in particular can change the global and gene-specific patterns of DNA methylation. They can modify DNA methylation patterns by influencing the expression or activity of (DNMTs). It's crucial to remember that different nanoparticle types and concentrations can affect DNA methylation in different ways ***(Hou et al., 2019; Wong et al., 2017)***.

A significant epigenetic process known as deoxyribonucleic acid methylation entails introducing a methyl group to self-complementary (CpG) or cytosine-guanine (CG) dinucleotides, which are DNA segments where a guanine nucleotide appears after a cytosine nucleotide in a base-by-base sequence. This process produces 50-methylated cytosine. The preservation of gene expression specific to a certain tissue or cell depending on the methylation/demethylation of CpG sites ***(Moore et al., 2013)***.

The class of enzymes that catalyze the methylation of deoxyribonucleic acid is known as DNA methyltransferases. DNMT1, 3A, and 3B are the three distinct forms of DNMTs, and each one has a distinct purpose. To preserve the methylation patterns during cell replication, DNMT1 mainly adds the methyl group to hemimethylated DNA locations. However, DNMT3A and 3B can de novo methylation activity. These enzymes are essential for the development of mammals and are vital for controlling certain gene expressions ***(Joseph et al., 2018)***.

It is essential to take DNA methylation into account when evaluating the possible toxicity of nanoparticles. This is because altered DNA methylation may have a substantial impact on the onset and course of some illnesses and pathological states. We can learn more about the mechanisms behind the harmful effects of nanoparticles by examining changes in this epigenetic pathway ***(Valente et al., 2023)***.

THE AIM OF THE WORK

This work aims to assess titanium dioxide nanoparticle exposure's effect on cell viability and epigenetic modifications to the genome, such as global methylation and DNA methyltransferase expression levels. One unique feature of the current work is the use of TEM and the finding of particles in the cells.

MATERIAL AND METHODS

Chemicals:

Ti O2 nanoparticles were purchased from Sigma Aldrich, Egypt (Aldrich code 718467). Surface area 35-65 m^2/g (BET), particle size 21 nm, CAS Number (13463-67-7). TiO_2 NPs were white and in the form of powder.

Titanium dioxide nanoparticles characterization:

Characterization was made by Transmission electron microscope (TEM) at EM Unit, Mansoura University, Egypt. TiO_2 NPs size ranges from 9 nm up to 22 nm by TEM **(Figure 1)**.

Study design:

At Mansoura University Faculty of Medicine, an in vitro cell line experiment was carried out. *The Mansoura Faculty of Medicine Institution Research Board (MFM-IRB), Mansoura University, code (MD.21.01.400), approved the study.*

Caco-2 cell lines are the primary cell type used to explore nanoparticle translocation. The morphological features of Caco-2 cells are comparable to those of normal enterocytes ***(Storniolo, 2020)***.

Caco-2 cell line and treatment conditions:

All the following procedures were done at the National Research Center, Cairo, Egypt. The cells were placed in a sterile environment using a laminar flow cabinet with biosafety class II level precautions. The cells were suspended in DMEM containing a 1 percent antibiotic-antimycotic mixture (which includes 10,000U/milliliter potassium penicillin, 10,000µg/milliliter streptomycin sulfate and 25µg/milliliter amphotericin B) and 1% L-glutamine. The cells were kept at a temperature of 37 ºC under 5% CO2. The medium used was replaced every 3 days ***(Yang et al., 2017)***.

For ten days, the cells were grown in a closed bacterial culture system with certain nutrients, pressure, temperature, aeration, and other environmental variables to maximize growth. Subsequently, they were planted on 6-well microliter plastic plates with fresh complete growth media at a concentration of $5x10^6$ cells/well. After that, the plates were kept at 37 °C with five percent CO_2 for 24 hours in a water-jacketed CO_2 incubator. Cell differentiation, confluency, and proliferation were finally finished after 5 days ***(Bonnet et al., 2019)***.

After aspirating the media and adding the fresh medium, the cells were cultured for 72 hours either in the absence of 100 microgram/milliliter TiO2 NPs or in the presence of them. As a positive control, doxorubicin was utilized at an IC50 of 30 micrograms/ml. After 72 hours of incubation, a medium was aspirated and cells were washed two times with PBS and kept as pellets.

The cell line was divided into:

Group 1 (Negative control group): Untreated Caco-2 cells.

Group 2 (Vehicle control group): Caco-2 cells treated with 0.5 % Dimethyl Sulfoxide (DMSO) ***(Barthomeuf et al., 2008)***.

Group 3 (Titanium dioxide nanoparticles treated group) (TiO_2 NPs treated group): Caco-2 cells treated with 100 µg/milliliter

TiO_2 NPs for 72 hours ***(Bai et al., 2015; Ma et al., 2017)***.

