Cytotoxic effects of CeO₂ NPs and β-Carotene and their ability to induce apoptosis in human breast normal and cancer cell lines

Ali A.A. Al-Ali, Khalid A. S. Alsalami*, Ahmed Muhssin Athbi
Department of Biology, college of Education for pure science, University of Basrah, Basrah, Iraq

Abstract
Cerium Oxide Nanoparticles (CeO₂ NPs) and β-carotene are natural-source products that have recently gained an increased interest as pharmaceutical additives because of their effectiveness in living systems, but the behavior of these substances varies according to factors and conditions. The above mentioned materials were evaluated in breast normal (HBL-100) and cancer cell lines (CAL-51 and MCF-7) by different techniques: MTT assay for studying cytotoxic effects, morphological changes, sqPCR, including gene expression of caspases 8 and 9, and P53. All experiments were conducted on cell lines by the use of the materials alone as well as their combination.

The results of the MTT assay showed that the two materials (CeO₂ NPs and β-carotene) had a toxic effect on the cell lines, and the toxicity of the materials was concentration-dependent. The half maximal inhibitory concentration (IC₅₀) values of CeO₂ NPs and β-Carotene, as observed by the effects in all cell lines, have significant differences.

Our findings showed that CeO₂ NPs has a higher toxicity on the HBL-100 and MCF-7 cell lines compared with β-Carotene. The toxic effects of each material alone and combined together were reflected on cell morphology by the appearance of irreversible cytopathic changes in the treated cells. The two materials had the ability to cause cellular death and DNA damage, inducing the external pathway of apoptosis. A significant increase in the gene expression of caspase 8 appeared at P≤0.05. Collectively, the CeO₂ NPs and β-Carotene have an inhibitory effect on both breast cancer and normal cell lines. We conclude the both materials have genotoxic effects on normal and cancer cell lines.

Keyword: CeO₂ NPs, β-Carotene, cell lines, cytotoxic effect

*Email: Khalidsasalami75@gmail.com
Introduction

Nature is the main source of bio-effective materials that are used for the treatment of different diseases, such as cancers [1]. Nano particles are natural materials which have a potential effect against tumor [2]. It is considered as an important candidate for the treatment of some oxidative diseases, such as neurodegenerative disease, ischemic cardiopathy, ocular disease, diabetes, and cancer [3]. Studies reported that CeO$_2$ NPs have various levels of cytotoxicity. Some studies indicated that CeO$_2$ NPs have low toxicity [4], while others suggested that CeO$_2$ NPs cause high toxicity that can lead to cellular death [5]. Other researches pointed to the role of CeO$_2$NPs in inhibiting certain types of cancer cell lines [6]. Despite these results, there is a paucity of information on the role of CeO$_2$NPs against different type of tumors.

Over recent years, phytochemicals were used as chemotherapy for different diseases [7]. The redox role of β-carotene was proven by previous studies [8]. Also, few previous investigations revealed that β-carotene has the potential as an antitumor compound [9]. Some work showed that the higher the concentration of β-carotene, the higher the rate of inhibition of cancer cells [7, 10].

The present study aimed to detect the roles of CeO$_2$ NPs and β-Carotene in breast cancer and normal cell lines, i.e. whether they have cytotoxic effects on cell lines or not. In addition, we aimed to investigate the combined effects of CeO$_2$ NPs and β-Carotene on the cell lines. The present study also aimed to investigate the possible ability of the two materials to induce apoptosis in breast cell lines.

Materials and methods

Cell maintaining

The cell lines, HBL-100 normal human breast epithelial cell line and tow human breast cancer cell lines that included CAL-51 and MCF-7, were obtained from the tissue culture laboratory in the College of Education for Pure Sciences of Basra University. The cells were maintained in RPMI 1640 culture medium with 10 % fetal bovine serum (FBS, Euroclone – Italy) and 100 mg/ml streptomycin/ampicilin at 37 °C and 5 % CO$_2$. After trypsinization, the cells were resuspended with RPMI with 10% FBS in a new T-25 flask, then incubated at 37 °C and 5 % CO$_2$.

