Nano toxicological studies of metallic oxide nanoparticles (zno, pb $(no_3)_2$)

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Abstract

Metal oxide nanoparticles are widely used in the paint and coating industry as well as in cosmetics, but the knowledge of their possible interactions with the immune system is very limited as well as we are far from understanding how nanoparticles affect the human health. Most knowledge is derived from studies on cell lines that are reproducible and well standardized assays. However, it is unclear how well these results translate to primary human cells. Only a few studies have tested the effect of nanoparticles on human Peripheral Blood Mononuclear Cells (PBMC). The aim of the present study is to investigate if commercially available ZnO and $Pb(NO_3)_2$ nanoparticles may affect different human immune cells, nano-sized vesicles that have a role in cell to cell communication. Here found that PBMC reacted with a dose dependent manner, increase in cell death via cell viability to ZnO nanoparticles indicating different sensitivity in different immune cells. Here, PBMC were found to be strongly affected by increased doses of ZnO, with 54 ± 6% dead cells at 100 µg/ml, whereas $Pb(NO_3)_2$ did not induce any significant cell death. Here concluded that subtoxic concentrations of ZnO nanoparticles do significantly alter PBMC characteristics, affect immune system and that evaluations of nanoparticles should be performed even at sub toxic concentrations on human blood cells when investigating potential effects on immune functions.

Keywords: Nanotoxicology, Colon Cancer, $Pb(NO_3)_2$, Tissue, Toxicology

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Introduction

Nanotoxicology was proposed as a new branch of toxicology to address the adverse health effects caused by nanoparticles. Despite suggestions that nanotoxicology should only address the toxic effects of engineered nanoparticles and structures we recommend that nanotoxicology should also encompass the toxic effects of atmospheric particles, as well as the fundamentals of virology and bacteriology. While significant differences exist between the health effects of nonbiological particles and viruses and bacteria, there are significant common aspects of intrusion and translocation[1].

Metal oxide nanoparticles have been extensively studied from a toxicology point of view and many studies have focused on zinc oxide (ZnO) and lead nitrate ($Pb(NO_3)_2$) nanoparticles due to their frequent use in sunscreens, cosmetics and paints.

As stated in a recent review, Nohynek 2010, ZnO nanoparticles do not penetrate the skin and thus they have been claimed to not pose a potential risk to human health when applied onto undamaged skin. However, due to semiconductor properties of ZnO nanoparticles, the industrial and electronic applications have gained much attention with other exposure routes and risks at hand. Human skin, lungs, and the gastro-intestinal tract are in constant contact with the environment. While the skin is generally an effective barrier to foreign substances, the lungs and gastro-intestinal tract are more vulnerable. These three ways are the most likely points of entry for natural or anthropogenic nanoparticles. Injections and implants are other possible routes of exposure, primarily limited to engineered materials. Due to their small size, nanoparticles can translocate from these entry portals into the circulatory and lymphatic systems, and ultimately to body tissues and organs. Some nanoparticles, depending on their composition and size, can produce irreversible damage to cells by oxidative stress or by organelle injury.

Diseases associated with inhaled nanoparticles are asthma, bronchitis, emphysema, lung cancer, and neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases. Nanoparticles in the gastro-intestinal tract have been linked to Crohn's disease and colon cancer. Nanoparticles that enter the circulatory system are related to occurrence of arteriosclerosis, and blood clots, arrhythmia, heart diseases, and ultimately cardiac death. Translocation to other organs, such as liver, spleen, etc, may lead to diseases of these organs as well. Exposure to some nanoparticles is associated to the occurrence of autoimmune diseases, such as: systemic lupus erythematosus, scleroderma, and rheumatoid arthritis.

Although recent development in nanotechnology resulting to development of variety of nanomaterials having diverse applications in daily life has attracted the interest of common people, much research work and scientific efforts are further required to meet the demand of public considering the changing scenario of society infrastructure, industrialization and global competitiveness requiring the need of new materials, whether it may be in health, medicine, industry, cosmetics etc to be used for the welfare of society and common people[2]

The extensive development of nanotechnology has given successful path in production of nanoparticles having applications in diverse areas like clinics, biomedicine, industry and cosmetics in general. It has been observed that the performance of products can increase when nanoparticles are incorporated in them but particles with nano-sized structure can

induce toxic effects or undesirable adverse effects too.

At the nano-meter length scale, the reduced size of particle increases the number of various essential properties such as their surface atoms/molecules as well as their surface area exponentially.This will lead to complex bio-physicochemical interactions when it exposed to physiological environments. Although nanoparticles exhibit unique significant beneficial properties, toxic effects of these nano products are also to be looked seriously. In this context, it may be further added that toxic effects of metal and metal containing nanoparticles have been studied and documented.