This dose is equal to half the maximal inhibitory concentration (IC50) which measures the potency of a substance's potential in inhibition of specific biological or biochemical function. (IC50) of TiO_2 NPs at 24 hours is 211.3 +/- 15.2 µg/mL ***(Sha et al., 2013)***.

Group 4 (Doxorubicin treated group) (DOX treated group): Caco-2 cells treated with doxorubicin 30 microgram/ml as a positive control group. This dose is equal to (IC50) ***(Wen et al., 2018)***.

Doxorubicin is a type of molecule that functions as an intercalating agent. Intercalating agents are molecules that have a similar structure to nucleotide base pairs. Due to this structural similarity, they can insert themselves into the DNA helix.

This incorporation of intercalating agents distorts the molecular structure of DNA and hinders the activity of enzymes that manipulate DNA. This includes DNA methyltransferases like DNMT1, which is the primary mammalian DNA methyltransferase enzyme responsible for DNA methylation ***(Pang et al., 2013)***.

The cell line was subjected to the following:

1-Cytotoxicity Assessment (MTT assay).

2-Evaluation of titanium dioxide nanoparticle translocation by transmission electron microscopy.

3-Measurement of global 5-methylcytosine (global methylation): Quantification of global DNA methylation by Colorimetric method.

4-Quantitative measurement of DNMT1, DNMT3A, and DNMT3B gene expression by reverse transcription-PCR (qRT-PCR): (gene expression analysis).

1-*Cytotoxicity Assessment:*

It had been used to measure metabolic activity within cells as an estimate for cell viability, proliferation, and cytotoxicity ***(Wang et al., 2010)***. The procedure was done according to ***(Ghasemi et al., 2021)***. (IC50) was calculated by Prism software 6 program.

2-*Evaluation of TiO_2 NPs translocation by TEM:*

The procedure was done according to ***Graham and Orenstein, (2007) and Tizro et al. (2019)***. Ultrathin sections were observed at 160 kV using a JEOL JEM -2100 at the EM Unit, Mansoura University, Egypt.

3-Measurement of global DNA 5-methylcytosine:

With a few modest modifications, the EpiSeeker Methylated DNA Colorimetric Quantification Kit was used to measure global DNA methylation in accordance with the manufacturer's recommendations. To produce single-stranded DNA (ssDNA), the bisulfite-treated DNA samples were denatured for ten minutes at 95°C. After that, they were gradually cooled to room temperature in preparation for additional analysis. To obtain 25 ng of DNA for DNA sample analysis, 5 µL of denatured ssDNA was diluted in 5X sodium saline citrate (SSC) buffer. Subsequently, 5 µL of every denatured DNA sample was immediately placed onto an electrode-Au that had been screen-printed, and it was left to adsorb for ten minutes ***(Fariasa et al., 2015)***.

Three washes of 10 mM phosphate buffered saline at a pH of 7.4 were performed on the electrodes. The electrode surface was treated with 10 ng/µL of horseradish peroxidase (HRP)-conjugated methyl-cytosine (HRP-5mC) antibody after it had dried by air. After that, unless otherwise indicated, it was incubated for 30 minutes at room temperature with gentle shaking to enable the antibody to bind with ssDNA. After that, three 10 mM PBS washes were performed on the electrode to eliminate any remaining unbound HRP-5mC antibody ***(Haque et al., 2017)***.

Lastly, the gold electrode surface was covered with 50 µL of 3,3,5,5'-tetramethylbenzidin substrate solution, which was then left to incubate in the dark for 15 minutes. Spectrophotometric ocular observation of the colour shift was made. After adding 2 µL of HCL solution (2.0 M), absorbance readings at 450 nm were taken to quantify the colour change ***(Fariasa et al., 2015)***.

4-Quantitative measurement of DNMT1, DNMT3A, and DNMT3B gene expression by reverse transcription-PCR:

a) DNA primers:

They were purchased from Hosted Virtual Desktops (HVD.com), Germany. Primers' sequences are shown in **table (1)**. Using the RNeasy Mini Kit and the DNase I digestion step, total RNA was extracted from colon cancer cell lines in accordance with the manufacturer's instructions. After being treated with one unit of RQ1 RNAse-free DNAse to eliminate any remaining DNA, the isolated total RNA was re-suspended in water that had been treated with DEPC and measured photospectrophotometrically at 260 nm. The ratio of 260/280 nm, which ranged from 1.8 to 2.1, was used to evaluate the purity of total RNA. Furthermore, formaldehyde-containing agarose gel electrophoresis was utilised to analyse the 28S and 18S bands using ethidium bromide dye in order to guarantee integrity. Aliquots were kept at -80° unless they were utilised right away for (RT) ***(Sroor et al., 2022)***.

b) RT Reaction:

To create cDNA, poly(A)+ RNA from colon cell lines was reverse transcribed using the RevertAidTM First Strand cDNA Synthesis Kit. Five micrograms of total RNA were mixed with 10 mM of each dNTP, 50 µM oligo-dT primer, 20 IU ribonuclease inhibitor (50 kDa recombinant enzyme to limit RNase activity), and 50 IU MuLV reverse transcriptase in 10x RT buffer (pH 8.3). After centrifuging the mixture at 1000 g for 30 seconds, it was moved to a thermocycler. Ten minutes at 25 degrees Celsius, an hour at 42 °C, and five minutes at 99 degree Celsius were used to denaturize the RT reaction.

