

# Comparative Study of Different Methods to Determine the Role of Reactive Oxygen Species Induced by Zinc Oxide Nanoparticles

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**Abstract**—Accumulation of reactive oxygen species (ROS) followed by an increase in oxidative stress is associated with cellular responses to nanoparticle induced cell damages. Finding the best method for assessing intracellular ROS production is the key step in the detection of oxidative stress induced injury. This study evaluates and compares four different methods for the measurement of intracellular ROS generation using fluorogenic probe, 2',7'-dichlorofluorescein diacetate (DCFH-DA). Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was utilised as a positive control to assess the reactivity of the probe. Spherically shaped zinc oxide (ZnO) nanoparticles with an average particle size of 85.7 nm were used to determine the diverse roles of ROS in nanotoxicity in Hs888Lu and U937 cell lines. The results showed that different methods exhibit different patterns of ROS measurement. In conclusion this study found that the time point at which the DCFH-DA is added to the reaction, the incubation time and the oxidative species that is responsible for the oxidation of DCFH, have impact on the intracellular ROS measurement.

**Index Terms**—DCFH-DA, nanotoxicity, nanoparticle, ROS, ZnO.

## I. INTRODUCTION

Reactive oxygen species are important intermediates constantly produced *in vivo* through a variety of normal metabolic processes as well as being common intermediates generated after exposure to drugs or ionizing radiation (Curtin, et al., 2002). The nanoparticles have small size and a large surface area that can lead to the sequential oxidation-reduction

reactions at nanoparticles surface to produce reactive species such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and hydroxyl radical (Donaldson, et al., 2003; Lin, et al., 2009; Oberdorster, 2004; Xia, et al., 2008; Xia, et al., 2006).

Fluorogenic probes have been widely employed to monitor oxidative activity. Among these, 2',7'-dichlorofluorescein diacetate (DCFH-DA) has been utilised as an assay to evaluate oxidative stress in cells (Wang and Joseph, 1999). This hydrophobic non-fluorescent molecule penetrates rapidly into the cell and is hydrolysed by intracellular esterase to give the dichlorofluorescein (DCFH), which is trapped inside the cells. In the presence of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and other ROS, DCFH molecule is oxidised to its highly fluorescent product 2',7'-dichlorofluorescein (DCF) (Foucaud, et al., 2007; Loetchutinat, et al., 2005; Rota, et al., 1999).

The DCF fluorescence intensity can be easily measured and its fluorescence intensity is proportional to the amount of ROS. This probe has been used extensively for quantifying nanomaterial-induced ROS in a range of cell types, some examples of which include LN2308 (glioma) cells exposed to ZnO nanoparticles (Ostrovsky, et al., 2009), ZnO induced ROS in Ana-1 (mouse macrophage) cells (Song, et al., 2010), ROS induced in A549 (human lung epithelial) cells after exposure to ZnO and silica nanoparticle (Lin, et al., 2006; Lin, 2009) and by ambient ultrafine particles, cationic polystyrene nanospheres, TiO<sub>2</sub> and fullerol nanoparticles in RAW264.7 phagocytic cells (Xia, 2006).

In a previous study (Najim, et al., 2014) we demonstrated that the presence of zinc oxide (ZnO) nanoparticles with an average particle size of 85.7 nm induced cytotoxicity towards human histiocytic lymphoma (U937), neuron-phenotypic cells (SH-SY5Y), neuroblastoma (SH-SY5Y) and normal lung (Hs888Lu) cell lines.

Moreover, our data have also indicated that the cytotoxicity of ZnO was concentration dependent. The mechanism of nanoparticles cytotoxicity is not clear, however the generation of reactive oxygen species (ROS) was considered as one of the main causes of the nanotoxicity by the nanoparticles as shown

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in previous studies (Li, et al., 2008; Oberdorster, 2004; Reeves, et al., 2008; Sharma, et al., 2009).

This study was designed to investigate the underlying mechanisms of nanoparticles induced cytotoxicity. While there is a number of different cellular injury responses to ROS generated from nanoparticles, we developed a rapid screening procedure for ZnO toxicity premised on oxidative stress injury. In this study four different methods for intracellular ROS detection were compared for their compatibility by using the same representative characterised probe, 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Grabinski, et al., 2007; Lin, 2009; Ostrovsky, 2009; Song, 2010; Wang and Joseph, 1999).