Cytotoxicity assay

The MTT assay was used to study viability of cell lines [11]. Briefly, the cells, after resuspension, were seeded in 96 well plates at 1x10$^4$ with 100 ul RPMI medium supplemented with 10 % fbs, then incubated at 37 °C with 5 % CO$_2$ in a humidified incubator for 24 h. The series dilutions of CeO$_2$ NPs (<5nm) were prepared by serum free medium.
while β-Carotene was prepared by serum free medium with less than 1% DMSO (Dimethyl sulfate). Volumes of 100 µl of CeO₂ NPs (<5nm) (0, 2.5, 10, 20, 30, 50, 90 µg/ml) and β-Carotene (0, 5, 15, 50, 150, 300, 500 µg/ml) were then added. Triplicates of each concentration of the treated and untreated cells were used, then the plates were incubated at 37°C with 5% CO₂ in a humidified incubator for 72 h. The cell viability was calculated as follows: t/c×100, where t is treatment and c is control. Experiments were repeated three times. Then, the half maximal inhibitory concentration (IC₅₀) was determined using Graphpad Prism 8.0.1 software [12].

Combination effect
To study the combined effect of β-C and CeO₂ NPs, the MTT assay was also utilized [11]. Then, the cells were treated with a combination of CeO₂ NPs + β-C with IC₅₀ values of 20+50 µg/ml and 50+300 µg/ml of CeO₂ NPs + IC₅₀ β-C. The plates were incubated at 37°C with 5% CO₂ in a humidified incubator for 72 h. The cell inhibition was calculated as in the formula: (c-t/c)×100, where t is treatment and c is control [12]. Then, the data were analyzed by the Compusyan Isobologram sofetware, Chou-Talalay method [13].

Morphologic study
The cells were seeded at 5x10⁴ cells/well in a slide chamber, incubate for 24 h, then treated with the IC₅₀ of CeO₂ NPs and β-C for 72 h. Then, the cells were stained with Haematoxylin & Eosin (H&E) for morphological changes study. Briefly, the old medium was first discharged. After the slides were passed through a serial concentration (70, 90, and 100%) of ethanol, they were treated with Haematoxylin and Eosin stains, respectively, for one minute each, covered with a cover slide, and examined by a light microscope [14]. The Acidin Orange / Ethidium Bromide (AO/EB) stains were used for the cytopathic study. Briefly, the medium was discharged and 150 µl of AO/EB was added for 20 second, then the slide was covered with a cover slide and examined by a fluorescent microscope [15].

DNA fragmentation
DNA fragmentation was performed by agarose gel electrophoresis [16]. Polluted cells were lysed by using the GENAID DNA extraction kit (Genaid Biotech Ltd., Taiwan). The extracted DNA was loaded on 1.5% agarose gel. The gel was powered at 85 volts for 55 min.

Gene expression analysis
The total RNA was extracted from untreated and treated cells by utilizing the TriRNA Pure Kit (GEBEZol™, USA). The cDNA was prepared by utilizing the BIONEER Accu Power® kit (BIONEER, USA). The expression of caspases 8 and 9 and p53 genes was evaluated by sqPCR. Briefly, the total volume per reaction (12.5 µl qPCR master mix, 1 µl forward primer, 1 µl reverse primer, 5.5 µl nuclease-free water, and 5 µl cDNA) was amplified using a thermo cycler, then PCR products were elecrophoresed using 1.5 agarose gel. After visualization, bands were analyzed using an Image Lab Bio-Rad V6 software. The expression of each sample was studied by dividing target gene by housekeeping gene values. The sequences of the primers are shown in Table 1.

