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Research Article

Cellular Interactions and Biological Responses to Titanium Dioxide Nanoparticles in HepG2 and BEAS-2B Cells: Role of Cell Culture Media

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We showed previously that exposure of human lung cells (BEAS-2B) to TiO_2 nanoparticles (nano-TiO_2) produced micronuclei (MN) only when the final concentration of protein in the cell-culture medium was at least 1%. Nanoparticles localize in the liver; thus, we exposed human liver cells (HepG2) to nano-TiO_2 and found the same requirement for MN induction. Nano-TiO_2 also formed small agglomerates in medium containing as little as 1% protein and caused cellular interaction as measured by side scatter by flow cytometry and DNA damage (comet assay) in HepG2 cells. Nano-TiO_2 also increased the activity of the inflammatory factor NFkB but not of AP1 in a reporter-gene HepG2 cell line. Suspension of nano-TiO_2 in medium containing 0.1% protein was sufficient for induction of MN by the nanoparticles in either BEAS-2B or HepG2 cells as long the final concentration of protein in the cell-culture medium was at least 1%. Environ. Mol. Mutagen. 55:336–342, 2014.

Key words: micronucleus; comet assay; flow cytometry

INTRODUCTION

Titanium dioxide nanoparticles (nano-TiO_2) have been used as an additive to sunscreens, paints, toothpastes, and food coloring due to their small size, white pigmentation, resistance to degradation, and high refractive index [Weir et al., 2012]. Nano-TiO_2 has produced inconsistent biological effects in vitro as well as in vivo. The majority of studies on nano-TiO_2 toxicity in cultured human cells have found the induction of cytotoxicity, genotoxicity, inflammation, and reactive oxygen species (ROS) [Iavicoli et al., 2011; Petković et al., 2011]. However, other studies have reported that nano-TiO_2 did not induce either DNA damage in human peripheral blood lymphocytes or DNA strand breaks in human lung cells [Bhattacharya et al., 2009; Hackenberg et al., 2011]. In vivo studies of nano-TiO_2 in the drinking water of mice reported the induction of 8-hydroxy-2′-deoxyguanosine adducts, micronuclei (MN), gamma-H2AX foci, and DNA deletions in the bone marrow and liver [Trouiller et al., 2009]. On the other hand, nano-TiO_2 failed to induce DNA strand breaks as assessed by the comet assay in rats after intra-tracheal exposure [Naya et al., 2012] and MN in mice after intravenous injection [Sadiq et al., 2012].

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Bio-distribution studies of nano-TiO_2 in vivo have indicated that the liver is a primary target organ of exposure after oral administration as well as after intraperitoneal or intravenous injection, making liver an important focus for nanotoxicity studies [Wang et al., 2007; Fabian et al., 2008; Chen et al., 2009]. Nano-TiO_2 exposure in vivo has resulted in liver dysfunction, inflammation, oxidative damage, hepatocyte apoptosis, and gene expression of associated response pathways [Liu et al., 2010; Cui et al., 2012].

In a previous study [Prasad et al., 2013b], we found that nano-TiO_2 suspended in a serum-free medium (KGM) supplemented with either 0.1% bovine serum albumin (BSA) or 10% fetal bovine serum (FBS) produced different agglomeration profiles, with the FBS-supplemented KGM eliciting smaller agglomerates than that supplemented with BSA. Additionally, the smaller particles in the medium supplemented with FBS were associated with increased cellular interaction, MN induction, and an increased fraction of cells in S-phase compared with the medium supplemented with BSA in human lung epithelial (BEAS-2B) cells. Although both media facilitated a similar induction of DNA damage as measured by the comet assay, MN were induced by the nano-TiO_2 only when the final concentration of protein (FBS) was at least 1% in the culture medium [Prasad et al., 2013b].