## II. MATERIALS AND METHODS

### A. Materials

#### *ZnO Nanoparticles*

The ZnO nanoparticles were synthesised using a method detailed elsewhere (Rusdi, et al., 2011). The ZnO nanoparticles were characterized using scanning electron microscopy (SEM), X-Ray diffraction (XRD) and transmission electron microscopy (TEM) techniques (Najim, 2014; Rusdi, 2011). ZnO average particle size used in these experiments was 85.7 nm.

The 100 mM stock solution of ZnO nanoparticles was prepared in 0.01 M phosphate buffer saline (PBS, Sigma, USA) and sonicated for 30 minutes. The stock solution was stored at 4°C until required. ZnO nanoparticles stock concentration was vigorously vortexed and then diluted with complete medium prior to each experiment, to obtain concentrations of 100, 200, 500 and 1000 µM.

#### *Cell lines and Culture Conditions*

Lymphoma U937 cell line was provided by Dr. Mohamed Saifulaman (Faculty of Applied Sciences, UiTM, Malaysia). Normal lung Hs888Lu cell line was purchased from the American Type Culture Collection (ATCC, The Global Bioresource Centre, Manassas, USA). The U937 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma, USA) with glucose (4500 mg/L), 1% non-essential amino acids (at a strength of 100 ×) (PAA Laboratory GmbH, Austria), 1% L-Glutamine (200 mM) (Sigma, USA), 1% Gentamicin (10 mg/mL) (PAA Laboratory GmbH, Austria) and supplemented with 10% fetal bovine serum (FBS, PAA Laboratory GmbH, Austria). Hs888Lu cells were adapted to grow in DMEM with glucose (4500 mg/L), 1% non-essential amino acids, 2% L-Glutamine (200mM), 1% of Penicillin/Streptomycin (10,000 units/mL of penicillin and 10 mg/mL of streptomycin) (PAA Laboratories GmbH, Austria), 1% sodium pyruvate (1 mM) (Sigma-Aldrich, USA) and supplemented with 10% FBS. All cell lines were maintained at 37°C in a 5% CO<sub>2</sub> atmosphere with 95% humidity (Incubator, Contherm Scientific Ltd, New Zealand).

### *Intracellular ROS Measurements*

In order to establish the role of oxidative stress induced by ZnO nanoparticles, intracellular reactive oxygen species (ROS) generation was measured. In addition, 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma, USA) was used to detect and quantify the ROS level within the cells (Wang and Joseph, 1999). DCFH-DA stock solution (in methanol) of 10 mM was diluted in Hank's balanced salt solution (HBSS, with Ca<sup>2+</sup> and Mg<sup>2+</sup>, without phenol red, Gibco, USA) to yield a 20 µM working solution. Cells were plated in 96-well plate and incubated for 24 hours at 37°C before the experiment. Subsequently, various methods were applied to assess the ROS production.

Hydrogen peroxide (30% H<sub>2</sub>O<sub>2</sub>, Merck, Germany) was used as a positive control in these experiments to assess the reactivity of the probe (Ostrovsky, 2009; Winterbourn and Sutton, 1984) and also to validate the study. H<sub>2</sub>O<sub>2</sub> final concentrations in media were 100 and 200 µM. Four different methods were used to determine their efficiency in the measurements of intracellular ROS, with variation in incubation time and termination of dye. The applied methods were a modification of that described by Wang and Joseph (1999), Grabinski (2007), Lin (2009), Song (2010), and Ostrovsky (2009). Prior to each experiment for ROS measurements, cells (1×10<sup>5</sup> cells/mL) were seeded in 96-well plates and left to grow overnight in humidified atmosphere containing 5% CO<sub>2</sub> at 37 °C incubator.

### B. Methods

#### *Method 1 (Coded as M1)*

On the day of the experiments, after removing the medium, the cells were washed twice with PBS and then incubated with 50 µL/well of 20 µM of DCFH-DA (working solution) for 30 minutes in a dark environment at 37°C incubator. Immediately following incubation, DCFH-DA was removed and the cells were washed with PBS and treated with various concentrations of H<sub>2</sub>O<sub>2</sub> (100 and 200 µM) or ZnO nanoparticles (ranging from 100 µM to 1 mM) for 24 hours. The fluorescence intensity from each well was measured with a 485 ± 10 nm excitation wavelength and a 520 ± 12.5 nm emission wavelength on a Glomax multi detection system (Promega, USA). Results were representative of six independent experiments and were expressed as percentages of the value observed with control (no H<sub>2</sub>O<sub>2</sub> or ZnO treatments) (Grabinski, 2007; Wang and Joseph, 1999).