| Table 1 - The sequences of the sets of primers |
|-----------------------------------------------|
| Sequence                                      | Target genes   | Reference |
|-----------------------------------------------|----------------|-----------|
| 5'-GGAGTTGCAGTGGTTCACCCATCGAGCT-3' 129bp      | Hs_Caspase 9_FWD4 | [17]      |
| 5'-CAACGTACAAAGACAGGCACCTT-3                 | Hs_Caspase 9_REV4 |          |
| 5'-CATCGGTCAGTGAGTACAGTCTCAGG-3 128bp        | Hs_P53_FWD4    | [17]      |
| 5'-TGATGTGTGGAGTACAGTCTCAGG-3                | Hs_P53_REV4    | [17]      |
| 5'-CATCGGTCAGTGAGTACAGTCTCAGG-3 128bp        | Hs_Caspase8_FWD |          |
| 5'-GGAGTTGCAGTGGTTCACCCATCGAGCT-3' 129bp      | Hs_Caspase8_REV |          |
| 5'-GGAGTTGCAGTGGTTCACCCATCGAGCT-3' 129bp      | R Hmn rRNA 18s  | [19]      |
| 5'-GGAGTTGCAGTGGTTCACCCATCGAGCT-3' 129bp      | F Hmn rRNA 18s  | [19]      |
Statistical Analysis

IC\textsubscript{50} values for β-Carotene and CeO\textsubscript{2} NPs for each cell line were compared using SPSS software for T test. IC\textsubscript{50} values for each material were compared between the cell lines using SPSS software for the One Way Analysis of Variance (ANOVA). The values of gene expression were analyzed using SPSS software for T test.

Results and Discussion
Cytotoxicity of CeO\textsubscript{2} NPs

The results of the current study showed that CeO\textsubscript{2} NPs (<5 nm) had effects on the viability of CAL-51, MCF-7, and HBL-100 cell lines. The viability of cells was decreased in a concentration-dependent manner, as shown in Figure 1 A. The values of IC\textsubscript{50} were 46.21 µg/ml for CAL-51, 43.23 µg/ml for MCF-7, and 39.82 µg/ml for HBL-100 cell lines (Figure 1-B). The results collectively showed that CeO\textsubscript{2} NPs (<5 nm) had a similar trend of cytotoxic effect on all cell lines (malignant and normal). This cytotoxic effect may be attributed to the small size of CeO\textsubscript{2} NPs. Our finding corresponds with previous studies [20, 21, 22]. Some studies showed that the small size of CeO\textsubscript{2} NPs had the ability to generate Reactive Oxygen Species (ROS) [23, 24, 25]. The mechanism of ROS generation is dependent on the chemistry of CeO\textsubscript{2} NPs (Ce\textsuperscript{3+} / Ce\textsuperscript{4+} valence ratio) [26, 27]. ROS plays multiple functions in cellular biology. ROS generation is a key factor in metallic NP-induced toxicity, as well as modulation of cellular signaling involved in cell death, proliferation, and differentiation [28]. The results revealed that CeO\textsubscript{2} NPs had significant cytotoxic effects when compared with the control and that these effects were dose-dependent. Diaconeasa and co-authors [29] observed significant difference in cytotoxicity of CeO\textsubscript{2} NPs on cancer and normal cell lines. Since the cellular responses to cytotoxic agents differ, the cytotoxicity of CeO\textsubscript{2} NPs varied considerably between different cell lines [30].

