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Original Research Article

Effects of Cerium Oxide and Selected Heavy Metals on the Induction of Cell Death Via Oxidative Stress-Mediated DNA Damage

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ABSTRACT

Oxidative stress is a condition that can lead to cell death in humans and is defined as an increase in reactive oxygen species (ROS) in the body. Several heavy metals, such as copper sulfate, lead acetate, and zinc acetate, are capable of causing oxidative stress. However, researchers have not yet identified the precise mechanism through which these heavy metals induce oxidative stress. Some heavy metals, including cerium oxide, iron, cobalt, vanadium, and arsenic exhibit antioxidant properties. This study was therefore conducted to compare the effects of cerium oxide and selected heavy metals on cell death induction via oxidative stress- mediated DNA damage. Cerium oxide, copper sulphate, zinc acetate, and lead acetate were tested for their scavenging abilities using DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) and alkaline DMSO (dimethylsulfoxide) methods. To determine whether the heavy metals could affect kidney cells, toxicity assays on Vero cells were performed. Ultraviolet light was used to induce oxidative stress in Vero and bacterial cells. To investigate the effects of heavy metals on UV radiation-induced oxidative damage, DNA nicking assays were carried out. The results revealed that all the heavy metals exhibited oxidative stress. Only cerium oxide neutralized free radicals and was observed to have antioxidant properties. Also, heavy metals normally produce oxidative stress when exposed to UV radiation, but heavy metals such as cerium oxide prevent oxidative damage. The findings of this study reveal that cerium oxide has great potential for the treatment of oxidative stress-induced DNA damage.

Keywords: Cell death, Cerium oxide, DNA damage, Heavy metals, Oxidative stress, Reactiveoxygen species

Introduction

Oxidative stress arises when the ability of the body to rapidly eliminate or repair damage caused by free radicals is compromised. Anions such as hydroxyl radicals and hydrogen peroxide are included in this category.[1] Other reactive aldehydes include nitric oxide and superoxide dismutase (SOD), which catalyze the rapid hydrogen peroxide conversion into water via glutathione peroxidase (GPX) and catalase (CAT).[2-9] Because of its therapeutic and sometimes negative impacts on biological systems, oxygen is also known as the Janus gas. Because of its reactivity, oxygen can lead to significant redox reactions, causing significant amounts of adenosine-5-triphosphate (ATP) to be synthesized via oxidative phosphorylation.

This is critical for the growth of complex multicellular creatures, but it is not sufficient. It also makes every biological molecule susceptible to attack, including DNA, proteins, and lipids. As a result, it exposes the human body to the potentially harmful effects of reactive oxygen species (ROS), which may lead to oxidative stress. Due to oxidative stress, biological markers including redox-sensitive transcription factors such as AP-1, p53, and NFB10 are activated to control inflammatory cytokines, cell differentiation, and death. Pro-inflammatory cytokines are also released along with other cytokines.[10,11] There is also an increase in the activity of protein kinases.

Cell survival and proliferation are primarily encouraged by ROS-induced stimulation of the extracellularly regulating-3-kinase (ERK1/2), whereas cell death is primarily caused by activation of the p38MAPK (p38) or stress-activated protein kinase-c-Jun amino-terminal kinase (SAPK-JNK). When ROS levels are out of balance, intracellular Ca^{2+} equilibrium is affected, causing Ca^{2+} to be released from the endoplasmic reticulum and other storage sites.[12-14] Both free or bound amino acids can be harmed by oxidative stress. Protein aggregation and cell death can also happen from aberrant protein folding. Mutations in mitochondrial DNA happen five to ten times more than in nuclear DNA due to significant damage.[15] A decrease in energy output and an increased risk of subsequent electron leakage that cause DNA oxidation could occur from changes to the mitochondrial DNA, which code for numerous proteins, including enzymes in the electron transport chain.[16-20] An intricate cellular network of DNA repair pathways is used by a collection of proteins and enzymes that are dedicated to DNA maintenance. Both endogenous processes and external stressors frequently result in DNA damage, making these pathways essential to cell survival. If left unrepaired, double- strand breaks (DSBs) in the DNA helix are one of the more cytotoxic lesions that result in cell death. Homologous recombination (HR) and non-