It is interesting to emphasize that the minute/nanostructure level size of a particle plays significant role and appears to be responsible in exerting undesirable side effects/toxic effects by a particular nanomaterial as the particles with nanostructure size can easily penetrate the basic biological structures due to which normal physiological functions of biological structures are get disturbed allowing them to produce toxic effects such as tissue inflammation as well as altered cellular redox balance along with the increased production of ROS (reactive oxygen species), causing abnormal function, tissue damage and cell death etc.

Experimental studies conducted on animal model and clinical studies on human subjects have clearly shown that inhaled small particles at nanostructure level can be removed less efficiently in comparison to larger particles through the mechanism of macrophage clearance in the lung which causes pulmonary damage. Further inhaled nanoparticles can also be translocated via lymphatic system, nervous system and circulatory system to most organs and tissues, including the brain [3].

Toxicity of Metal Nanoparticles

From a toxicological point of view many studies have been focused on nanomaterials especially on metal oxide nanoparticles. For example zinc oxide and lead nitrate nanoparticles have been considerably studied because these nanoparticles are frequently used in cosmetics, sunscreens and paints. ZnO nanoparticles when come in contact with skin have ability not to penetrate it, thus these nanostructured materials have been asserted not to possess a potential risk to public health when it applied on undamaged skin. However, metal oxide nanoparticles have semiconductor properties due to this reason; the electronic and industrial applications have received much attention with other exposures routes as well as risks at hand.

Size of particles plays a key role in producing toxicity in a biological system. However, significant increases in solubility of metal oxide nanoparticles have also been reported with decreasing particle size. The solubility of a material also greatly influences the toxicity of different NPs. The dissolution rate is dependent on a particle's size, its chemical and surface properties, and is further influenced by the surrounding media. Brunner 2006, reported that more soluble NPs like ZnO and $Pb(NO_3)_2$ shows higher acute toxicity than NPs of much low solubility such as TiO_2 and CeO_2.

ZnO NPs are used in paints, sunscreens, food packaging, solar cells, drug delivery and other therapeutics. The wide applications are because of its high optical transparency, large band gap, near UV-emission and antimicrobial properties. The growing use of NPs has also enhanced the likelihood of exposure of NPs to humans and environment. Toxicity of ZnO nanoparticles have been reported in bacteria, human cells and also in vivo models. On entering the biological system, the toxicity of nanoparticles occurs due to their higher surface-to-volume ratio which provides an enhanced available area for physical and chemical interaction with the cells. The increase in reactive surface area is the reason for toxicological insult by the production of ROS and oxidative stress.

Lead is continuously to be of concerns to human health because it is used in ceramics fabrication and in metal field in different countries. At low concentration, the toxic effect of Pb causes several neurological, behavioral and metabolic disorders. In nanotoxicology, many research studies conducted on metal concentration indicated that even low concentration of lead level in human blood associated with diminished skeletal growth, impairment in the psychological progress and disturbances in the cardiovascular function. Lead poisoning results from the food ingestion or contamination of water with lead. It can also enter into the body through the inhalation of lead based paint, leaded petrol, soil and dust. Hence, to understand the nano lead chemical and physical properties and its toxicity compare to lead metal. Lead metal affecting very nearly on all organs as well as system in body. There is ample evidence to indicate that consumption of food and water contaminated with lead, occasional ingestion of contaminated soil, dust, or lead-based paint have been found major causative factors for lead poisoning.

Since metal nanoparticles produce considerable toxic effects or undesirable adverse effects on biological systems/products during their manufacturing stage or even at their disposal stage, studies to understand the biochemical basis of toxicity of metal nanoparticles and its abatement measures are of great concern in the field of metal oxide nanoparticles. Different methods are available in order of toxicity assessment imposed through nanoparticles on to the various organisms. The methods for toxicity assessment can be categorized as in vivo and in vitro.

In Vivo, In Vitro Toxicity Assessment

In vivo tests are time-consuming, expensive, and involve ethical issues. Sayes et al., investigated the reliability of in vitro systems at predicting the pulmonary toxicity in vivo manner of fine ZnO particles and ZnO nanoparticles in rats, and concluded that in vitro cell culturing systems do not precisely forecast the nanomaterial. Toxicologists have explored the impacts of a variety of nanomaterials or nanoformulations in animal experiments. However, in vivo studies with nanomaterials, unlike studies involving chemicals or compounds, are interlaced with many challenges. The in vivo dose used for experiments should be derived from the quantity of nanoparticles exposed in the actual scenario.

