ORIGINAL ARTICLE

The cytotoxicity effect of bismuth oxide particles synthesized hydrothermally using different reaction temperatures *in vitro*

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ABSTRACT

Introduction: Bismuth oxide (Bi_2O_3) particles gained attention in preclinical research especially in medical imaging. Bismuth oxide with its long circulation time is an alternative to the current iodine contrast media which directly possesses high X-ray attenuation coefficient. Exploration of bismuth compound is hampered owing to challenges in synthesizing control for in vivo stability.

Materials and Methods: This study aimed are to characterize Bi2O3 particles synthesized at 60, 90 and 120 °C via hydrothermal method and investigated cytotoxicity of cell viability assay, cell morphology analysis, intracellular reactive oxygen species (ROS) assay and expression of ER stress genes by real-time PCR.

Results: Results indicated that the size of rod-shaped Bi_2O_3 particles increased with rising synthesizing temperatures. The cytotoxicity of Bi_2O_3 particles in Chang liver cells was size-dependent. Bigger-sized Bi2O3 particles resulted in lesser toxicity effects. mRNA expressions of GRP78 and C/EBP homologous protein (CHOP) were down-regulated in all treated Chang liver cells due to the increasing size of Bi_2O_3 particles. Bi_2O_3 particles synthesized at 120 °C was found to be less toxic than iodine.

Conclusion: Data suggested that the response of Chang liver cells to Bi_2O_3 particle cytotoxicity has a significant relationship with its reaction temperatures. This outcome is important in hazard assessment of Bi_2O_3 particles as a new contrast media and provides better understanding in synthesizing control to enhance its biocompatibility.

KEYWORDS:
Bismuth oxide, hypothermal, cytotoxicity, Chang liver cells

INTRODUCTION

Nanotechnology in medicine could be mankind's giant leap towards having better diagnosis and prognosis of diseases. Due to current limitations of the existing contrast media, researchers focused on searching and fabricating for more compatible metal-based particles relevant in this context.[1] Several compounds of metallic elements with high atomic numbers such as gold, iron, silver and bismuth (Bi) were investigated as potential contrast media. Among all these, bismuth (Bi) is known to have high atomic number (Z = 83) with strong X-ray attenuation power and thus seen as a suitable candidate to replace iodinated contrast media.[2]

Bismuth sulphide nanodots have gained special attention as contrast media due to its characteristics including low toxicity, strong X-ray attenuation power and low price. This material was successfully prepared in a big scale and targeted and used to contrast breast cancer.[3] Bismuth selenide nanoplatlets were also employed for photodynamic treatment of cancer using animal model and bismuth oxide/oxychloride nanotubes were used in stem cell imaging. Unlike bismuth, gold nanoparticles already known with its ability to synthesise, size-control and morphology. Therefore, these factors have caused limitations to bismuth expansion being used as contrast media.[4] The ongoing researches on bismuth modification serve as contrast media have produced nano and micro-sized bismuth particles with various physicochemical properties. The vast differences in physicochemical properties between smaller size particles with bulk material have sparked concerns and debates about its potential risk to human health.[5] Shape, zeta potential, surface chemistry and particle solubility depend on biocompatibility variables, including cytotoxicity, reticuloendothelial system (RES) recognition, cellular uptake and clearance. In-depth understanding of nano and micro-sized particles physicochemical properties and behaviour in biological environment are crucial if better contrast media are to be designed.[6]

Nevertheless, biocompatibility and possible toxicity related to synthesization method and physicochemical properties of a particle remains a challenge. The hydrothermal method is commonly used in synthesizing Bi nanostructures; however, this method is only able to synthesize nanoparticles with wide size distribution and poor mono-dispersity.[7] Yang et al.[8] highlighted the morphology of Bismuth Oxide (Bi_2O_3) particles synthesized by using the hydrothermal method can

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be controlled by reaction temperature. The diameter of Bi_2O_3 particles can be increased by raising the reaction temperature.[8]