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homologous end-joining (NHEJ) are the two main types of DSB repair used by mammalian cells. Genome instability brought on by errors in these repair pathways or incorrect DSB repair can lead to cancer. The choice to repair a DSB using these pathways is influenced by a variety of variables, and accumulating evidence indicates that these primary repair pathways cooperate and compete with one another at DSB sites to promote effective repair and genomic integrity.[21] The human body has developed a complex system of antioxidant defense system to shield itself from this attack. However, persistent disruptions in this balance may cause oxidative damage. Oxidative stress is the term for an imbalance between pro-oxidants and antioxidants that, if left unchecked, can result in tissue damage.[22] Several health issues, particularly cancer and CNS disorders, have recently been linked to oxidative stress.[23-25] The present study was aimed at comparing the effects of cerium oxide and selected heavy metals in the induction of cell death via oxidative stress-mediated DNA damage.

Materials and Methods

Sources of chemicals, microorganisms, and cell lines
Cerium chloride heptahydrate, copper sulfate, zinc acetate, and lead acetate were supplied by Sigma Aldrich (Coimbatore, India, Batch no: 18618-55-8). *Candida albicans,* which was used to synthesize cerium oxide were obtained from Sigma Aldrich India (Batch no: 19503). The Vero cell type. a cell line derived from the kidneys of an African monkey was purchased from NCCS Pune in India.

DPPH for radical scavenging assay
A 96-well microtitre plate was used for the experiment. To 200 µL of 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) solution, 10 µL of DPPH solution, and 10 µL of a test sample or the standard solution were transferred individually. The range of their final concentrations was between 1000 and 1.95 g/mL. At 490 nm, the absorbance of each well on the plates was determined using the ELISA reader, and the plates were then incubated at 37°C for 20 minutes. The remaining DPPH in each well was estimated. Free radical-scavenging capacity (IC50) was estimated as the percentage of DPPH molecules that can be eliminated by the sample.[26,27]

Scavenging of the superoxide radical by alkaline DMSO
The test heavy metals were dissolved in DMSO (0.3 mL) at various concentrations, yielding a final volume of 1.4 mL. Variable quantities of NBT (0.1 mg) were added to a nitroblue tetrazolium reaction mixture before the reactions were carried out. To complete the final volume, 1 mL of alkaline DMSO was added. The absorbance was measured at a wavelength of 560 nm.[28–31]

UV-radiation-based DNA damage assessment test
DNA nicking was performed using a mixture of 10 µL of DNA, 10 µL of the various concentrations of test solutions, and 4 µL of loading dye (0.25% bromophenol blue dye in 50% glycerol). The control standards were not analyzed under UV light. The mixtures (24 µL) were electrophoresed at 50 V for 2 hours on Tris–Borate–EDTA buffer (made by dissolving 2 mL of TBE in 50 mL of distilled water), followed by ethidium bromide staining.[32–35]

MTT assay for Vero cell lines
To better understand the origin of the Vero cell line, it was confirmed to originate from the kidneys of African monkeys. They were chosen because heavy metals are primarily excreted through the kidney, where they may severely harm kidney cells through oxidative stress- induced DNA damage.[36] They were kept at 37°C until they were needed. The tissue culture monolayer was tilted into 25 cm^2 flasks for storage, cleaning, and disposal. A fresh TPVG solution was added at room temperature and allowed to stand for the next few minutes. The TPVG solution was withdrawn, leaving the flask containing the monolayer empty, and incubated for 3 to 5 minutes at 37 °C. Using a suction cup and a tap, the cells were gently removed from the surface of the Vero cells. Ten milliliters of Dulbecco's Modified Eagle medium were pipetted into the flask along with 10% serum to help break up cell clumping. With the aid of a hemocytometer, the total number of remaining cells was counted. The bottles were filled halfway with a medium containing an appropriate number of cells (0.5-1.0 x 105 cells/mL). Subsequently, the required amount of neonatal calf serum (NBCS; 10% growth medium and 2% maintenance medium) was added. As the cells moved through a 37°CCO_2 incubator environment, morphological and contamination alterations in the cells were routinely observed. Vero cells were added to the monolayer after they had grown to a certain size.[36,37]