Cell viability assays showed that β-Carotene has the ability to decrease the proliferation of MCF-7, CAL-51, and HBL-100 cell lines, and these effects were increased by increasing the concentration. Figure 1-C shows that the percentage of viability peaked in lowest concentrations compared with highest concentration in the three cell lines. Therefore, the present study suggest that the high concentrations (150, 300, and 500 µg/ml) of β-carotene have higher cytotoxic effects than those of the low concentrations (5, 15, and 50 µg/ml). Another study showed a similar finding on LNCAP cell line [7]. β-Carotene has multiple-potent biological effects, including its pro-oxidant role in stimulating ROS production in high concentrations [7], cell cycle arrest, DNA damage [31], and cell death[30]. Figure 1-D shows that the IC\textsubscript{50} values of β-Carotene were 58.45, 165.30, and 82.47 µg/ml in CAL-51, MCF-7, and HBL-100 respectively. The statistical analysis showed a significant difference at P≤ 0.05 between these values, except the IC\textsubscript{50} values of HBL-100 and CAL-51 cell lines. These results may be due to the nature of cell lines in relation to steroid hormones (estrogen, androgen, and HER2) [32, 33]. = β-Carotene leads to the inhibition of receptors of steroid hormones [34]. Hence, the sensitivity of CAL-51 and HBL-100 cell lines is higher than that of MCF-7. Table 2 shows that the IC\textsubscript{50} value of β-Carotene was significantly higher than that of CeO\textsubscript{2}NPs on MCF-7 and HBL-100 cell lines, while no significant difference in IC\textsubscript{50} values of these materials was observed on CAL-51 cell line at P≤ 0.05. This indicates that CeO\textsubscript{2} NPs was more toxic than β-Carotene on MCF-7 and HBL-51 cell lines.
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Iraqi Journal of Science, 2022, Vol. 63, No. 3, pp: 923-937

The results of Compushyan Isobologram analysis of the combination index (CI) for three concentrations of both CeO₂ NPs and β-Carotene for 72 h indicate synergistic effects with the second concentration (IC₅₀) in all cell lines. While, for the first concentration (20+50µg/ml), such effects were observed in MCF-7 cell line only, since the CI value was <1 (Tables 3, 4, 5 and Figure 2). In fact, each of β-carotene and CeO₂ NPs were found to generate ROS [35] and apoptosis [8, 36]. Therfore, the synergistic effects occurred upon combining those materials, as noticed by the increased levels of ROS and apoptosis. This finding might have occurred due to the association between cell cycle arrest induced by β-carotene [37] and the cytotoxic effect caused by CeO₂ NPs [7]. We suggest that the synergistic action was caused by independent behavior of each substance in cell inhibition.

Figure 1-A: viability ratios of HBL-100, Cal-51, and MCF-7 cell lines treated with CeO₂ NPs (<5nm) for 72 h. B: IC₅₀ of CeO₂ NPs (<5nm) of HBL-100, Cal-51, and MCF-7 cell lines. C: Viability ratios of HBL-100, Cal-51, and MCF-7 cell lines treated with β-Carotene for 72h. D: IC₅₀ of β-Carotene for HBL-100, Cal-51, and MCF-7 cell lines.

Table 2: A comparison between the IC₅₀ values of CeO₂ NPs (<5nm) and β-Carotene against breast cancer and normal cell lines.

| Cell lines | CeO₂ NPs<5nm (mean±Std. Error) | β-Carotene (mean±Std. Error) | Sig. |
|------------|-------------------------------|-------------------------------|------|
| MCF-7      | 43.24±2.89                    | 165.3±8.45                   | *    |
| HBL-100    | 39.82±2.12                    | 82.48±12.61                  | *    |
| CAL-51     | 46.21±5.40                    | 58.4±0.76                    |      |

*The stars refer to significant differences.

The combined effects of CeO₂ NPs + β-Carotene

The results of Compushyan Isobologram analysis of the combination index (CI) for three concentrations of both CeO₂ NPs and β-Carotene for 72 h indicate synergistic effects with the second concentration (IC₅₀) in all cell lines. While, for the first concentration (20+50µg/ml), such effects were observed in MCF-7 cell line only, since the CI value was <1 (Tables 3, 4, 5 and Figure 2). In fact, each of β-carotene and CeO₂ NPs were found to generate ROS [35] and apoptosis [8, 36]. Therfore, the synergistic effects occurred upon combining those materials, as noticed by the increased levels of ROS and apoptosis. This finding might have occurred due to the association between cell cycle arrest induced by β-carotene [37] and the cytotoxic effect caused by CeO₂ NPs [7]. We suggest that the synergistic action was caused by independent behavior of each substance in cell inhibition.
Morphological changes of cells