When in vivo treatment is given for any test substance, it should be ensured that the vehicle is isotonic and nontoxic, and that the nanoparticle is well dispersed in the vehicle. Since nanoparticles are very susceptible to agglomeration owing to their increased relative surface area, they may not form a stable suspension in the physiological solutions suitable for in vivo exposure. The poor dispersion of nanoparticles during in vivo exposure negatively affects their biological distribution and subsequent activity. Therefore, the results from such studies may be misleading and

Will differ from study to study.

In vitro experimentation has always been the first choice for toxicologists, since it is time- and cost-effective. Although it cannot replace animal experimentation completely, but it does help to ensure that they are only used when absolutely necessary, and it sometimes provides mechanistic information on the toxicity of nanoparticles after in vivo studies.

The risk assessments of different aspects of nanotechnology are still in its early stages. Therefore, most of the studies pertaining to nanoparticle toxicology that has been carried out so far have been preliminary and confined to the classical in vitro toxicity assessment methods established for drugs and chemicals. Due to these properties, nanoparticles interfere with normal test systems, and this interference has been well documented in the literature. Examples of such properties include: large surface area and leading to increased adsorption capacity; different optical properties that interfere with fluorescence or visible light absorption detection systems.

The study about in vitro toxicity assessment of nanoparticles was under taken to find out the recent deficient knowledge of the cellular response of nano sized particle exposure.

The study evaluated the acute toxic effects of metallic oxide nanoparticles (ZnO, $Pb(NO_3)_2$) proposed for future use in industrial production methods using in vitro in human blood. The use of metal oxide nanoparticles are widely distributed towards the coating industry and paint and also in cosmetics, whereas the knowledge about their potential interactions with human being is necessary.

The aim of the study is to investigate the result about human health, when commercially available nanomaterials may affect various human blood cells. Various types of nanoparticles synthesized by biological methods were evaluated for their potential toxicity. It may be noted that some manufactured nanomaterials are redox active while some transport across cell Membranes. These properties/actions of nanoparticles on biological system are of great concern towards the toxicity studies of these nanoparticles.

Peripheral Blood Mononuclear Cell (PHMC)

Although inflammatory effects of nanoparticles (in vitro and in vivo) have been studied by leading scientists and results are well documented in scientific literature, there is little knowledge about the mechanism by which nanoparticles interact with human system and produce effects.

Studies on cell lines which are reproducible and standardized assays have thrown some light in this direction. However, it is unclear how well these results translate to primary human cells.

Only some study has examined the effect of nanomaterials on human PBMC (Peripheral Blood Mononuclear Cells). A Peripheral Blood Mononuclear Cell (PBMC) is any peripheral blood cell having a round nucleus. These cells consist of lymphocytes (T cells, B cells, NK cells) and monocytes, in case of humans; the major part of the peripheral blood mononuclear cell (PBMC) population is made up of lymphocytes, followed by monocytes, and only a few percentages of dendritic cells. With the help of gradient centrifugation of whole blood, these peripheral blood mononuclear cells could be extracted. This centrifugation technique will separate the whole blood sample in to three distinct layer ie., plasma, a top layer, followed by a PBMCs layer and Polymorphonuclear Cells (such as neutrophils and eosinophils) and erythrocytes, a bottom fraction.

Cell viability

The toxic effect of nanoparticles is measured by applying biological technique “Cell viability”. Many studies have been focused towards carbon nanoparticles/nanotubes (CNTs). Among different nanoparticles carbon nanoparticles (CNTs) are frequently used for studying toxicity and assessment of viability/ lethality of cells. Only few studies have been developed towards the toxicity assessment of metal oxide nanoparticles. In this present study, two different metal oxide nanoparticles have been exposed to human blood to examine their toxicity employing cell viability technique.

In the emerging field of Nanoscience and technology, a significant area of research deals with the production of nanostructured material of various sizes, controlled monodispersity and different chemical compositions. The engineering and science of nanosystems is one of the fastest growing and most challenging sectors of nanotechnology. Indeed, the shape controlling properties of nanoparticle is a current addition in to the demand list being made up of novel emerging production methods.