#### *Method 2 (Coded as M2)*

On the day of the experiments, after removing the medium, the cells were washed twice with PBS and then incubated with 50 µL/well of 20 µM of DCFH-DA (working solution) for 30 minutes in a dark environment at 37°C incubator, followed by incubation with various concentrations of H<sub>2</sub>O<sub>2</sub> or ZnO nanoparticles for 24 hours (Lin, 2009). The fluorescence intensity from each well was measured as described in the first method.

### Method 3 (Coded as M3)

For the treatments, the steps in method 2 were followed. After the treatment with H<sub>2</sub>O<sub>2</sub> or ZnO nanoparticles, the cells were washed twice with PBS to eliminate DCFH-DA that did not enter the cells (Song, 2010). One hundred  $\mu$ L of media was added to each well and the fluorescence intensity was measured as described in method 1.

### Method 4 (Coded as M4)

On the day of the experiments, after removing the medium, the cells were washed twice with PBS and then treated with various concentrations of H<sub>2</sub>O<sub>2</sub> or ZnO nanoparticles. After 24 hours of exposure to ZnO nanoparticles, the cells were washed twice with PBS and then incubated with 50  $\mu$ L/well of 20  $\mu$ M of DCFH-DA (working solution) for 30 minutes in a dark environment at 37°C incubator. The fluorescence intensity from each well was measured as described in method 1 (Lin, 2006; Ostrovsky, 2009).

## III. STATISTICAL ANALYSIS

GraphPad PRISM<sup>®</sup> version 5.0 program was used for statistical analysis. Means and standard deviations were determined from six independent experiments. All comparisons were made using two-tailed Student's *t*-test and when positive indicated by asterisks (\**p*<0.05, \*\**p*<0.01 and \*\*\**p*<0.001).

## IV. RESULTS AND DISCUSSION

### A. Comparison of intracellular ROS detection methods

To obtain optimal results from intracellular ROS assay, two different cell lines such as normal lung (Hs888Lu) and lymphoma (U937) were used, because the quality of DCFH-DA relies on the cell types and culture conditions. In the presence of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and other ROS as well as heme protein catalysts such as peroxidases or cytochrome *c*, DCFH is oxidized to highly fluorescent DCF in cells (Burkitt and Wardman, 2001; Ischiropoulos, et al., 1999; LeBel, et al., 1992; Ohashi, et al., 2002). Thus, H<sub>2</sub>O<sub>2</sub> was used as a positive control in this study to assess the reactivity of the probe and also to validate the method.

DCF- fluorescence intensity was measured (proportional to the intracellular ROS) in Hs888Lu and U937 cells exposed to H<sub>2</sub>O<sub>2</sub> or ZnO according to the methods described earlier (Fig. 1 and Fig. 2). The results showed that different methods exhibit different pattern of ROS measurement. Higher amount of intracellular ROS was detected using method 2. H<sub>2</sub>O<sub>2</sub> content increased the intensity of DCF- fluorescence in a dose-dependent manner. There were significant differences between intracellular ROS production in different cell lines. Using this method the percentages of fluorescence intensity were 164% and 1011% in Hs888Lu cell lines (*p*<0.05) and U937 cell lines (*p*<0.01) respectively after exposure to 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> (Fig. 1 and Fig. 2).

Following methods 1, 3 and 4, the results did not indicate any change in DCF- fluorescence intensity thereafter in ROS measurements in selected cell lines exposed to H<sub>2</sub>O<sub>2</sub> (Fig. 1 and Fig. 2).