Bismuth metal is considered to be non-toxic, but bismuth-induced encephalopathy is still being reported. Turkez et al.[9] reported that 5.0 mg/L of Bi_2O_3 is able to induce oxidative stress in blood. Meanwhile administration of 100 mg/L colloidal bismuth subnitrate can induce liver damage *in vivo*.[10] Abudayyak, Öztaş, Arici, & Özhan[11] recently reported Bi_2O_3 particles induced cytotoxicity effect in mammalian cells via generation of reactive oxygen species (ROS) which leads to increased oxidative stress. However, the relationship between generated ROS by Bi_2O_3 particles and detailed mechanism of endoplasmic reticulum (ER) stress has not been clearly determined. Therefore, ER stress was used in this study as an early biomarker to provide new insights toward understanding Bi_2O_3 particles cytotoxicity *in-vitro*.

The aim of this study is to investigate the cytotoxicity mechanism and the effect of different reaction temperatures used to synthesize the Bi_2O_3 particles. The study outcomes should focus towards minimizing the toxicity risks of Bi_2O_3 particles and so that it as a potential contrast media.

MATERIALS AND METHODS

Chemicals and Reagents

Minimum Essential Media (MEM) for cell culture medium was purchased from *Nacalai Tesque* Inc., Japan. Fetal Bovine Serum and 1X TrypLE express enzyme were purchased from Gibco. 100 X Penicillin-Streptomycin was purchased from BioWest, USA. Phosphate buffer saline (PBS) tablets, dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. OxiSelect™ Intracellular ROS Assay Kit Green Fluorescence was purchased from Cell Biolab, USA. All Real-Time Quantitative Reverse Transcription PCR (RT-qPCR) kits were purchased from Qiagen, USA. Primers and probes were purchased from IDTDNA, USA.

Bi_2O_3 Particles Preparation

Bi_2O_3 particles used in this research were each synthesized at 60 °C, 90 °C and 120 °C using hydrothermal method. They were in powder form and slightly yellow in color. The particles were produced by NanoBiotechnology Research and Institute (NanoBRI), Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia (USM), Penang, Malaysia. All Bi_2O_3 particles were suspended in serum-free Minimum Essential Media (MEM) at 1 mg/ml concentration. The suspensions were vortexed at 800 rpm for 20 minutes at room temperature to form homogenous suspension. The stock solutions of all Bi_2O_3 NPs were diluted to 100 µg/ml working solution to perform cytotoxicity evaluations.

Characterization Bi_2O_3 Particles

Morphology and structure of Bi2O3 particles synthesized at 60 °C, 90 °C and 120 °C were characterized at Microscopy Imaging Center, Faculty of Pharmacy, Universiti Teknologi MARA, UiTM Puncak Alam. Bi_2O_3 powders were diluted in ethanol and placed on the sample holder. The sample holders were dried prior to measurement and all Bi_2O_3 particles were observed using FEI Tecnai G2 Transmission Electron Microscope (TEM) under 17000x magnification power. The sizes of all Bi2O3 particles were measured using Zetasizer 1600 from Malvern at Nanopharmacy Unit, Biopharmaceutics and Pharmacokinetics Research Laboratory, Faculty of Pharmacy, UiTM Puncak Alam.

Cell Culture

HeLa [Chang Liver] (ATCC® CCL13™) cells were obtained from American Type Culture Collection. The cells were maintained in a medium consist of Minimum Essential Media (MEM), 10 % Fetal Bovine Serum and 1 % Penicillin-Streptomycin Cells in T-75 flask (Corning, USA) with 37 °C humidified atmosphere of 5 % CO2 and 95 % air for 3 days or until reached 80 % confluency before exposure to Bi_2O_3 particles. Confluent cells were detached from culture flask using 1X TrypLE express enzyme for 5 minutes. Cells suspension was counted using Vi-CELL™ Cell Counter (Beckman Coulter, USA) and seeded at a density of 1 x 104 cells per well in 96-well microplate (Corning, USA) for cell viability and ROS assays.