Cell death induction by AO/EtBr
Acridine orange was used to stain both living and dead cells with a bright orange color. Cells that had lost their membrane integrity were stained with ethidium bromide, while cells that were alive were always green. Due to DNA breakage and nuclear breakdown inside their nucleus during the cell death process, apoptotic beginning cells have brilliant green patches that give them the appearance of being green. Necrotic cells, in contrast to early apoptotic cells, had contracted nuclei that were prone to shattering.[38] Secondary apoptotic cells, for instance, would condense in ethidium bromide and turn orange. One animal cell (5000 l/well) was injected and grown in each of the six wells of a 6-well plate. A total of six wells were utilized to test for the components with the greatest concentrations. The cells were then cultivated for a range of times, including 24, 48, and 72 hours. Acridine orange and ethidium bromide were then added to the suspension, and the suspension was then covered with a coverslip using a fluorescent staining solution that contains 100 mg/mL of each dye and 100 mg/mL of ethidium bromide. In less than 20 minutes, fluorescent microscopes were used to observe the apoptotic cell shape and count the number of cells. Staining with AO/EB was done at least three times using acridine orange and ethidium bromide (AO/EB).[39,40]

Results and Discussion

Assessment of the scavenging capacities of test heavy metals
Heavy metals were subjected to preliminary radical scavenging studies using the DPPH radical scavenging assay as well as superoxide radical scavenging using the alkaline DMSO technique to determine their scavenging abilities. Heavy metals were created in amounts ranging from 25 to 100 g/mL, depending on the heavy metal. Figures 1 and 2 show the experimental evidence for the heavy metal radical scavenging capacities at 100 g/mL in radical scavenging tests, alkaline DMSO method superoxide radical scavenging, and heavy metal radical scavenging abilities. Highly significant inhibition was obtained in the DPPH scavenging tests and superoxide radical scavenging using an alkaline DMSO technique, followed by the nitric oxide inhibition assay. Cerium oxide, among other chemicals, had the highest scavenging activity when compared to copper sulfate, zinc acetate, and lead acetate. In this research, all of the heavy metals utilized were tested for their ability to scavenge the superoxide radical.

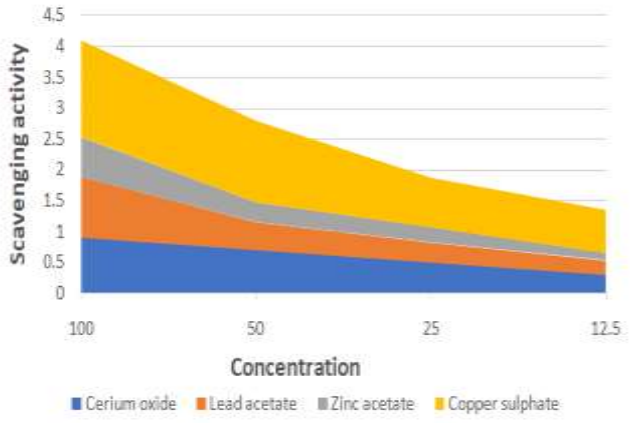

Figure 1: The DPPH scavenging assay. DPPH: 2,2-diphenyl-1-picryl-hydrazyl-hydrate

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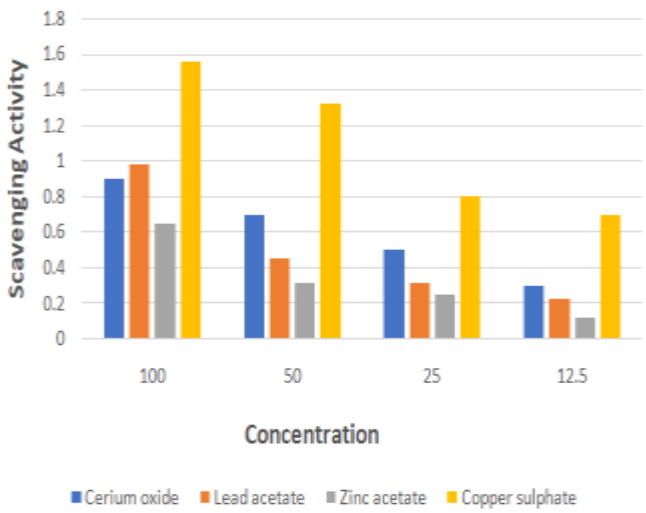