Given the variable results in the literature with nano-TiO_2, we investigated in this communication whether the association between protein concentration in the treatment medium, agglomeration, cellular interaction, and genotoxicity in BEAS-2B cells observed previously [Prasad et al., 2013b] would also be found in HepG2 cells. Thus, we prepared nano-TiO_2 dispersed in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 0.1% BSA (referred to as DB) and DMEM supplemented with 10% FBS (referred to as DF) and measured the same endpoints in HepG2 cells as we had in BEAS-2B cells. Because DMEM is used to culture HepG2 cells, but KGM for BEAS-2B cells, we also explored whether differences in medium composition (beyond protein concentration) influenced the biological response of these two cell lines to nano-TiO_2. Finally, we also evaluated nano-TiO_2 for induction of inflammatory factors (NFkB and AP1) in a HepG2 cell line containing luciferase-reporter genes as we had done previously with silver nanoparticles [Prasad et al., 2013a].

MATERIALS AND METHODS

Chemicals

We purchased TiO_2 nanoparticles (86% anatase and 14% rutile as listed by the manufacturer) from Degussa (now Evonik, AEROXIDE® TiO_2 P25, Parsippany, NJ), and we sonicated the particles using a probe sonicator (Cole Parmer, Vernon Hills, IL). We characterized the nanoparticles as described previously [Prasad et al., 2013b]; we purchased glucose and potassium chloride from Sigma-Aldrich (St. Louis, MO).

Particle Preparation and Characterization

We suspended nano-TiO_2 in DMEM supplemented with 0.1% BSA (DB) or DMEM supplemented with 10% FBS (DF), respectively, at 1 mg/ml and probe sonicated at 7 W for 2 min on ice. Likewise, we suspended the particles in KB or KB-Plus, which were composed of keratinocyte growth medium (KGM) supplemented with 0.1% BSA or KGM supplemented with 3.5 g/l glucose, 3.87-mM KCl, and 0.1% BSA, respectively. We made subsequent dilutions of nano-TiO_2 suspended in DB, DF, KB, and KB-Plus media to yield 50 and 100 μg/ml for treatment.

Cell Culture and Treatments

We maintained HepG2 cells (ATCC, Manassas, VA) in DMEM + 10% FBS and BEAS-2B cells (ATCC) in serum-free keratinocyte basal medium supplemented with KGM SingleQuots (Lonza). We assessed cell viability by the CellTiter-Blue® Cell Viability Assay (Promega, Madison, WI) with modifications [Prasad et al., 2013b]. This assay is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin). Nonviable cells rapidly lose metabolic capability and, thus, do not generate a fluorescent signal.

Flow Cytometry

We determined the side scatter (i.e., cellular interaction) of nano-TiO_2 by flow cytometry in both cell lines in three independent experiments as described previously [Prasad et al., 2013b; Zucker et al., 2013]. Side scatter can result from cellular uptake of the nanoparticle, binding of the particle to the cell surface, or association of the particle within the cell membrane. We have used the term cellular interaction to encompass all of these possibilities. However, dark-field microscopy showed that nano-TiO_2 were most likely inside BEAS-2B cells and other cells as demonstrated in our previous studies [Prasad et al., 2013b; Zucker et al., 2013]. The alignment and calibration of the flow cytometer was assessed by measuring the coefficient of variation (CV) using 2- to 3-μm polystyrene beads [Zucker et al., 2013].

Comet, Cytokinesis-Blocked MN, and Reporter-Gene Assays

We performed assays for comets and MN as described previously [Prasad et al., 2013b], doing three independent experiments for MN and two for the comet assay in both HepG2 and BEAS-2B cells for each nano-TiO_2 concentration tested in each treatment medium for 24 h. We also determined the cytokinesis-blocked proliferation index (CBPI), which is a measure of cytostasis and an additional indicator of cytotoxicity [Prasad et al., 2013b]. We used a HepG2 cell line containing luciferase-reporter genes to measure transcriptional activation of the inflammatory factors nuclear factor-kappa-light-chain-enhancer of activated B cells (NFkB) and activator protein 1 (AP1) as described previously for studies we did with silver nanoparticles [Prasad et al., 2013a].