Cell Viability Assay

In vitro cytotoxicity of Bi2O3 particles synthesized at 60, 90, 120 °C and iodine were determined by MTT assay. Seeded cells in 96-well microplate were incubated for 24 hours (37 °C, 5 % CO2 and 95 % air) before it is exposed to 100 µl of 0-100 µg/ml of all Bi2O3 particles and iodine for 24-, 48- and 72-hours treatment period (37 °C, 5 % CO_2 and 95 % air) to study the concentration and time-dependent cytotoxicity effect. After incubation period, cells viabilities were determined using MTT colorimetric reagent. 50 µl of MTT solution was added to each well and incubated for 4 hours (37 °C, 5 % CO_2 and 95 % air). MTT solution was discarded and 200 µl of Dimethyl Sulfoxide (DMSO) was then added in each well. The fluorescence intensity was measured at 550 nm by using Tecan F200 Infinite 200-TWT Microplate Reader. Results were expressed in percentage of cell viability relative to control cells. First, the percentage of cell death was calculated, and then subtracted with 100 % to obtain the cell viability percentage as shown in the equation below.

$$\text{Cell death (\%)} = \frac{(\text{Absorbance of control cells} - \text{Absorbance of treated cells}) \times 100\ \%}{\text{Absorbance of control cells}}$$

$$\text{Cell viability (\%)} = 100\% - \text{Cell death (\%)}$$

Cell Analysis using Light Microscope

Seeded cells were plated in 6-well plate (Corning, Inc., USA). Cells were incubated overnight at 37 °C humidified atmosphere of 5 % CO_2 and 95 % air, and then rinsed with Phosphate Buffer Saline (PBS) before being treated with 2 ml of 100 µg/ml of Bi_2O_3 samples for 24 hours. The cells were observed using light microscope at 10 x magnification power. The images of the cells were captured using Dino-Eye Premier AM4023X digital eyepiece (AnMo Electronics Corporation., Taiwan) with DinoCapture software version 2.0.

ROS Intracellular Assay

Seeded cells in 96-well microplates were incubated 24 hours before treatment with all Bi2O3 particles and iodine. Cells were stained with 100 µL 1 mM (1 X) of the fluorescent dye

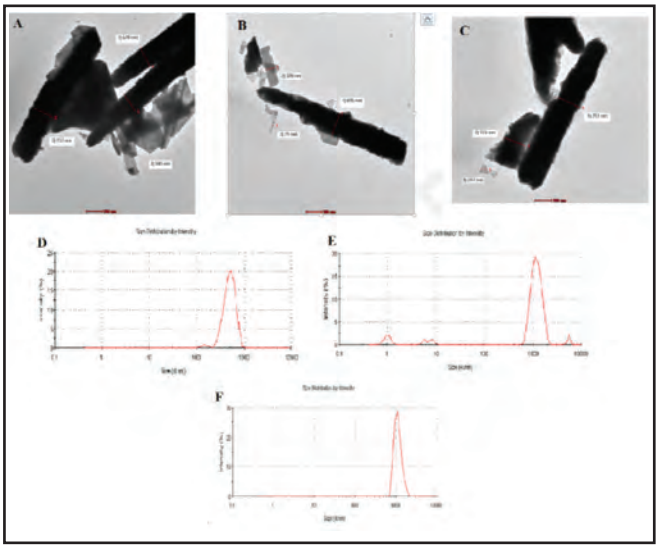

Fig. 1: TEM images of Bi_2O_3 particles synthesized at 60 °C

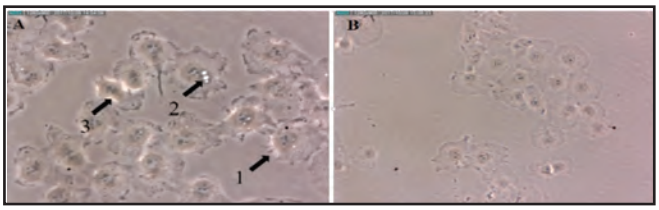

Fig. 3: Morphological changes observed by light microscope of (A) Chang liver cells treated with Bi_2O_3 particles in comparison to (B) untreated normal cells. Solid arrow (1) membrane blebbing, (2) cytoplasmic vacuole, (3) cell shrinkage