Before the reaction tubes containing the RT preparations were used for (qRT-PCR) cDNA amplification, they were flash-cooled in an ice chamber ***(Yang et al., 2017)***.

c) qPCR:

The colon cell line cDNA copy number was determined using the StepOneTM Real-Time PCR System. The PCR reactions were set up in 25 mL reaction mixtures using 6.5 millilitres of distilled water, five millilitres of cDNA template, 12.5 mL of 1× SYBR® Premix Ex TaqTM, 0.5 millilitres of 0.2 mM sense primer, and 0.5 millilitre of 0.2 mM antisense primer. The reaction plan was divided into three phases. The first process required three minutes at 95 degree Celsius. The 40 cycles that made up the second stage were as follows: 15 seconds at 95 degree Celsius, a duration of thirty seconds at 55 degree Celsius, and thirty seconds at 72 degree Celsius. There were 71 cycles in the third phase. From sixty degrees Celsius to ninety-five degrees Celsius, the temperature rose by almost half degrees Celsius every ten seconds. To confirm the quality of the primers used, a melting curve analysis was carried out at 95 degrees Celsius at the conclusion of each sqRT-PCR. A control of distilled water was included in every experiment ***(Sroor et al., 2022)***.

Following each qPCR, a melting curve analysis was carried out at 95 degree Celsius to evaluate the primer quality. To ascertain the target's relative quantification with respect to the reference, the $2^{-\Delta\Delta CT}$ method was applied (***Yang et al., 2017)***.

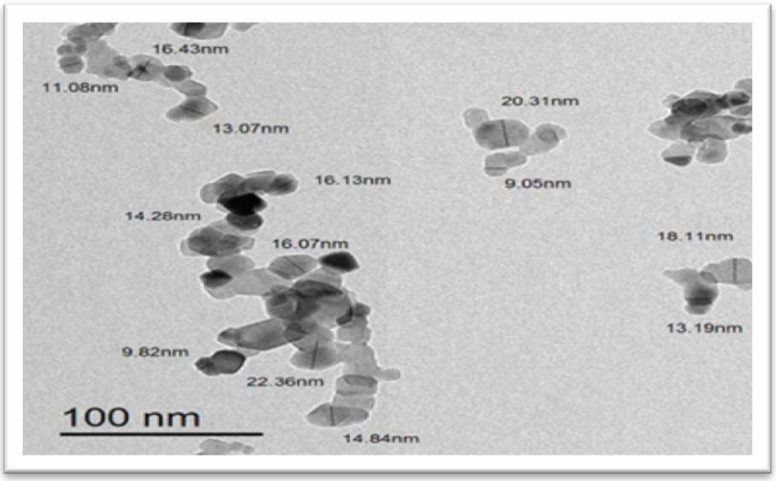


Figure (1): Transmission Electron Microscope (TEM) image showing TiO2 NPs size scale bar=100 nm.

Table (1): Primers' sequences used for quantitative reverse transcriptase- polymerase chain reaction (qRT-PCR) of the colon cell line.

Gene	Primer sequence (5´ –3´)	NCBI Reference
DNMT1	F: ACC AAG AAC GGC ATC CTG TA R: GCT GCC TTT GAT GTA GTC GG	NM_001379.4
DNMT3A	F: AGA AGC CGC TGT TAC CTC TT R: ATC TGC AAG CTG TCT CCC TT	NM_001320893.1
DNMT3B	F: CAA ACC CAA CAA CAC GCA AC R: ATC TTC CAG GCT GCT CTT GT	NM_001207056.2
GAPDH	F: CAC ATC GCT CAG ACA CCA TG R: TCC CGT TCT CAG CCA TGT AG	NM_001357943.2

* *F: forward primer; R: a reverse primer. DNMT1: DNA methyltransferase 1; DNMT3A: DNA methyltransferase 3 alpha; DNMT3B: DNA methyltransferase 3 beta; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; A: adenine base; G: guanine base; T: thymine base; C: cytosine base; NCBI: National Center for Biotechnology Information. NM: accession number links to the mRNA record in the Nucleotide database.*

STATISTICAL ANALYSIS:

The computerized SPSS version 22.0 (SPSS Armonk, NY: IBM Corp.) was used to tabulate, code, and analyze the data. After utilizing the Shapiro-Wilk test to verify normality, parametric data were presented using mean±SD deviation. The probability (p) value was used to assess the results' significance. A p-value of less than 0.05 was deemed statistically significant. When comparing more than two independent groups, the One Way ANOVA test was employed, and the Post Hoc Tukey test was utilized to identify pairwise comparisons. Using the R2 calculation, multiple linear regression was utilized to evaluate predictors of continuous normally distributed outcomes.