In view of sustainable development of society and making environment free from hazardous chemicals being used in helm of various human activity, there is a growing need to the development of environmentally benign production processes of nanoparticle that avoid the use of any toxic chemicals in to the synthesis protocol. Considering above observations in view the present study has been conducted towards synthesis of ZnO and $Pb(NO_3)_2$ nanoparticles, their characterization and antibacterial activity against few pathogenic bacteria. The study also deals with toxicological evaluation of these synthesized nanomaterials using peripheral blood mononuclear cell of human blood. It is hoped that the present study might be useful in developing novel nanomaterials having applications in important sectors such as health, medicine, environment and industry.

Assessment of Nanoparticle Toxicity

Various methods are available for the toxicity assessment imposed by nanoparticles on the organisms. The inflammatory effects of ZnO and $Pb(NO_3)_2$ nanoparticles have been addressed both in vivo and in vitro. The methods for toxicity assessment can be categorized as in vivo and in vitro.

In vivo toxicity studies

In vivo tests are time-consuming, expensive, and involve ethical issues. Sayes et al., investigated the reliability of in vitro systems at predicting the in vivo pulmonary toxicity of fine ZnO particles and ZnO nanoparticles in rats, and concluded that in vitro cell culture systems do not precisely forecast the Nanomaterial toxicologists have explored the effects of a variety of nanomaterials in animal experiments. However, in vivo studies with nanomaterials, unlike studies involving chemicals or compounds, are interlaced with many challenges. The in vivo dose used for experiments should be derived from the quantity of nanoparticles exposed in the actual scenario. However, determining the quantity of nanoparticles in air, water, soil or any consumer product is a technical challenge due to their tiny size

and the small quantity present. Even if the dose of nanoparticles is known, exceeding a certain dose in experiments is not advisable due to increased agglomeration of nanoparticles.

Method for in vivo toxicity assessment is the examination of changes in the serum chemistry and cell type after exposure of nanoparticles. Histopathology of the cell, tissue or organ after exposure is used to determine the toxicity level caused by a nanoparticle. Histopathology examination has been used to nanoparticles' exposed tissues such as lung, eyes, brain, liver, kidneys, heart and spleen. The advancement of toxicity assessment includes use of micro-electrochemistry and microfluidics.

When in vivo treatment is given for any test substance, it should be ensured that the vehicle is isotonic and nontoxic, and that the nanoparticle is well dispersed in the vehicle. Since nanoparticles are very susceptible to agglomeration owing to their increased relative surface area, they may not form a stable suspension in the physiological solutions suitable for in vivo exposure. The poor dispersion of nanoparticles during in vivo exposure negatively affects their biological distribution and subsequent activity. Therefore, the results from such studies can be misleading and will differ from study to study.

In Vitro Toxicity Studies

In vitro experimentation has always been the first choice for toxicologists, since it is time- and cost-effective. Although it cannot replace animal experimentation completely, but it does help to ensure that they are only used when absolutely necessary, and it sometimes provides mechanistic information on the toxicity of nanoparticles after in vivo studies.

The risk assessment of different aspects of nanotechnology is still in its early stages. Therefore, most of the studies pertaining to nanoparticle toxicology that have been carried out so far have been preliminary and confined to the classical in vitro toxicity test methods established for drugs and chemicals. However, the methods that are used in traditional toxicology cannot be applied to nanoparticle toxicology, as nanoparticles display several unique physicochemical properties. Due to these properties, nanoparticles interfere with normal test systems, and this interference has been well documented in the literature. Examples of such properties include: high surface area, leading to increased adsorption capacity; different optical properties that interfere with fluorescence or visible light absorption detection systems; increased catalytic activity due to enhanced surface energy; and magnetic properties that make them redox active and thus interfere with methods based on redox reactions.

In vitro nanoparticle toxicity assessment is one of the important methods. The advantages include lower cost, faster and minimum ethical concerns. Assessment can be subdivided into proliferation assay, apoptosis assay, necrosis assay, oxidative stress assay and DNA damage assays.

Proliferation Assays

This assay is used to measure the cellular metabolism by assessment of metabolically active cells. MTT is the most commonly used tetrazolium salt for in vitro toxicity assessment of nanoparticles. The technique is advantageous due to quick yields, reproducible results and minimum manipulation of the model cells. The assay is based on the measurement of tetrazolium salt and it can sometimes lead to altered measurement due to changes in the culture media additives, media pH, ascorbate and cholesterol. Thymidine incorporation is a method used for assessment of cellular proliferation, but this method is avoided due to toxicity and relatively high cost. Alamar Blue is used to measure the cellular redox potential and advantageous as compared to MTT assay due to simpler sample preparation. But the success of the Alamar Blue is hindered due to unavailability of the biochemical mechanisms of the assay and reaction of non-porous silicon with Alamar Blue in the absence of the cells.