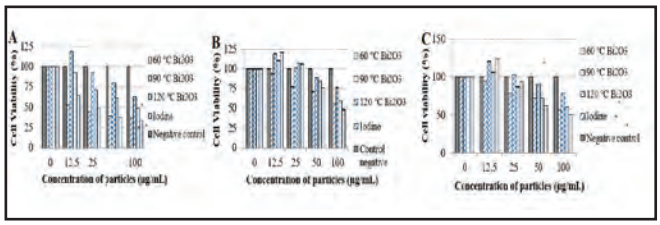

Fig. 2: Cell viability of Chang liver cells on 24 hours (A); 48 hours (B) and 72 hours (C) exposure to Bi_2O_3 particles synthesized at 60, 90, 120 °C and iodine. Values were expressed as mean ± SEM (n =4), ($p<0.05$).
*Indicates significant differences when compared to control negative, ($p<0.05$)

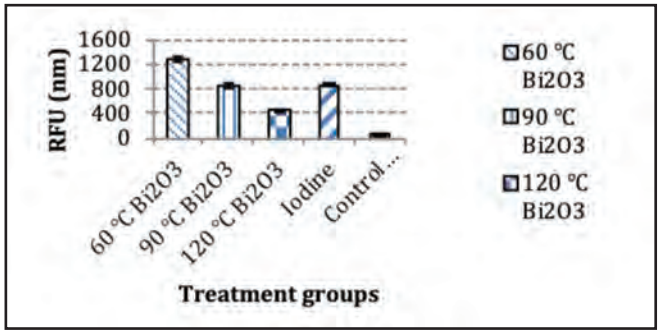

Fig. 4: Intracellular ROS production of Chang liver cells exposed to Bi2O3 particles synthesized at 60, 90, 120 °C and iodine. Values were expressed as mean ± SEM (n = 4), ($p<0.05$). [a]Indicates significant differences when compared to control negative, ($p<0.05$). [b]Indicates significant differences when compared to iodine, ($p<0.05$)

2,7-dichloro-dihydrofluorescein diacetate (DCFH-DA) for 1 hour at 37 °C. After staining, cells were washed and treated with 100 µL of 100 µg/mL of all Bi_2O_3 particles and iodine for 24 hours. After the incubation period, 100 µL of fresh MEM cell culture media was added after cells were washed. 100 µL of 2X Cell Lysis Buffer was added into each well before transferring 150 µL of lysate into to a 96-well black plate. The fluorescence intensity was measured at 480 nm excitation/ 530 nm emissions.

Real-time PCR

The mRNA expressions of ER-stress genes (GRP78 and CHOP) were determined using real-time PCR. Total RNA was extracted from treated Chang liver cells using RNeasy Mini Kit (Qiagen, USA). 1000 ng of RNA was reverse transcribed to cDNA using Quantinova Reverse Transcription Kit (Qiagen, USA). Real-time PCR was done according to manufacturer's protocol in 20 µL final volume of reaction, consisting of 2X Quantinova Probe PCR Mastermix (Qiagen, USA), template cDNA, primers and probes (Prime Time qPCR assay, IDTDNA, USA). The reaction parameters were one cycle at 95 °C for 2 mins, followed by 40 cycles with each cycle at 95 °C for 5 seconds and 60 °C for 30 seconds. GAPDH was used as the housekeeping gene.

Statistical Analysis

All values were expressed as mean ± standard error mean (SEM). Statistical significance of obtained data was determined by using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Significance of data was designated as $p<0.05$.

Ethics Approval and Infirmed Consent

No ethical clearance needed for this study.