RESULTS

Cytotoxicity assessment results:

The application of TiO_2 NPs on Caco-2 cells revealed a dose-dependent cytotoxicity. The cell viability was decreased significantly with increased TiO_2 NPs concentration. (IC50) was (420.4 µg/ml) as shown in **table (2) and figure (2)**.

Evaluation of titanium dioxide nanoparticles translocation by transmission electron microscopic:

Ultrathin sections of group 1 (negative control group), which were not given medication or exposed to TiO2 NPs, revealed normal cell morphology. As demonstrated in **figure (3)**, the cells remained confluent with intact apical membranes. They were also appropriately organized and had uniform microvilli, without any TiO2 NPs present in the cells of the control group.

When ultrathin sections of group 3 were examined, titanium dioxide nanoparticles were found in vesicles, microvilli, and cells that were located beneath the apical membrane. In comparison to group 1, cells exposed to the TiO2 NPs group did not exhibit any discernible intracellular pathogenic alterations **(Figure 4)**.

Measurement of global 5-methylcytosine (global methylation):

When comparing group 3 and group 4 to group 1, the degree of DNA methylation was considerably lower in both cases ($p2<0.05$ and $p3<0.05$, respectively). Furthermore, compared to group 2, there was a statistically significant decrease in DNA methylation in groups 3 (treated with TiO2 NPs) and 4 (treated with DOX) ($p4 < 0.05$ and $p5 <0.05$, respectively). Nevertheless, concerning DNA methylation levels, there was no statistically significant difference between groups 1 and 2 ($p1=0.092$) or between groups 3 and 4 ($p6=0.292$) **(Figure 5)**.

Quantitative measurement of DNMT1, DNMT3A, and DNMT3B gene expression by reverse transcription PCR (gene expression analysis):

In all examined cell line groups, a comparison of the mean levels of DNMT1, 3A, and 3B gene expression was presented in **figure (6)**.

When compared to groups 1 and 2, it was found that DNMT1 gene expression levels were considerably higher in groups 3 and 4.

Nonetheless, there was no statistically significant difference in the DNMT1 gene expression levels between groups 1 and 2. Regarding DNMT3A gene, the expression levels were decreased significantly in groups 3 and 4 when compared with group 1.

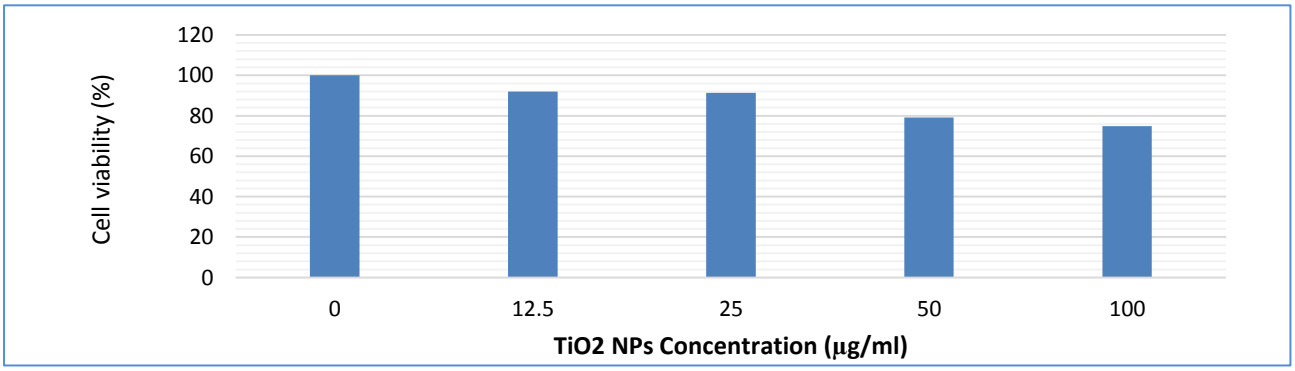
Also, there was a statistically significant decrease in expression levels of DNMT3A gene in groups 3 and 4 when compared with group 2.
Nonetheless, there was no statistically significant difference in DNMT3A expression levels between group 2 and group 1. Furthermore, groups 4 and 3 had DNMT3A expression levels that were comparatively low and did not show statistical significance from one another.