Apoptosis Assay

Apoptosis is one of the major markers observed in the in vitro assessment of nanoparticle toxicity. Generation of excessive free radical is considered the cause of apoptosis and DNA damage. Evidence suggested that apoptosis and DNA damage can be caused by oxidative stress in cell culture systems. Many studies have reported the apoptosis induced by nanoparticles. In vitro studies indicated that silver nanoparticles caused apoptosis in mouse embryonic stem cells. In another investigation, the release of apoptosis markers viz. Caspase-3 and caspase-9 were examined on the treatment of larval tissues of Drosophila melanogaster with silver nanoparticles at concentrations of 50 and 100 μg/ml for 24 and 48 h. The results suggested the involvement of silver nanoparticles in the apoptotic pathway of D. Melanogaster.

There are a number of methods for assessment of apoptosis. These include Annexin-V assay, Comet assay and inspection of morphological changes. Annexin-V and Propidium Iodide (PI) are typical cell death markers used in toxicity assessment. The assay works on the principle that when Annexin-V bounds to phosphatidyl serine, it shows increased fluorescence and hence indicates the externalization of the plasma membrane. This externalization of the plasma membrane is induced by activation of the caspase-dependent pathway. PI is an impermeable dye which stains the nucleus only when the integrity of the cell membrane is lost, which can be related to the late stage of apoptosis.

Necrosis Assay

Necrosis is measured by the integrity of the membrane and it is commonly used to determine the viability of the cells. Membrane integrity is measured by uptake of the dye such as Neutral Red and Trypan Blue. The need for a reliable, rapid, inexpensive and reproducible quantitative in vitro assay for screening of nanoparticles is generally acknowledged. The Neutral red readily diffuses through the plasma membrane. It concentrates in the lysosomes and binds by electrostatic hydrophobic bonds with anionic sites in the lysosomal matrix. Alterations of the cell surface lead to lysosomal fragility. Such changes brought about by the action of xenobiotics or nanoparticles can result in a decreased uptake and binding of Neutral red. It is thus possible to distinguish between viable and dead cells.

Another method is called Trypan blue exclusion test. The dye trypan blue enters dead cells and is excluded from living cells. A trypan blue exclusion assay was performed for the evaluation of cell membrane stability.

Oxidative Stress Assay

Exposure of nanoparticles leads to the production of reactive ROS and reactive nitrogen species (RNS). The method for detection of ROS and RNS involves the reaction of 2, 2, 6, 6-Tetramethylpiperidine (TEMP) with O2-stable radical which can be detected using X-band Electron Paramagnetic Resonance (EPR). The application of these methods is hindered due to their high cost. Fluorescent probe molecules have emerged as an alternative and cost-effective approach. But there are limitations with fluorescent probes as they are inefficient due to their ability to react with a variety of reactive species. Oxidative stress can also be assessed by measuring lipid peroxidation C11-BIODIPY assay and TBA assay for malondialdehyde.

Human Peripheral Blood Mononuclear Cells (PBMC)

The inflammatory effects of ZnO and $Pb(NO_3)_2$ nanoparticles have been addressed both in vitro and in vivo but we are far from understanding how nanoparticles affect the human immune system.

Most knowledge is derived from studies on cell lines that are reproducible and well standardized assays. However, it is unclear how well these results translate to primary human cells. Only a few studies have tested the effect of nanoparticles on human Peripheral Blood Mononuclear Cells (PBMC). The use of PBMC allows for the simultaneous analyses on the effects of nanoparticles on several different important immune cells such as T cells, B cells, monocytes and Natural Killer (NK) cells. Thus, exposure of PBMC to silver nanoparticles showed specific effects on monocytes while T cell proliferation remained unaffected. Dendritic Cells (DC) are the Antigen Presenting Cells (APC) in the body with the highest capacity to initiate and direct immune responses and most likely to encounter and take up nanoparticles in vivo.

The inflammatory effects of nanoparticles have been addressed both in vitro and in vivo but we are far from understanding how nanoparticles affect the human health. Most knowledge is derived from studies on cell lines that are reproducible and well standardized assays. However, it is unclear how well these results translate to primary human cells. Only a few studies have tested the effect of nanoparticles on human Peripheral Blood Mononuclear Cells (PBMC). A Peripheral Blood Mononuclear Cell (PBMC) is any peripheral blood cell having a round nucleus. These cells consist of lymphocytes (T cells, B cells, NK cells) and monocytes, in humans, lymphocytes make up the majority of the PBMC population, followed by monocytes, and only a small percentage of dendritic cells. These cells can be extracted from whole blood by gradient centrifugation, which will separate the blood into a top layer of plasma, followed by a layer of PBMCs and a bottom fraction of polymorphonuclear cells (such as neutrophils and eosinophils) and erythrocytes.