RESULTS

Bi_2O_3 Particles Characterization

The TEM images in Figure 1A-1C shows the Bi2O3 particles synthesized at 60, 90 and 120 °C were in rod shape and well crystallized particles. The mean size of all Bi2O3 particles were analysed using zetasizer shown in Figures 1(D-F). The size distribution graphs showed Bi2O3 particles synthesized at 60, 90 and 120 °C had an average diameter of 813.8 nm, 1280 nm and 1627 nm respectively.

(A), 90 °C (B) and 120 °C (C); mean hydrodynamic size of Bi2O3 particles synthesized at 60 °C (D), 90 °C (E) and 120 °C (F) Cell Viability Analysis

MTT assay was done to measure the cytotoxicity of Bi_2O_3 particles synthesized at 60, 90 and 120 °C on Chang liver cells metabolic activities. Figures 2(A-C) shows the percentage viability of Chang liver cells treated with Bi_2O_3 particles and iodine in the range of 0-100 µg/mL concentrations for 24, 48 and 72 hours. After 24 hours exposure, cells treated with Bi_2O_3 particles synthesized at 60 °C showed the least percentage of viable cells (33.4 %), followed by iodine (37.2 %) and Bi_2O_3 particles synthesized at 90 and 120 °C respectively showed 48.3 % and 63.1 %. The cytotoxicity trend was found to be similar throughout all three-incubation time. The viability of cells increases gradually with cells treated with Bi_2O_3 synthesized using higher reaction temperatures with declining particles cytotoxicity. With prolonged incubation time, the viability of treated cells increased. The cytotoxicity displayed was concentration-dependent as the viability of cells decreased when exposed to higher concentration of Bi_2O_3 particles and iodine at 24 hours. Cells treated with 100 µg/mL of Bi_2O_33 particles and iodine displayed the highest cytotoxic effect and showed significant differences with untreated cells at 24, 48 and 72 hours (p<0.05). Therefore, 100 µg/mL concentration was used to further evaluate the cytotoxicity effect Bi_2O_3 particles synthesized at 60, 90 and 120 °C against iodine in treated cells, via generation of ROS and expression of ER stress genes.

Evaluation of Morphology of Chang Liver Cells Upon Exposure to Bi_2O_3 Particles

The morphology examination in this study revealed the morphological changes of Chang liver cells treated with 100 µg/mL Bi_2O_3 particles after 24 hours. Chang liver cells showed distinct morphological changes indicating unhealthy cells in comparison to untreated control cells (Figures 3A and 3B). The size of treated cells shown in Fig 3A appeared to be larger and irregular compared with untreated cells with formed blebs and cytoplasmic vacuoles.

The effect of 100 µg/mL Bi_2O_3 particles synthesized at 60, 90 and 120 °C was further evaluated by inducing intracellular ROS in Chang liver cells. The cells were treated with 100 µg/mL upon 24 hours exposure and stained with DCF-DA. The fluorescence intensity of cells stained with DCF-DA increased with the presence of ROS. Figure 4 shows the level of ROS gradually decreased with Bi_2O_3 particles synthesized at higher temperatures and showed significant differences in comparison to untreated cells. Cells treated with Bi_2O_3 particles synthesized at 60 °C showed the highest ROS production (1277.02 nm), followed by iodine (865.20 nm), Bi_2O_3 particles synthesized at 90 and 120 °C respectively (835.87 nm and 466.28 nm. When compared to iodine, Bi_2O_3 particles synthesized at 60 and 120 °C showed significant differences in ROS production of (p<0.05).

ER Stress Responses Induced by Bi_2O_3 Particles

RT-PCR was performed to determine the cytotoxicity of 100 µg/mL Bi_2O_3 particles synthesized at 60, 90 and 120°C at molecular level by assessing ER stress genes in Chang liver cells. The mRNA expression of GRP78 and CHOP was measured in cells treated with Bi_2O_3 particles and iodine 24 hours after incubation time. Figures 5A and 5B showed mRNA expression of GRP78 and CHOP were both suppressed in all treated cells in comparison to untreated cells. CHOP gene was observed to be more suppressed than GRP78 gene. Cells treated with Bi_2O_3 particles synthesized at 60 °C exhibited highest suppression of GRP78 and CHOP genes, followed by cells treated with Bi_2O_3 particles synthesized at 90 and 120 °C and iodine when compared to untreated cells.