When compared to groups 1 and 2, the expression levels of the DNMT3B gene were considerably lower in groups 3 and 4.
Moreover, there was a statistically significant difference in expression levels of DNMT3B levels in group 2 when compared to group 1. However, DNMT3B expression levels in group 3 and group 4 were relatively low without statistical significance differences between them ($p < 0.05$).

Table (2): MTT assay using different concentrations of TiO2 NPs treated caco-2 cells:

Conc in µg/ml	Mean±SD	Viability	IC50 (µg/ml)	Overall Significance
0	0.364±0.01100	100	(420.4 µg/ml)	F= 52.995
12.5	0.335±0.00964	92.032967		P < 0.001*
25	0.332667±0.01069	91.391941		
50	0.288333±0.00473	79.212454		
100	0.272667±0.00651	74.908425		

Conc: concentration, SD: standard deviation. IC50: half maximal inhibitory concentration, µg: microgram, ml: milliliter. MTT: cytotoxicity assay, TiO2 NPs: titanium dioxide nanoparticles.



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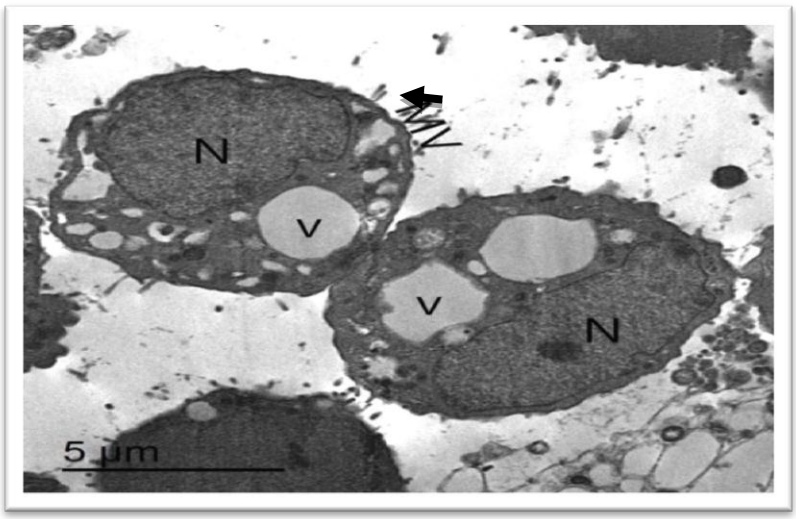

Figure (3): The electron micrograph of group 1 (the negative control group) reveals normal morphology within the cells. The cells maintain their confluency and adhere to the dishes with undamaged apical membranes. They are organized correctly and have uniform microvilli (MV), but they do not contain TiO2 NPs. Nucleus: N, vesicles: V (scale bar: 5 µm).

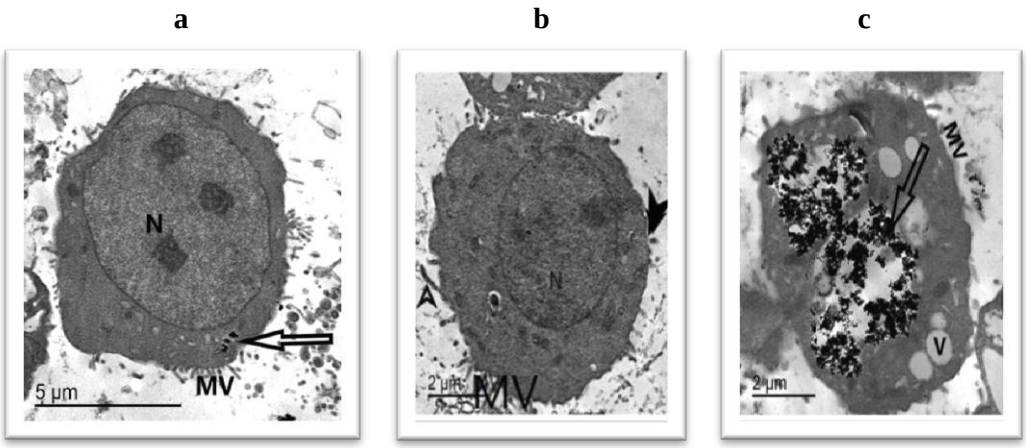

Figure (4): Group 3 (TiO2 NPs treated group) is shown in electron micrographs, where titanium dioxide nanoparticles were found in the following areas: (a) inside cells under the apical membrane (arrow; scale bar 5µm); (b) in microvilli (head arrow); (c) in vesicles (arrow); (scale bar 2µm). There were no discernible alterations in intracellular pathology between group 3 cells and group 1 cells. Microvilli is MV, while nucleus is N.