The microscopic examination of HBL-100, CAL-51, and MCF-7 cells showed different cytopathological changes after 72 h of treatment with the materials (CeO$_2$ NPs >5nm and β-carotene). The morphological changes were similar considerably between cell lines treated with the materials, as shown for HBL-100 (Figure 3 (A), CAL-51 (Figure 3 (E), and MCF-7 (Figure 4 (C) cells. The atrophy, along with the spherical and irregular shapes, observed in cells are among the hallmarks of the cytopathic effect. The cytoskeleton contributes significantly to the maintenance of cell shape. Previous studies showed that CeO$_2$ NPs can interfere with cytoskeleton organization [38]. Also, β-carotene caused modifications in many cytoskeleton proteins, such as Lamin B1, Tubuline, Flotillin, and Cavalin2 [39]. Hence, any change in the organization of the cytoskeleton causes changes in the shape of the cell [40]. These events may interpret the cytomorphological changes in the cells. Other changes observed in this study include the large spaces that appeared in cell cultures, which are due to atrophy, shrinkage, and decomposition of cells (Figure 4 A, E). These ongoing morphological changes were brought about by the exposure to CeO$_2$ NPs and β-Carotene, accompanied with
Karyopyknosis and hyperchromy of the nuclei (Figures 3 (B and F) and 4 (D)). Karyopyknosis is one of the characteristics of cell that is suffering apoptosis [24, 41]. Moreover, necrosis is another cell injury which was observed (Figure 3 F and 3 D) which might be attributed to free radicals (ROS) [42].

Cytoplasmic vacuolation was also observed in treated cells (Figures 3 C) and 4 A and E as a defensive step to isolate CeO$_2$ NPs and $\beta$-Carotene from the internal environment [43]. Nucleic fragmentation and apoptotic bodies were noticed in cells treated with $\beta$-Carotene, changes that indicated the induction of apoptosis [41]. In addition, multinucleated cells appeared in some cases (Figure 4 E). This may be due to the fusion of cells with each other or the impairment in cytokinesis [44].

The damage was further progressive when the cells were treated with the combination of the substances. The cells suffered from diverse cytormorphological changes (Figures 3 D and 4 B and F).

**Figure 3**- Morphology of HBL-100 and Cal-51 cell lines stained with H&E - A: Untreated HBL-100 cells grown in monolayer with normal morphology 400x; B: treated cells at IC$_{50}$ of CeO$_2$ NPs for 27h, showing changes in cell shape (arrow), necrosis (arrow head), karyopyknosis (double arrow), decay of some cells (circle), and large spaces between cells (stars), 1000x; C: treated cells at IC$_{50}$ of $\beta$.C., showing vaculation of cell cytoplasm (double arrow), nuclei decomposition (arrow), large spaces between cells (stars), and decomposition (circle); D: cells treated with the combination of IC$_{50}$ (CeO$_2$ NPs + $\beta$.C.), showing cytoplasmic vacuolation (arrows), kidney-shaped nucleus (dabble arrows), necrosis (head arrows), large spaces between cells (stars), and decaying cells (circle). E: untreated Cal-51 cell line grown in monolayer with normal morphology, 1000x; F: treated cell line at IC$_{50}$ of CeO$_2$ NPs, showing atrophy of hyperchromic cells (arrows), necrosis (head arrows), nucleic fragmentation (double arrow), and large spaces between cells (stars).
Figure 4 - Morphology of HBL-100 and Cal-51 cell lines stained with H&E. A: treated Cal-51 cell line at IC₅₀ of β.C., showing changes to spherical-shape (arrow), necrosis (head arrow), formation of apoptotic bodies (double arrow), and presence of large spaces between cells (stars), 1000x; B: cells treated with the combination of IC₅₀ (CeO₂ NPs + β.C.), showing conversion of cells to spherical-shape (double arrows), karyopyknosis (arrows), necrosis (head arrows), and large spaces between cells (stars); C: untreated MCF-7 cell line grown in monolayer with normal morphology, 400x. D: treated cells at IC₅₀ CeO₂ NPs, showing vacuolative degeneration (double arrows), change in cell shape (black arrows), necrosis (head arrows), karyopyknosis, hyperchromy of some cells (white arrows), absence of large spaces between cells (stars), and cell decomposition (circle); E: treated cells at IC₅₀ of β.C., showing karyopyknosis (black arrows), multinucleated cells (double arrow), and necrosis (arrow head); F: treated cells with the combination of IC₅₀ (CeO₂ NPs + β.C.), showing degradation (arrows), necrosis (head arrows), nuclei degradation, hyperchromy (arrows), large spaces between cells (stars), and decomposition (double arrow).
Cell death