DISCUSSION

The exploration of finding new novel x-ray contrast media formulation is motivated by the growing concerns over the biocompatibility of iodinated agents especially in patients with iodine intolerance and compromised renal functions.[12] Bismuth is thought to be another potentially useful material for contrast media, after gold which attenuates X-rays strongly, inexpensive and most biocompatible heavy metal. However, the development of bismuth as a contrast media is hampered due to insufficient study on its synthetic approaches in comparison to gold.[13] Bismuth compound has a diverse chemistry and it is least well established among all heavy stable elements in terms of coherent or comprehensive database. Bismuth compound can be prepared using various starting materials, mixtures or preparations which contribute to ill-defined formulas, vague name designations as well as indefinite characterization and properties.[14] As the use of engineered nanomaterials expand as therapeutics and as diagnostic tools, the administration of these materials into the human body should be of high concern due to its toxicity. At nano and microscopic scale, the physical and chemical properties of materials can be dramatically altered hence they warrant thorough assessments of occurrence of unexpected toxicities.[15] In this study, the toxicity profile of Bi_2O_3 particles was assessed to determine the effect of different reaction temperatures using the hydrothermal method on the principle of biological interaction and responses of Bi_2O_3 particles in vitro.

Results in Figures 1A-1C show Bi_2O_3 particles synthesized at all different temperatures are rod-shaped. Similar to previous study, our findings indicate that Bi_2O_3 particles synthesized hydrothermally using alkaline solution yielded well-crystallized rod-shaped particles.[16] Another study, [17] also reported that well-crystallized Bi_2O_3 particles were successfully synthesized using sodium hydroxide (NaOH) in a hydrothermal reaction. This may suggest that strong alkali such as NaOH produces well-defined particles because more OH-ions were produced during the formation of particles. Our findings also revealed that the morphology of Bi_2O_3 particles synthesized hydrothermally can be controlled by the reaction temperature. The size of Bi_2O_3 particles increased with rising reaction temperature. This explains that reaction temperature may influence chemical reaction rate of particles and rising the reaction temperature may increase the formation rate that will later produce bigger particles.[18]

The viability of Chang liver cells assayed by MTT exposed to Bi_2O_3 particles in this study is size-dependent in which the particles size is directly proportional to the synthesizing temperatures. As presented in Figures 2A-2C, cells exposed to Bi_2O_3 particles with a diameter of 813.8 nm synthesized at 60 °C showed the lowest percentage of cell viability. As the particles get bigger and with the rising synthesizing temperature, the viability of treated cells increased. Several

recent articles focused on carbon black particles, silver and silicon dioxide (SiO2) nanoparticles were also in positive agreement with the study results reported that smaller particles contributed to a greater cytotoxicity strengthened the evidence and established a better understanding on size-dependent cytotoxicity in vitro.[19] The cytotoxicity of Bi_2O_3 particles synthesized at all different temperatures was also found to be concentration-dependent.

Figures 2A-2C showed viability of treated cells decreased when exposed to Bi_2O_3 particles and iodine with higher concentrations. However, after 24 hours, the viability of Bi_2O_3 particles was observed to increase with prolonged incubation time. This finding may suggest that Bi_2O_3 particles have a property of acute toxicity and mitigated over time causing the cells to recuperate at extended incubation time. These results were found to be similar with the study reported using bismuth ferrite nanoparticles (BFO NPs) which possessed a nature of acute toxicity upon exposure to neuronal cells.[20] Substantially lower toxicity of bismuth synthesized at 90 and 120°C when compared with iodine was observed shown by the higher percentage of cell viability of treated cells. The results suggested that Bi_2O_3 particles synthesized at 90 and 120°C which possessed bigger particles size due to higher reaction temperatures has outperformed clinically used iodine as contrast media in Chang liver cells culture. Likewise, a study[21] highlighted that bismuth sulphide PVP nanoparticle (BPNP) to be less toxic in hepatocytes and showed superior profile in comparison to clinically used iodine. Notably, on exposure, the shape and size of cells appeared bigger and irregular with the presence of cytoplasmic vacuoles and membrane blebbing indicating unhealthy cells in comparison to untreated control cells. Such alteration in morphology or shape of cells is considered as a significant effect following exposure to toxicant.[22]