Figure 2: Alkaline DMSO scavenging assay. DMSO: Dimethylsulfoxide

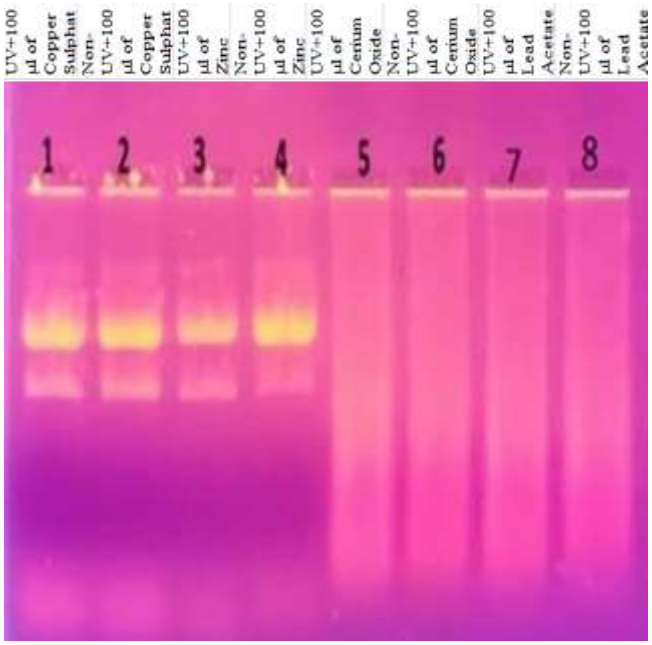

Figure 3: Evaluation of UV-induced DNA damage

Table 1: Compound composition for UV-induced DNA damage

Lanes	Compound	DNA
1	UV+100µl of Copper Sulphate	10µl pBR 322 DNA
2	Non- UV+100µl of Copper Sulphate	10µl pBR 322 DNA
3	UV+100µl of Zinc acetate	10µl pBR 322 DNA
4	Non- UV+100µl of Zinc acetate	10µl pBR 322 DNA
5	UV+100µl of Cerium Oxide	10µl pBR 322 DNA
6	Non- UV+100µl of Cerium Oxide	10µl pBR 322 DNA
7	UV+100µl of Lead Acetate	10µl pBR 322 DNA
8	Non- UV+100µl of Lead Acetate	10µl pBR 322 DNA

Superoxide radicals are among the most harmful to cell components since they are the precursor to more reactive species. The ability of antioxidants in the extract to scavenge superoxide is determined by a reduction in absorbance at 560 nm. By comparison, cerium oxide has a high potential in the alkaline DMSO technique (Figure 2).

UV-radiation-based DNA damage assessment

The effects of ultraviolet radiation on DNA damage were examined using the preliminary results of this study. The results revealed that certain heavy metals, such as cerium oxide and lead acetate, produced the least amount of oxidative and DNA damage when used at lower levels and without being exposed to ultraviolet light (Figure 3). However, when other heavy metals were exposed to UV radiation, they caused DNA damage and oxidative stress.

Cytotoxic effects of the test heavy metals

All heavy metals used in this study were assessed for cytotoxicity using Vero cell lines. It was possible to synthesize the chemicals at quantities ranging from 12.5 to 100 mg/mL. Except for cerium oxide, the heavy metals appeared to have a cytotoxic effect on the Vero cell line after a 24-hour exposure period. The cytotoxic effects of the compounds as measured against Vero cell lines are shown in Figure 4. It was intended to create lethal concentrations of the compounds, which would be used in cell line-based investigation. The results suggest that even small amounts of heavy metals can result in the death of normal cells.