Statistical Analyses

For the cytotoxicity and reporter-gene assays, we normalized the data to in-plate vehicle controls and presented the results as the mean fold change over vehicle + standard error of the mean. We fitted the concentration-responses to four-parameter, non-parametric curves using a least squares (ordinary) fit method with GraphPad Prism 5 (San Diego, CA). For reporter-gene, comet, MN, and CBPI assays, we performed the

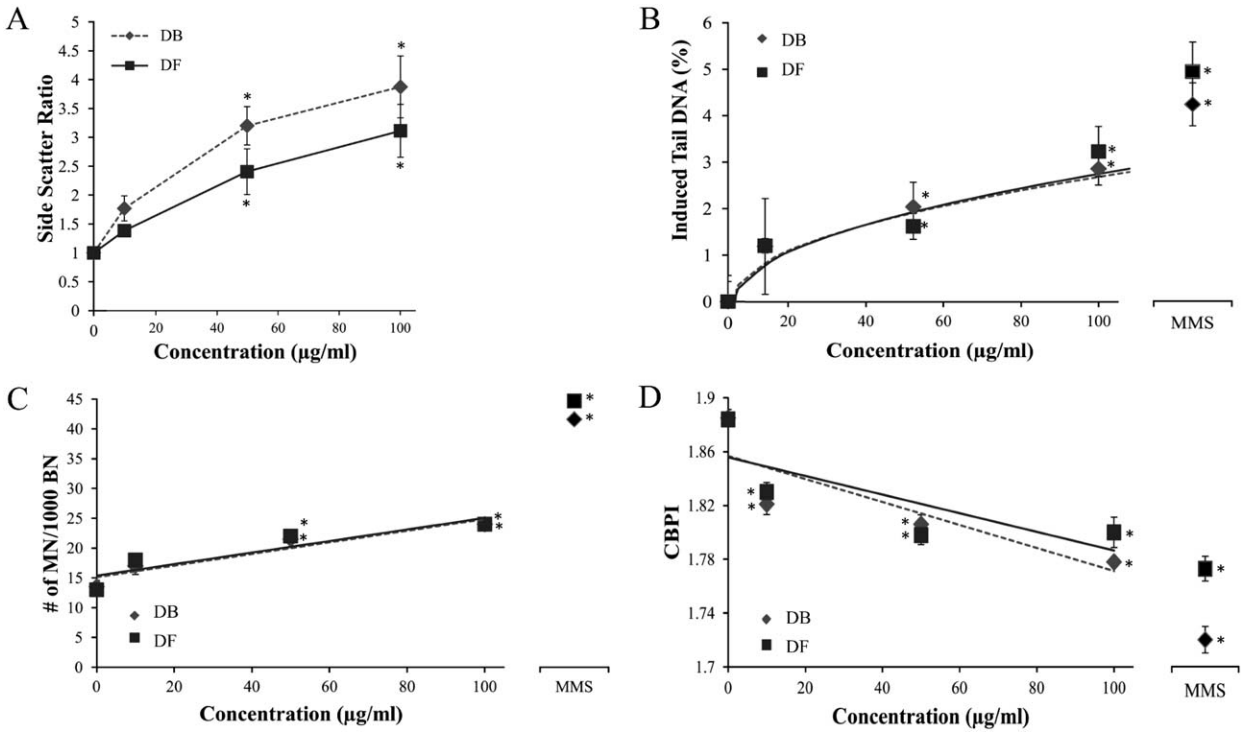

Fig. 1. Characterization of nano-TiO_2 in DB and DF media after exposure of HepG2 cells for 24 hr. **A**: Cellular interaction was assessed by flow cytometry; the side-scatter ratio was calculated as the mean of the treated histogram/mean of the untreated histogram. Data are expressed as mean ± SD. DNA damage induced by nano-TiO_2 assessed by the (**B**) comet and (**C**) MN assays; (**D**) shows the CBPI, which is a measure of cell-cycle progression. The positive control was 100-µM MMS. For the comet assay, the exposure to the positive control was for 1 hr, whereas it was 24 hr for the MN assay and CBPI. Data are expressed as mean ± SD; $^*P < 0.05$ from untreated control, $n = 3$ for comet assay, and $n = 2$ for MN assay and CBPI.

statistical analyses as described previously [Prasad et al., 2013b]. We presented the results as the mean ± SD, and we used a one-tailed analysis of variance (ANOVA). Results were considered statistically significant if $P < 0.05$. If the ANOVA analysis resulted in a significant effect, then we performed a one-tailed Dunnett's test to compare each concentration to the control, which considers multiple comparisons. We performed a similar analysis for the side-scatter ratio (where the control was set at 1), again using a multifactor ANOVA followed by a one-tailed Dunnett's test.