Cell viability and lethality

Cell viability and lethality are the two parameters which are used to measure the toxicity caused by the nanoparticles. Among the various nanoparticles, carbon nanoparticles (CNTs) are used most frequently for assessment of viability and lethality of cells. They are widely used in chemical, industrial and biomedical applications due to their unique properties. They are synthesized as single-walled carbon nanotubes (SW-CNTs) and multi-walled carbon nanotubes (MW-CNTs). The anti-microbial properties of CNTs have been observed by studies in various bacteria due to the mechanical damage caused by the nanotubes. A recent study has indicated that functionalized CNTs affect soil bacterial diversity. The toxicity studies on a micro crustacean (Daphnia magna), freshwater microalgae (Raphidocelis subcapitata and Chlorella vulgaris) and a fish (Oryzias latipes) revealed inhibited the growth of the algae C. vulgaris and R. subcapitata with effective SW-CNT concentration 30.96 mg/ml and 29.99 m/l, respectively. The nanoparticles synthesized in the form of iron oxide were also reported toxic in murine macrophage cells, human macrophages, human hepatocellular carcinoma cells and rat mesenchymal stem cells. Metal oxide nanoparticles reported toxicity at 25-200 μg/ml for 2 h exposure on murine macrophage cells. The study observed effects include the decrease in cell viability. Another study reported a reduction in the cell viability when murine macrophage cells were treated with 0.1mg/ml iron oxide nanoparticles for 7 days. Another toxicity study which was performed on rat mesenchymal stem cells at 0.1 mg/ml for 2 days reported a decrease in cell viability. In the present study, the toxicity of the zinc and lead nanoparticles at different concentration was evaluated using human peripheral blood mononuclear cells (PBMC) and reported cell viability.

In vitro culture of human lymphocytes

In the present study, exposed primary human PBMC (peripheral blood mononuclear cells) to commercially available synthesized lead nitrate and zinc oxide (Pb(NO3)2 and ZnO) nanoparticles with the hypothesis that they may affect the human blood cells. Santosh 2013, PBMCs (Peripheral blood mononuclear cells) consist of chiefly monocytes and lymphocytes.

Preparation and characterization of Nanoparticles

Primarily synthesized nanoparticles ($Pb(NO_3)_2$ and ZnO NPs) through the use of fungus (Aspergillus niger) used in this assay. Lead nitrate and zinc oxide ($Pb(NO_3)_2$ and ZnO NPs) nanoparticles with the reported size of 45nm and 59.60 nm (microscopic characterization) was evaluated by the instrument JEOL JSM 1oocx (Jeol Ltd., 1400, Tokyo Japan).

Synthesized nanoparticles were stored as powdered form for further use. In prepared nanoparticles deionised water was carefully added and they were properly transferred to the centrifuge tubes. Centrifugation was carried out for 10min at 3000rpm and this process was repeated several times. Then after every centrifugation process the pallet was washed properly with deionised water. Finally the pellet was stored in a small size plate and for drying, it was kept in oven at 40 ° C for 8hrs till it was dried totally and zinc oxide nanoparticle was obtained in the form of powder.

Isolation of Peripheral Blood Mononuclear Cells Material and Reagents

Freshly collected heparinised blood, Ficoll Histopaque (Sigma-Aldrich, catalog number: 10771), Sterile PBS or Dulbecco's Modified Eagle Medium (DMEM) (catalog number: P-04-03590), Pencillin-streptomycin solution (Sigma- Aldrich, catalog number: P4333), W. B. C. diluting fluid (Qualigen, catalog number: 42425), Fetal bovine serum (catalog number:

1302-P100402, Pan Biotech), Trypan blue (catalog number: 193, Mediatech), DMEM supplemented with 10% FBS and 1% of Pencillin-streptomycin solution.

Equipment

Centrifuge machine with swing-out bucket rotors (Eppendorf, catalog number: 5810 R), Heparin vials (BD Biosciences, catalog number: 367886), Sterile 15ml centrifuge tube, Auto pipettes, 200 µl and 1ml tips, 24 well cell culture plate (TPP Techno Plastic Products), Haemocytometer, Tissue culture hood, CO2 incubator and Microscope.