Evaluation of apoptotic induction by the test heavy metals

The AO/EtBr fluorescent labeling technique was employed to detect cellular alterations in morphology after 24, 48, and 72 hours of exposure to heavy metals at their CTC_{50} values, respectively. Meanwhile, untreated Vero cells were color-coded blue throughout the experiment, necrotic cells appeared red. Major alterations in apoptotic induction were found at each time point following the application of heavy metal fractions to Vero cells for durations of 24, 48, and 72 hours. The results demonstrated that there was no evidence of apoptosis (Figure 5). The results from the DPPH and alkaline DMSO assays showed that at higher concentrations, all of the heavy metals may cause more oxidative stress. It was determined that concentration is linked to oxidative stress. When the highest concentrations of heavy metals were exposed to ultraviolet radiation, cerium oxide exhibited less oxidative stress than other heavy metals. Therefore, cerium oxide has high scavenging activity than other heavy metals. This is due to cerium oxide's ability to switch between the (+3) and (+4) oxidation states. Additionally, the existence of a mixed-valance state aids in the elimination of reactive oxygen species from the environment. The cytotoxicity of other heavy metals and cerium oxide on Vero cell lines was investigated. It was decided to use a normal cell line to better understand the impact of heavy metals on healthy cells, specifically regarding the production of oxidative stress. All heavy metals, except for cerium oxide, were toxic to Vero cell lines after only 24 hours of exposure. This series of research also showed that heavy metals, even at low concentrations, can destroy healthy cells. Cerium oxide showed higher viability of 80% at 530 nm and 78% at 630 nm, indicating that Vero cells were not subjected to as much oxidative stress. Other heavy metals displayed viability of 61 and 59% at 530 and 630 nm, respectively, highlighting the production of severe oxidative stress in Vero cells. This resulted in a considerable increase in cell survival for both cerium oxide and lead acetate $(CH_3COO)_2$, indicating that both compounds had a long-term effect on lowering oxidative stress in the Vero cell line. Most heavy metals, including cerium oxide, have been demonstrated to interact with proteins and DNAs, and produce pathogenic genes in laboratory animals. The morphological alterations that take place in cells upon exposure to heavy metals at the CTC_{50} concentration of the heavy metal were examined using the fluorescent labeling agent, AO/EtBr. Orange staining was utilized in the samples to differentiate among apoptotic cells containing condensed nuclei with apoptotic bodies in the samples.[41] The necrotic cells changed color to red at that point, whereas the Vero cells that were not treated remained green. It was observed that there were considerable modifications in the development of apoptotic and necrotic cell death in Vero cells.

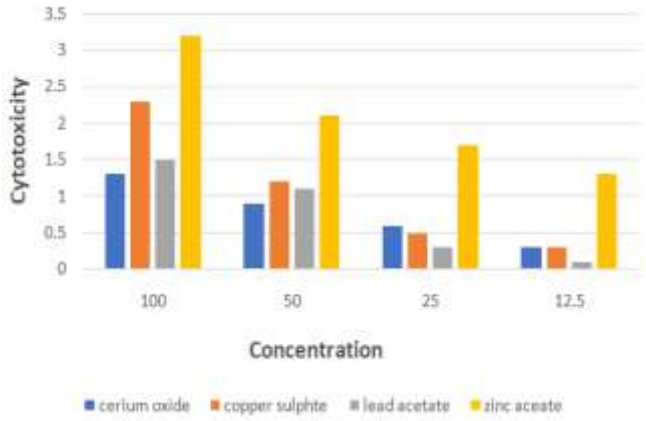

Figure 4: MTT cytotoxicity assay. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

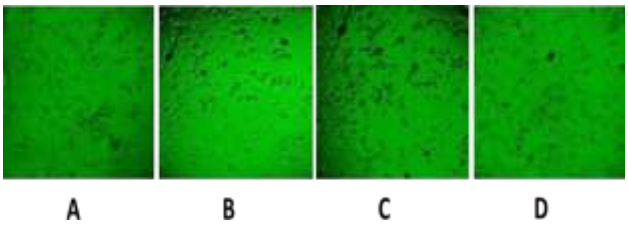

Figure 5: Apoptosis induction by AO/EtBr staining method.
A: CeO_2(Cerium oxide); B: $ZnC_4H_6O_4$(Zinc acetate);
C: $CuSO_4$(Copper sulphate);D: $Pb(C_2H_3O_2)_2$(Lead acetate)
DNA damage.

Conclusion

The efficacy of the test heavy metals and cerium oxide for their oxidative stress-induced DNA damage via the ability to scavenge free radicals, cytotoxic properties, induction of oxidative stress, and potential interactive effects with proteins and DNAs were demonstrated. The findings of this study reveal that cerium oxide has shown promising results in the treatment of oxidative stress-induced DNA damage. Furthermore, these investigations have opened the possibility of further research on the creation of drugs based on heavy metals for the treatment of numerous disorders.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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