RESULTS

Physicochemical Characteristics

The hydrodynamic diameter (a measure of protein agglomeration), the polydispersion index (a measure of the variance in size measurements), and the zeta potential (a measure of electrokinetic potential indicating the degree of repulsion between particles) showed no significant differences relative to increasing concentrations of nano-TiO_2 or between the two particle-suspension media (data not shown).

Viability in HepG2 Cells

No concentration of nano-TiO_2 tested in either treatment medium elicited a significant decrease in viability of the HepG2 cells compared with the untreated controls using the Cell-Titer Blue® Cell Viability Assay (data not shown). The positive control cytotoxicant, $AgNO_3$, elicited significant decreases in cell viability from untreated controls at all concentrations tested, with a calculated $IC_{50} = 0.3643$ µg/ml.

Cellular Interaction in HepG2 Cells

We assessed cellular interaction by measuring the side-scatter ratio by flow cytometry of treated to untreated cells. Figure 1A shows that an increasing concentration of nano-TiO_2 caused a significant increase ($P < 0.05$) in the side-scatter ratio in both media relative to the controls; however, there was no significant difference in this ratio between the two media.

Comet Assay in HepG2 Cells

As depicted in Figure 1B, DNA damage as measured by the comet assay in HepG2 cells was increased significantly with increasing concentrations of nano-TiO_2 in both treatment media, indicating a genotoxic effect (DB: $P = 0.0029$; DF: $P = 0.0002$). The responses at the two