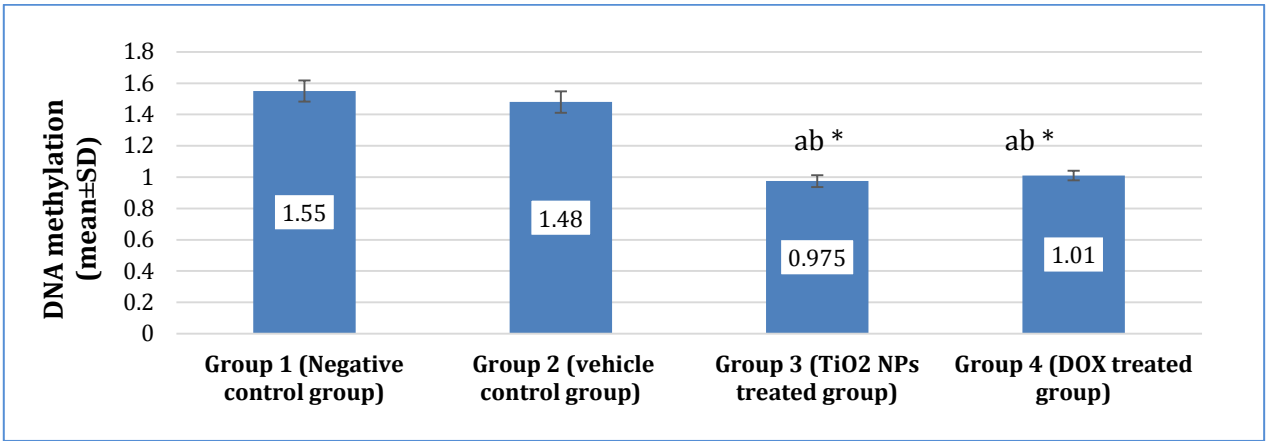

Figure (5): Comparison between mean values of global 5-methylcytosine in human epithelial colorectal adenocarcinoma cell lines (Caco-2 cells) in all studied cell line groups. a: statistical significance compared to negative control group, b: statistical significance compared to vehicle control group. *: significance at (p ≤0.05).

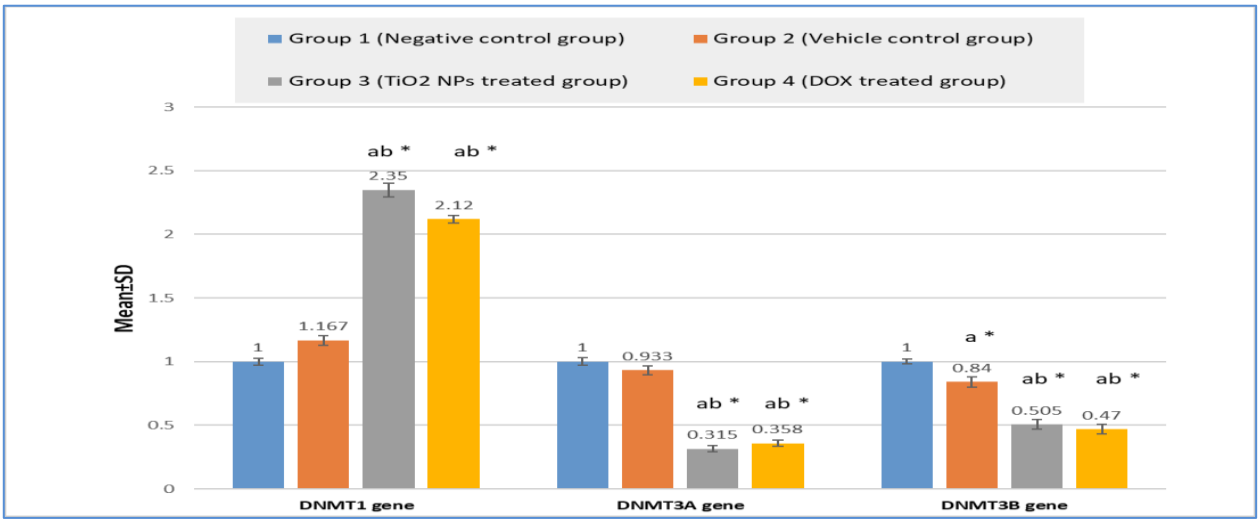

Figure (6): Comparison between mean levels of DNA methyltransferase 1 (DNMT1), DNA methyltransferase 3 alpha (DNMT3A), and DNA methyltransferase 3 beta (DNMT3B) gene expression among all studied cell line groups. *: significance at (p <0.05). a: statistical significance compared to the negative control group, b: statistical significance compared to the vehicle control group.

DISCUSSION

Even at low concentrations of titanium dioxide nanoparticles which are safe for cells, certain cells and tissues can experience epigenetic changes as shown by different research. These changes could lead to changes in the expression or activity of DNMTs as well as changes in global and gene-specific DNA methylation patterns ***(Wong et al., 2017; Hou et al., 2019)***.