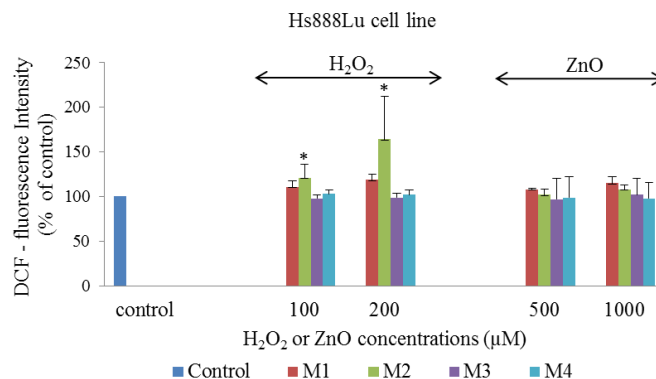


Fig. 1. Comparison of four methods (M1, M2, M3 and M4) to determine the intracellular ROS in Hs888Lu cell line. DCF-fluorescence intensities in Hs888Lu cell lines are exposed to different concentrations of H<sub>2</sub>O<sub>2</sub> or ZnO. The control represents ROS measurement in non-treated cells.

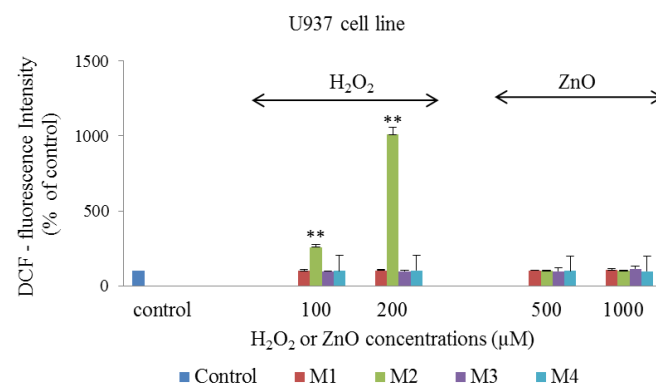


Fig. 2. Comparison of four methods (M1, M2, M3 and M4) to determine the intracellular ROS in U937 cell line. DCF-fluorescence intensities in U937 cell lines are exposed to different concentrations of H<sub>2</sub>O<sub>2</sub> or ZnO. The control represents ROS measurement in non-treated cells.

After comparison of ROS-induction by H<sub>2</sub>O<sub>2</sub> using the selected methods as a representative analysis of optimization experiments, ZnO nanoparticles – induced ROS in human cell lines was quantified according to the methods described earlier.

Following all methods 1, 2, 3 and 4, results did not show increase in DCF-fluorescence intensity in all cell lines compared to the control after exposure to 100 and 200 $\mu$ M ZnO nanoparticles (data not shown), but results showed 10% increase in DCF-fluorescence intensity in all cell lines compared to the control after exposure to ZnO nanoparticles (500 and 1000 $\mu$ M). Therefore, the results did not indicate significant changes in fluorescence intensity thereafter in ROS

measurements in a nanoparticle dose-dependent study (Fig. 1 and Fig. 2). In addition, hydrogen peroxide produced significant increase in DCF-fluorescence compared to ZnO nanoparticles under the experimental conditions of method 2.

Following methods 1, 3 and 4 results indicated that the  $H_2O_2$  and ZnO were weak inducer of DCF-fluorescence in both cell lines (Fig. 1 and Fig. 2). In contrast, previous studies (Grabinski, 2007; Ostrovsky, 2009; Song, 2010) were demonstrated different pattern of the ROS measurement with the selected methods. In previous studies, mouse keratinocyte (HEL-30) cells exposed to  $H_2O_2$  in a dose-dependent manner showed an increase in the ROS production according to the method 1 by Grabinski (2007). According to method 3, Song and co-workers (2010) have illustrated an increase in intracellular levels of ROS after exposure to all particle size of ZnO nanoparticles (30 nm, 100 nm) in mouse macrophage (Ana-1) cell line. Ostrovsky and co-workers (2009) have reported an increase in the fluorescence intensity of DCF in the normal human astrocyte and human glioma (U87) cell lines exposed to  $H_2O_2$  according to method 4. In addition, they found an increase in the fluorescence intensity of DCF after 24 hours exposure to 10 mM ZnO nanoparticles in the human glioma (U87) cells. The ZnO nanoparticles induced a smaller increase in the fluorescence intensity of DCF in the normal astrocytes cells as a compared to  $H_2O_2$  (positive control) (Ostrovsky, 2009).