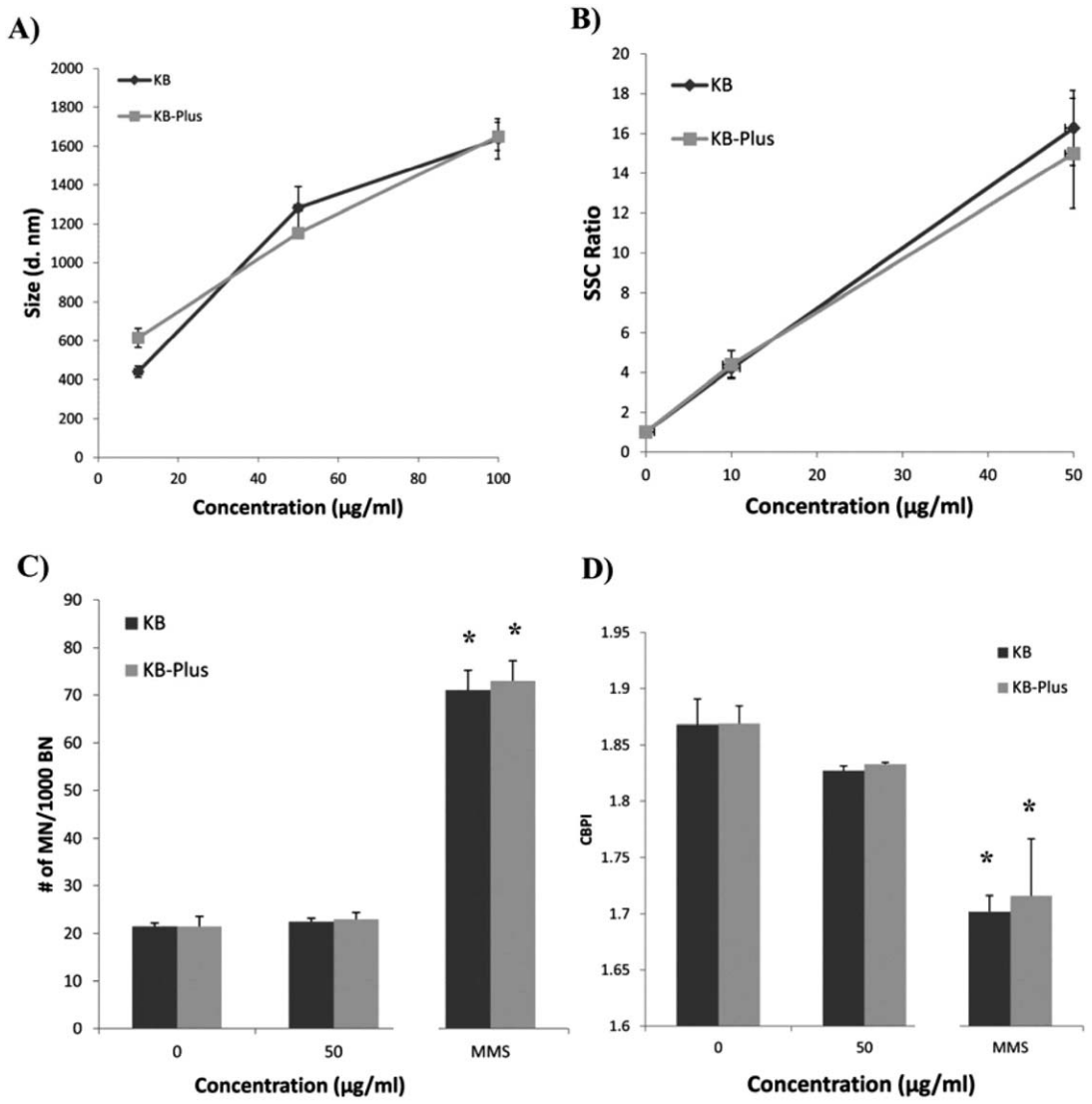

Fig. 2. The effects of glucose and ionic concentrations in cell-culture medium on agglomeration, cellular interaction, and genotoxicity in BEAS-2B cells. Nano-TiO_2 dispersed in KB and KB-Plus were examined for (**A**) size by dynamic light scattering of nano-TiO_2, (**B**) cellular interaction in BEAS-2B cells as assessed by side-scatter ratio by flow cytometry, (**C**) MN formation, and (**D**) CBPI. The positive control used for MN formation and CBPI experiments was 100-μM MMS. Data are expressed as mean ± SD, $n = 3$ for dynamic light scattering flow cytometry, and $n = 2$ for MN and CBPI; $^*P < 0.05$ from untreated control.

highest concentrations were significantly greater than the control; however, the type of medium used did not influence the level of DNA damage.

Cytokinesis-Blocked MN Assay in HepG2 Cells

MN frequencies were increased significantly with increasing concentrations of nano-TiO_2 in both treatment media (Fig. 1C, DB: $P = 0.0004$; DF: $P < 0.0001$). All concentrations produced frequencies of MN that were significantly higher than the control, and the CBPI was decreased at all concentrations, indicating a slowing of cell-cycle progression with increasing concentration of nano-TiO_2 (Fig. 1D).

Agglomeration, Cellular Interaction, and MN with KB and KB-Plus in BEAS-2B Cells

To examine the differences between KB medium used for BEAS-2B cells from our previous study and DB used in this study with HepG2 cells, we supplemented KGM medium with additional glucose (3.5 g/l) and KCl (3.87 mM) at concentrations similar to those found in DMEM to create KB-Plus medium. The dynamic light-scattering measurements (Fig. 2A), side-scatter ratio (cellular interaction) (Fig. 2B), MN (Fig. 2C), and CBPI (Fig. 2D) were not significantly different between the two media with BEAS-2B cells. The results with KB medium on all parameters measured were consistent with our previous study [Prasad et al., 2013b].