Only a small number of these investigations have been published, even though exposure to TiO2 NPs has been demonstrated to change DNA methylation in multiple test systems ***(Tachibana et al., 2021)***. The current study aimed to assess the epigenetic modifications of the genome, specifically DNA methylation. Using the MTT assay, this study demonstrated dose-dependent cytotoxicity of TiO2 NPs on Caco-2 cells. 92 percent of the cells are viable at 12.5 µg/ml of TiO2 NPs. As the concentration of TiO2 NPs increase, there was a considerable drop in cell viability; at 100 µg/ml, 74.9% were viable cells.

Similarly, TiO2 NPs were discovered to be hazardous for lung fibroblast cells in India, according to ***Patil et al. (2016)***. According to the study's findings, 1 µg/ml was the highest non-toxic concentration for both NPs. Within 24 hours, there wouldn't be significant cytotoxicity at a sublethal dosage of less than 2 µg/ml. However, cell viability dropped to fifty percent at a lethal dose of TiO2 NPs larger than 2 µg/ml.

In a study by ***Malakootian et al. (2021)***, the researchers employed the MTT test for assessment of TiO2 NPs cytotoxicity, using a range of nanoparticle concentrations from 25 to 200 µg/ml. The outcomes demonstrated that cell viability was ninety-five at a concentration of 25 µg/ml and was not affected by above twenty percent at 100 µg/milliliter. At 200 µg/milliliter, on the other hand, cell viability dropped to sixty percent We can infer that TiO2 NPs exhibit notable dose-dependent cytotoxicity based on the mean difference in cell viability at various nanoparticle doses. It should be noted that differences in non-toxic concentrations among studies are primarily due to variations in the types of cells being studied ***(Ghosh et al. 2013)***.

Regarding the examination of ultrathin sections of group 3 by TEM, they showed titanium dioxide nanoparticles within Caco-2 cells under the apical membrane, within microvilli, and in vesicles, and no intracellular pathological differences were seen in group 3 relative to group 1.

In this context, previous research has indicated that aggregate TiO_2 NPs were present in cytoplasmic vesicles ***(Allouni et al., 2012; Zhu et al., 2012; Halamoda-Kenzaoui et al., 2019)***.

Vales et al. (2015) study showed TiO2 NPs were taken up by BEAS-2B cells, which are non-tumorigenic lung epithelial cells originating from human lung tissue, as shown by TEM.

Similar to this, ***Freire et al. (2021)*** in Argentina looked at how TiO2 NPs internalized in the human lung cancer cell line (A549) and found that the particles were inside membrane-bound vesicles. However, it was shown by ***Koeneman et al. (2010)*** in the USA and ***Fisichella et al. (2012)*** in France that TiO2 NPs were not internalized in Caco-2 cells.

Furthermore, the degree of DNA methylation was decreased significantly after exposure to TiO_2 NPs and DOX than group 1. According to a recent Iranian study by ***Malakootian et al. (2021)***, human PBMCs exposed to TiO2 NPs at different doses (25, 50, and 100 µg/milliliter) showed lower levels of DNA methylation than negative control samples. The study found that at non-toxic doses, TiO2 NPs can induce epigenetic modifications and DNA hypomethylation. These results highlight the possible harm that TiO2 NPs may cause to people's health.

Pogribna et al. (2020) observed that DNA methylation in four different cell lines (Caco-2, HepG2 (liver), NL20 (lung), and A-431 (skin)) was affected by titanium dioxide nanoparticles (at a concentration of 100 µg/milliliter for 24 and 72 hours). The results of the study assertively demonstrate that TiO_2 NPs exposure leads to decreased DNA methylation in all cell lines tested.

In accordance, lung fibroblast cells exposed to TiO2 NPs showed reduced global DNA methylation, per a study by ***Patil et al. (2016)*** in India. Similarly, human bronchial and

alveolar epithelial cell lines showed decreased global methylation levels following treatment with TiO2 NPs, according to research by ***Ma et al. (2017)***.

The methylation state of genomic DNA may be affected differently by exposure to varied concentrations of NPs, according to a hypothesis based on research done by ***Lee et al. (2023)***. This could therefore lead to varying degrees of toxicity at varying dosages and a response from cells. However, there is no statistical significance in the methylation status concerning the time of incubation. According to the research, NP exposure causes a decrease in DNA methylation as soon as 24 hours have passed. Further methylation decrease does not occur with further exposure over time.