Sample collection

PBMCs (Peripheral Blood Mononuclear Cells) consist of chiefly monocytes and lymphocytes. For the preparation of PBMC, blood sample were obtained from healthy blood donors (Harsa Nursing Home, Lucknow) collected in sterilized heparinized vials. The collected sample was maintains at 4°C and then sent to the laboratory within 1 hour.

Procedure

Mononuclear cells were isolated following to Ficoll's protocol. Human venous blood sample of about 4ml was collected in heparinised vials. The blood sample was mix well through gently inverting the tubes for several times. The sample was centrifuged for 10 min at 100 rpm then washed the cells with the use of 10ml of sterile Dulbecco's modified eagle medium that was without FBS. This process was repeated two times. Haemocytometer was used to count the cells: 10 µl of cell suspension was added to the 190 µl of W.B.C. diluting fluid then mix it well. The obtained cell suspensions were loaded in a haemocytometer then count the cells. Dulbecco's modified eagle medium that was supplemented with 10% FBS and 1% of Pencillin-streptomycin solution was used to adjust the concentration of cell at 1×10^6 cells/ml. From the blood sample (4 ml) the approximate yield of cells varies between 107-108. 500 µl of cell suspension was seeded in a 24 well culture plate. When PBMCs incubated at 37 ° C, the lymphocytes and monocytes were become attached to the plastic. Long-time incubation was result in a firm attachment. Due to the lack of glass adherent property of lymphocytes, mostly they were remained in suspension. Lymphocytes were removed through mildly flushing the wells using buffer and/or medium. Because of such process they firmly get attached to the surface of culture plates.

In order to monitor the viability of the isolated peripheral blood mononuclear cells, in to the cell suspension (200 µl) was added 200 µl of 0.4% of Trypan blue solution. Then incubate the mixture for 15 min. After that Trypan blue staining was used to ensure the cell viability and was scored under a microscope using haemocytometer. Under Trypan blue staining the cells were taken up blue stain were considered as dead cells. The cell suspension that was having more than 90% viability was used for culture.

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Exposure of PBMC to Nanoparticle Material and Reagents

- PDB
- ZnO nanoparticles (powdered form)
- Ultrasonic homogenizer
- LPS (Lipopolysaccharide)
- 1 x Annexin V binding buffer

The available Annexin V Binding Buffer was 10X concentrated. This buffer was composed of 1.4M NaCl, 0.2 µm sterile filtered 0.1M Hepes have pH 7.4 and 25 mM CaCl2 solution. But the buffer concentration need in this experiment was 1X. It was prepared prior to staining cells by diluting the 10X concentrate 1:10 using distilled water.

Procedure

In vitro culture of human lymphocytes was performed by Santosh K Panda and Balachandran Ravindran with slight modification. Powder of ZnO NPs was dissolved in Milli-Q water (1mg/ml). Through the use of an ultrasonic homogenizer the solution was ultrasonicated with a stepped micro tip. The prepared nanoparticle solutions were later on added to PBMC at 1×10^6 cells/ml. Cell culture in media alone or with LPS (0.1µg/ml) were used as control. For viability studies, nanoparticles were added in various concentrations ranging from 0.5µg/ml to 100µg/ml in 24-well plates with 1ml of culture medium per well for 24 h.

Cell viability, Annexin-V and PI staining

Cell viability of PBMC, exposed to metal oxide nanoparticles for 24h, was assessed using Annexin-V and PI (Propidium iodide) staining (BD). Approximately 1×10^6 peripheral blood mononuclear cell were stained with Annexin-V and PI following the protocol and assessed the viability. The reaction tubes contain harvesting cells was added 100 µl of Annexin-V binding buffer. Then added 4µl of PI (Sigma, Cat# P-4864-10ML) which was diluted with 1:10 in Annexin-V binding buffer and yield a final Propidium Iodide (PI) concentration of 2µg/ml in each sample. Then the tubes were incubated for 15 minutes in the dark at room temperature. To wash the cells added 500µl Annexin-V binding buffer and the samples were centrifuged for 10 minutes at 300 rpm and decanted the supernatant. Thereafter the cells were resuspended in 500µl 2% formaldehyde and 500 µl 1 x Annexin-V binding buffer to create a fixative 1% formaldehyde solution. The tubes were mixed through gentle flicking. Then the samples were fixed on ice for 10 minutes. After that in each sample was added 1ml 1 x PBS and mixed gently through flicking. The tubes were centrifuged at for eight minutes and decanted the supernatant and repeated this step. Then added 16µl of 1:100 diluted RNase A (Sigma, R4642) to give 50 µg/ml, a final concentration. The sample was incubated at room temperature for 15 min. Then added 1ml and mixed gently through flicking. Again the tubes were centrifuged at 400 rpm for 8 minutes. Finally the samples were ready to

be analyzed. Cells positive for Annexin-V were considered as apoptotic, double positive and PI positive cells were considered as late apoptotic or necrotic, whereas cells negative for both PI and Annexin-V were considered as live.