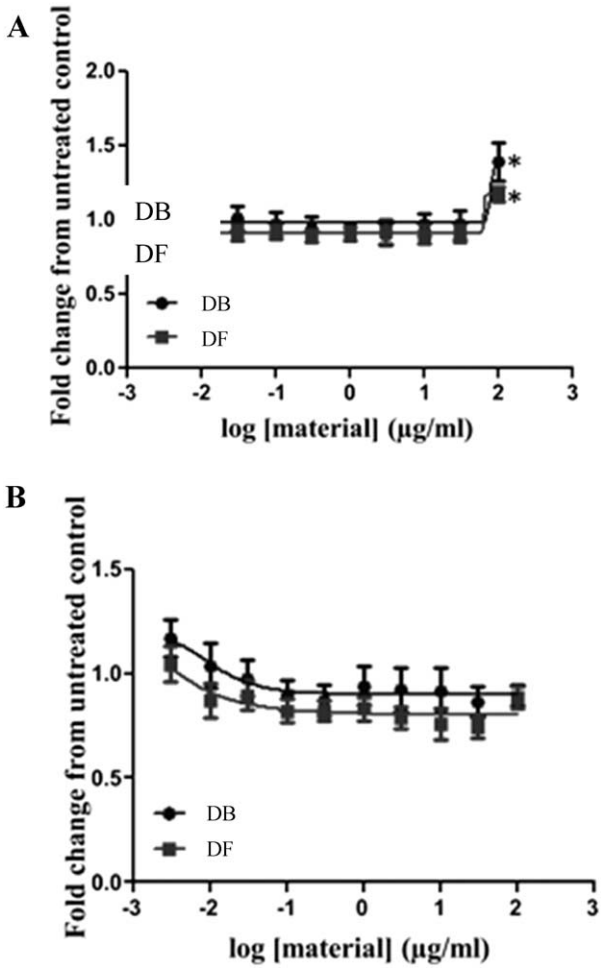

Fig. 3. Inflammatory responses of HepG2 cells after exposure to nano-TiO_2 for 24 hr; transcriptional activation of (**A**) NFkB and (**B**) AP1. For both luciferase reporter-gene assays, hydroquinone (HQ) was used as a positive control (data not shown). Data are expressed as mean ± SE; *$P < 0.05$ from untreated control, $n = 3$ for each experiment.

Transcriptional Activation of NFkB and AP1

In Figure 3A, 100 μg/ml of nano-TiO_2 increased NFkB activity in HepG2 cells in both DB and DF media compared to untreated control cells ($P < 0.05$). However, there was no significant difference in activity between cells in the two media ($P = 0.07$). There was no significant transcriptional activation of AP1 in HepG2 cells exposed to nano-TiO_2 for 24 hr (Fig. 3B). The positive control for these experiments, hydroquinone (HQ), activated significantly both NFkB and AP1 relative to untreated control cells (data not shown).

DISCUSSION

The liver represents an important target organ for engineered nanoparticles entering an organism through a variety of exposure routes. In this study, we set out to measure cellular interaction and biological responses (cytotoxicity, genotoxicity, DNA damage response, and inflammation) in HepG2 cells after exposure to nano-TiO_2 in DB and DF media. Although nano-TiO_2 did not induce cytotoxicity in either medium, the particles were absorbed in both media and interacted with the HepG2 cells in both treatment media as indicated by flow cytometry data, inducing DNA damage, MN induction, and activation of NFkB but not AP1.

The agglomeration, cellular interaction, cytotoxicity, and genotoxicity of nano-TiO_2 in HepG2 cells have been studied by others; however, no single study has concurrently examined all parameters. For example, Lankoff et al. [2012] found that nano-TiO_2 formed agglomerates of various sizes depending on the treatment protocol, with smaller agglomerates having higher cellular uptake than larger agglomerates. The authors also showed that cell type (lung, liver, or monocytes) influenced the biological effects of nano-TiO_2. Shukla et al. [2013] showed that nano-TiO_2 induced a wide variety of endpoints beyond MN in HepG2 cells, including reduced cellular glutathione levels as well as increased lipid peroxidation, ROS generation, and expression of p53, BAX, Cyto-c, Apaf-1, caspase-9, and caspase 3. A discussion of how these and other variables may affect health risks from nano-TiO_2 is beyond the scope of this paper; however, as reviewed by Skocaj et al. [2011], a number of issues require further clarification to understand better the safety of nano-TiO_2.