Liver is a major organ affected by ROS.[23] The results in Figure 4 shows significant increase in intracellular ROS in all cells exposed to Bi_2O_3 particles and iodine compared to untreated cells. Another study[24] reported elevated intracellular ROS level in HaCat cells after exposure to BiOBr NPs besides than disturbance in cell cycle and cell apoptosis.

Endoplasmic reticulum is a cellular organelle specializes in folding and modifying of protein. This organelle is highly sensitive to external stimuli and homeostasis changes within the cell. Accumulation of unfolded proteins contribute to a condition called ER stress that may activate a series of adaptive response known as unfolded protein response (UPR).[25] Consistent ER-stress may affect cellular signaling processes such as reduction-oxidation (redox) homeostasis, initiating inflammation and apoptosis.[25] Recently, several studies proposed that ER stress-related responses as an early biomarker for nanotoxicity.[26] The ability of Bi_2O_3 particles to induce ER stress in Chang liver cells was measured by the mRNA expression of GRP78 and CHOP. GRP78 serves as an ER stress sensor to control the activation of UPR. During ER stress, GRP78 will be dissociated from the ER lumen to activate pathways leading to UPR survival and apoptosis responses.[27] When the stress in ER prolongs, a UPR downstream effector called CHOP will promote apoptosis through downregulation of B-cell leukaemia/lymphoma 2 (Bcl-2) and depletion of cellular glutathione.[28] The results in Figures 5A and 5B show the low expression of both ER stress gene in treated cells compared to untreated cells, despite high levels of elevated ROS were observed. Smallest Bi_2O_3 particles synthesized using lowest reaction temperature showed the least expression of ER stress genes in treated cells. Corroborating with MTT assay and intracellular ROS data, ER genes showed lesser suppression with bigger size of Bi_2O_3 particles contributed by higher reaction temperature. The results may suggest particles show size-dependent effect that was observed in the suppression trend of GRP78 and CHOP. In agreement with another study[29], they highlighted that 90 nm and bulk-sized ZnO NPs induced less ER stress than

The 30 nm ZnO NPs. suppression of ER stress genes in this study can be inferred that ER stress was inhibited to ensure cell survival from assault induced by Bi_2O_3 particles.[30] Furthermore a study[31] explained that adaptation to physiologic stressors may be harmonize by the expression of all three bunches of UPR and eventually perturb the stress equilibrium occurs in the ER. This phenomenon suggests that suppression of ER stress genes involve in the mechanism to block further cell damage and inhibit ER stress-induced apoptosis.

CONCLUSION

In this study, the cytotoxicity of Bi_2O_3 particles synthesized using different reaction temperatures and iodine on Chang liver cells were comparatively investigated. The results revealed that different reaction temperatures of Bi_2O_3 particles may provoke size-dependent cytotoxicity by reduced cell viability, causing morphological changes to treated cells, induced intracellular ROS level and suppressed ER stress genes expression in order to curtail the stress imbalance. In comparison to other clinically used contrast media, Bi_2O_3 particles synthesized at 120 °C has biggest particles size and was found to be least cytotoxic in Chang liver cells. Nevertheless, additional studies are warranted including histological examination of susceptible organs to support the findings from this study in assessing the safety profile of bismuth-based contrast media.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests that would prejudice the impartiality of this scientific work.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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