Effect on cell viability

To examine the effect of $Pb(NO_3)_2$ and ZnO NPs on the PBMC viability, performed a Annexin V and P I assay. In the present study, it was found that PBMC were strongly affected through increased doses of zinc oxide, with 54 ± 6% dead cells at 100 µg/ml, whereas $Pb(NO_3)_2$ did not stimulate any valuable or serious cell death. The amount of late apoptotic or necrotic and apoptotic PBMC were assessed after 24h of exposure to various concentrations of $Pb(NO_3)_2$ or ZnO NPs, through Annexin V and P I assay and analyzed respectively. Results are offered as % of cell death. In the present study it was analyzed that the total population of PBMC were more sensitive to ZnO nanoparticle induced cell death in comparison of $Pb(NO_3)_2$ NPs, emphasizing the significance of the study of cell populations when assessing exposure to different nanoparticles. Song 2010, ZnO NPs are soluble in nature and have ability to release zinc ions into the medium of cell culture. Although in humans zinc is present which is an essential trace element, where as an excess amount of zinc ions could be cytotoxic and can stimulate apoptosis in cell few types.

To identify potential subtoxic effects of ZnO nanoparticles on PBMC function, unveiled cells to a comparatively high, as non-toxic concentration of 10µg/ml. This value is likely to be much greater than what can be in needed from an environmental exposure. Even though there is only scanty data available on the value of nano-ZnO used in user yields, it has been stated that commercial sunscreen endues nanoparticulate ZnO at amounts limitations from 4 to 30% wt/wt. Gulson 2010, now a days using highly susceptive stable Zn isotopes (Zn68) as tracers have demonstrate that only succinct amounts of Zn from ZnO particles in sunscreens can passage through the defensive layers of undamaged skin unveiled to the sun in a original-life environment and be explored in urine and blood.

Conclusion

According to the result it was observed that the total population of PBMC was more sensitive to ZnO nanoparticle than $Pb(NO_3)_2$ nanoparticle and ZnO nanoparticle induced significant cell death. It is emphasizing the importance to study various cell populations when evaluating its exposure to metal oxide nanoparticles. Due to solublizing property of zinc oxide nanoparticles it can be dissolved easily and released essential zinc ions into the solution / cell culture medium. Although in human body essential trace element of zinc is present but zinc ions an excess amount could be toxic and have ability to induce apoptosis in few cell types [4].

Subtoxic effects of $Pb(NO_3)_2$ and ZnO nanoparticles on the function of PBMC was obtained through using the exposure of nanomaterials at different concentration. At the concentration of 10µg/ml where exposed cells were relatively high, found that no any significant change known as non-toxic concentration. Although this concentration amount is likely to become much higher in comparison of that can be expected through an environmental exposure. The use of nano-ZnO amount in consumer products there is only scarce data are available, according to few studies it has been obtained that commercial sunscreen contains the amount of Nano particulate ZnO ranging from 4 to 30% wt/wt.

To obtain more knowledge about the effect of ZnO concentration, few studies have been carried out recently. The use of Zn isotopes such as Zn68 i.e., highly sensitive and stable, as tracers shown that only few amounts of zinc from zinc oxide particles in sunscreens have ability to pass through / penetrate the undamaged skin's protective layer that exposed to the sun in a real- life environment and can be detected in urine and blood. The application period of after 5-days, total amount of tracer detected in blood was 1/1000th; it was total endogenous zinc in the blood compartment. According to the result, at the concentration range of 10 µg/ml neither ZnO nor $Pb(NO_3)_2$ nanoparticles altered surface marker expression on PBMC.

In summary, it was found that PBMC reacted in a dose dependent manner and increase in cell death via cell viability to ZnO nanoparticles indicating different sensitivity in different cells. Here, PBMC population were found to become strongly affected through increased doses of zinc oxide, with 54 ± 6% dead cells at 100 µg/ml, whereas $Pb(NO_3)_2$ did not stimulate any significant death of cells at this concentration range. Here it was concluded that the subtoxic concentrations of zinc oxide nanoparticles do significantly alter PBMC characteristics. Our results characterized the importance of toxicological readouts on blood cells of human when checking out whether nanoparticles even at subtoxic concentrations can affect biological functions [5, 6,7].

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