In our study, we have confirmed several of the effects shown in these previous studies, where small agglomerates of nano-TiO_2 induced MN as long as the medium contained a sufficient concentration of protein. We also found that nano-TiO_2 activated NFkB equally in both media, but nano-TiO_2 did not activate AP1 in either media. We note that nano-TiO_2 activated NFkB only at the highest concentration (100 μg/ml). Gangwal et al. [2011] suggested that in vitro concentrations in the range of 30–400 μg/ml were representative of a 45-year working lifetime exposure; however, these concentrations are well above the average exposure scenario [Oberdorster, 2012]. In addition to addressing the in vitro toxicity of nano-TiO_2, it will also be important to extrapolate in vitro exposure concentrations to concentrations experienced by people.

Nano-TiO_2 did not elicit a significant decrease in cell viability in HepG2 cells in either medium at any concentration tested. This was similar to our previous study in BEAS-2B cells, where we saw no significant decreases in cell viability after a 24-hr exposure to nano-TiO_2 in three treatment media [Prasad et al., 2013b]. Studies using other cell types, such as A549 cells, have displayed loss of viability in response to nano-TiO_2 treatment [Lanone et al., 2009; Iavicoli et al., 2011; Lankoff et al. 2012]. Cell type differences likely play a role in the manifestation of nano-TiO_2 toxicity; therefore, choice of the model

TABLE I. Summary of Data on the Role of Protein Concentration on Genotoxicity of TiO_2

		Concentration (%) and type of protein			
Cells	Medium	Particle-suspension medium	Final in cell-culture medium	Comet	MN
BEAS-2B[a]	KGM	0.1% BSA	0.01% BSA	+	−
		10% FBS	1% FBS	+	+
BEAS-2B[b]	KGM	0.1% BSA	0.01% BSA	+	−
		0.1% BSA-Plus	0.01% BSA-Plus	+	−
HepG2[b]	DMEM	0.1% BSA	1% (1 part BSA:100 parts FBS)	+	+
		10% FBS	10% FBS	+	+

[a]Data from Prasad et al. [2013b].
[b]Data from current study.

system will dictate what effects, if any, will be observed following nano-TiO_2 exposure.

Previous research with gold nanoparticles found that ion and glucose concentrations may impact the nano-bio interface and subsequent cellular responses [Maiorano et al., 2010]. However, we did not find that altered concentrations of these components affected the genotoxicity of nano-TiO_2. Nonetheless, a study by Xu et al. [2012] investigated the selective binding of the components of DMEM, DMEM supplemented with FBS, and PBS to zinc oxide and copper oxide nanoparticles. They identified the formation of nanoparticle-bio complexes, independent of proteins, resulting from the adsorption of medium components, including ions such as Na^+, K^+, and Cl^-.

Table I summarizes the data from our current and previous study [Prasad et al., 2013b]. Collectively, our results show that nano-TiO_2 suspended in at least 0.1% protein and placed in cell-culture medium having a final concentration of at least 1% protein facilitated the production of MN in both BEAS-2B and HepG2 cells; however, a final protein concentration in the culture medium of 0.01% did not. Our data do not permit us to infer whether lung and liver cells in vitro are differentially susceptible to induction of MN by nano-TiO_2 because of the different types of media required to grow the two cell types; nor for the same reason did our data permit us to address the quality of protein (FBS vs. BSA) required for induction of MN. Nonetheless, differences between cell types may account for some differences in biological response to nano-TiO_2 [Lankoff et al., 2012].

In conclusion, exposure for 24 hr of HepG2 or BEAS-2B cells to nano-TiO_2 resulted in increased cellular interaction as measured by side scatter using flow cytometry, DNA damage in the comet assay, MN induction, and transcriptional activation of NFkB but not AP1 when the particles were suspended in at least 0.1% protein and the culture medium contained a final concentration of at least 1% protein. Medium containing a final protein concentration as low as 0.01% did not permit the induction of MN by nano-TiO_2. Further studies are needed to clarify whether other factors influence the genotoxicty of nano-TiO_2.

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AUTHOR CONTRIBUTIONS

RP, RF, CB, RZ, and DD designed the study; RP, MK and RZ collected the data; RR, SS, RZ, and AK analyzed the data; RP and SS prepared draft figures and tables; All authors contributed to the writing of the manuscript based on their specific expertise. All authors approved the final manuscript.

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