Researchers in previous works used dilution of DCFH-DA in HBSS free of  $Ca^{2+}$  and  $Mg^{2+}$  (Li, et al., 2002; Reinisch, et al., 2000). Thus, the above methods (M1, M2, M3 and M4) were repeated using HBSS without  $Ca^{2+}$  and  $Mg^{2+}$ . The results (Data not shown) were very similar to the results shown in Fig. 1 and Fig. 2. This may imply that  $Ca^{2+}$  and  $Mg^{2+}$  in the medium of reaction were not contributing to the formation of DCF and thereafter to the intracellular ROS measurement.

It must be pointed out that the importance of the mechanisms and the possibilities that lead to the formation of highly fluorescent DCF must be carefully understood. Previous research works focused on the effects of endogenous and exogenous ROS in a variety of cell types, using 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Grabinski, 2007; Lin, 2009; Loetchutin, 2005; Ohashi, 2002; Ostrovsky, 2009; Song, 2010; Xia, 2008; Xia, 2006).

Although there is clear evidence that DCFH-DA is deacetylated to form DCFH by cellular esterase which is then oxidised to DCF by oxidising species in most cell lines. Some limitations of utilising this probe exist. Not all cell types possess sufficient esterase activity to produce the DCFH needed for accurate measurements of ROS. This may limit the availability of DCFH and result in an underestimation of intracellular ROS levels (Brubacher and Bols, 2001). In addition, DCF, oxidised fluorescent product of DCFH, is membrane permeable and can leak out of cells over time (Ubezio and Civoli, 1994). Incomplete DCF trapping may complicate interpretation of the data and hinder the precise evaluation of intracellular oxidation.

It remains unclear which reactive oxygen species are responsible for the oxidation of DCFH in cells, even though it is often assumed to be  $H_2O_2$  (in the presence of cellular peroxidases and similar catalysts). It was reported that other biologically relevant ROS, including peroxytrite and hydroxyl radicals ( $\cdot OH$ ), can oxidize DCFH (Kooy, et al., 1997; Myhre, et al., 2003; Possel, et al., 1997). Others have found that DCFH showed rather low sensitivity towards oxidation by NO,  $O_2^{\cdot -}$  (Myhre, 2003; Rota, 1999). Rota and co-workers (1999) implied that DCFH cannot be used to measure superoxide free radical formation in cells because the oxidation of this compound leads to the formation of superoxide. Moreover, some oxidants require small quantities which rapidly increase DCF formation, whereas other oxidants may need higher concentrations and more time (Myhre, 2003). In addition,  $H_2O_2$  cannot oxidise DCFH directly. The oxidation occurs as a result of the reaction of  $H_2O_2$  with cellular peroxidase, cytochrom *c*, or  $Fe^{2+}$  (LeBel, 1992; Rota, 1999; Royall and Ischiropoulos, 1993).

An important question is which reactive oxygen species induced from ZnO nanoparticles responsible for the oxidation of DCFH in biological systems? One of the possible mechanisms of free radical induction by ZnO nanoparticles was proposed by Ai and co-workers (2003) which is that sequential oxidation-reduction reaction may occur at ZnO particle surface to produce reactive species such as  $H_2O_2$  and hydroxyl radical (Ai, 2003). Some limitations of utilising this probe exist, even though it is often assumed that  $H_2O_2$  (in presence of cellular peroxidases and similar catalysts) and hydroxyl radicals ( $\cdot OH$ ) can oxidise DCFH (Ai, 2003).

## V. CONCLUSION

This comparative analysis found different results for the measurements of ROS depending on which method was used, although same concentrations of  $H_2O_2$  and DCFH-DA, same cells lines and cell culture conditions were used. The methods are varying in the step sequence of exposure to DCFH-DA and  $H_2O_2$  or ZnO, elimination of DCFH-DA and termination of DCF. There are several significant factors affecting the measurements such as the timing of loading DCFH-DA to the reaction, duration of DCFH-DA incubation and the oxidative species responsible for the oxidation of DCFH. All these will have an impact on the formation of DCF and thereafter on the intracellular ROS measurement. Our data show that the higher amounts of intracellular ROS were detected after exposure to  $H_2O_2$  using method 2. In addition, there is no evidence of ROS production due to ZnO nanoparticle toxicity in lymphoma and normal lung cell lines.

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