Ménézo et al. (2016) reported a noteworthy link between methylation processes, epigenesis, and oxidative stress, which ultimately results in a decrease in DNA methylation. Oxidative stress happens when defense mechanisms fail to effectively protect biological material and DNA from the formation of ROS. DNA methylation is hampered by oxidative stress, as discovered in 2009 by ***Tunc and Tremellen.***

One important metabolic route that controls methylation recycles homocysteine, and governs glutathione synthesis is the one-carbon cycle. This route plays a critical role in both DNA methylation and oxidative stress; hence it is important to address ***(Menezo et al., 2016).***

Oxidative stress can harm the body's antioxidant system. GSH and GSTs are essential parts of the body's defense mechanisms against this. Using GSH, GSTs catalyze nucleophilic substitutions in the nitrogen, sulfur, or electrophilic carbon atoms of toxic compounds. This finally protects the organism from injury by preventing the hazardous substances from reacting with essential cellular proteins and nucleic acids ***(Josephy, 2010)***.

In several in vitro studies conducted in Sweden, the USA, Singapore, and Spain, the use of engineered nanoparticles has been associated with various biological effects. These findings were reported by ***Costa and Fadeel*** in ***(2016); Hardy and Tollefsbol*** in ***(2011); Khanna et al.*** in ***(2015)*** and ***Lozano-Fernández et al.*** in ***(2014)***.

In the current study, exposure to TiO_2 NPs in Caco-2 cell lines also altered expression levels of DNMT1, 3A, and 3B. DNMT1 gene expression levels showed a significant increase in group 3 (TiO_2 NPs treated group) and group 4 (DOX treated group) when compared with group 1 and group 2.

DNMT3A gene expression levels showed a significant decrease in group 3 and group 4 when compared with group 1. The expression levels of DNMT3B gene were reduced significantly in group 3 and group 4 compared with group 1.

Consistent with previous findings, the levels of DNMT3B expression were reduced in A549 cells. However, after exposure to TiO_2 NPs, the expression level increased in 16-HBE cells, as reported by ***Ghosh et al. (2017)***. Similarly, in a study conducted by ***Pogribna et al. (2020)*** in the USA, it was found that the expression levels of DNMT1 and 3A were decreased in Caco-2 cells treated with TiO_2 NPs (100 mg/mL for 24 and 72 hours) compared to the negative control group. However, the expression levels of DNMT3B remained unchanged.

In a study conducted in India, it was found that treating lung fibroblast cells (MRC5) with TiO_2 NPs led to a decrease in the expression levels of DNMT1, 3A, and 3B genes ***(Patil et al., 2016)***. Similarly, in a study conducted in China, it was found that porcine embryos treated with 1.5% DMSO showed a significant decrease in the expression levels of DNMT1, 3A, and 3B. At the same time, global methylation signals increased ***(Cheng et al., 2022)***.

Alterations in both global and gene-specific DNA methylation patterns can occur by TiO2 NPs exposure. It can also impact the expression or activity of DNMTs, which are responsible for modifying the DNA methylation pattern. It is noteworthy that the changes in DNA methylation can vary based on the type of nanoparticles and cells that are being used ***(Hou et al., 2019)***.

DNA methylation is a vital epigenetic mechanism that relies on (DNMTs) for its establishment and maintenance. There are three types of DNMTs involved in this

process, namely DNMT1, 3A, and 3B. DNMT1 functions as a maintenance methylase, targeting hemi-methylated DNA strands. Meanwhile, DNMT3A and 3B are responsible for de novo methylation and the establishment of new methylation, respectively ***(Hervouet et al., 2018)***. Nevertheless, our study has some limitations. The first is the short-term run of the experiment (72 hours). At the same time, in real conditions, exposure to titanium dioxide nanoparticles containing products is for several months or years which may lead to more DNA affection. The second limitation is that exposure of Caco-2 cells to TiO_2 NPs just once not at different times as DNA changes may be reversible as reported in some studies.

CONCLUSION

This study determined the possible toxicity of TiO2 NPs on DNA methylation. The most frequent finding was global hypomethylation. Significant changes were also seen in the expression levels of the DNA methyltransferases DNMT1, 3A, and 3B. The results of this study add to the growing research by showing that TiO2 NPs affect the methylation status of cells.

RECOMMENDATIONS

- Restricting the use of products containing TiO2 NPs to those that are necessary.
- Wear gloves and protective clothing when handling products that contain TiO2 nanoparticles, particularly when performing occupational work.
- Organising health campaigns to enlighten the public about items containing TiO2 NP and their harmful consequences.
- Additional research on locating additional genes impacted by DNA methylation modifications in response to TiO2 NPs will be required.

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Prof. Doaa Abdel Wahab and Dr. Linah Hassan Ali Habl conceived of the presented idea. Dr. Linah Hassan Ali Habl carried out the experiment. Dr. Dalia Alsaied Moustafa Ahmed wrote the manuscript. Dr. Mahmoud El-Sayed Awad and Prof. Doaa Abdel Wahab Ahmed El Morsi helped supervise the manuscript.

Data availability statement:
The data supporting the study conclusions are accessible within the journal, according to the authors.

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