The AO/EB assay was used to observe the differential uptake of fluorescent DNA-binding stain AO/EB [15]. The untreated cells were stained with green color, indicating intact cells (Figures 5 A, E and 6 C ), while some of the cells treated with the IC_{50} of CeO_{2} NPs, β-Carotene, and their combination for 72 h were stained with yellow or red color, which indicates cell death (Figures 5 (B, C, F) and 6 (A,B, D, E,F )). The cytomorphology that processed by AO/EB protocol is at least shown to, if not ensure than, mechanism of materials, cytotoxic effect on cell lines,. In addition, the results confirm necrosis incidence in all cell lines. The staining with red indicates that the necrotic and apoptotic cells suffered from a defect in permeability, which caused the penetration of EB through the plasma membrane and nuclear envelope, consequently leading to their contact with the nuclear material of the cell [45]. The yellow cells are in an ongoing pathway to death, appearing as pro-apoptotic or pro-necrotic cells [13]. Untreated cells were stained with green color of AO stain, an indication of the integrity of their membranes [45].

Figure 5- AO/EB staining of cells. A- untreated HBL-100 cells; B- cells treated with IC_{50} of CeO_{2} NPs; C - cells treated with IC_{50} of β.C; D- cells treated with the combination of IC_{50} (CeO_{2} NPs & β.C.). E- untreated CAL-51 cells; F- treated cells at IC_{50} of CeO_{2} NPs. The arrow refers to the nucleus the double arrow refers to the cytoplasm , the green arrow refers to the living cells , and the red arrow refers to the dead cells (400x magnification).
DNA fragmentation

Figure (7 A, B, C) shows the DNA fragmentation in MCF-7, CAL-51, and HBL-100 cells treated with CeO$_2$ NPs and its combination with β-Carotene. Our findings demonstrate that there was DNA cleavage at $\leq 100$ bp in all treated cells. In addition, there was a smear on the agarose gel for all treated cells. The DNA fragmentation did not appear in untreated cells and cells treated with β-Carotene. Moreover, the results indicate that the CeO$_2$ NPs overlapped with the DNA and altered the metabolic function, then leading to defects in cellular components [46]. The small-sized CeO$_2$ NPs induced primary DNA lesions. This DNA damage was reported to be induced by oxidative stress [24]. These results suggest DNA damage that indicates the induction of apoptosis.

Figure 6- AO/EB staining of cells A- Treated Cal-51 cells with IC$_{50}$ of β.C, 400x; B- Cells treated with the combination of IC$_{50}$ (CeO$_2$ NPs + β.C.); C- Untreated MCF-7 cells; D- cells treated with the IC$_{50}$ of CeO$_2$ NPs; E- Cells treated with the IC$_{50}$ of β.C.; F- Cells treated with the combination of IC$_{50}$ (CeO$_2$ & β-C), 1000x. The arrow refers to the nucleus, the double arrow refers to the cytoplasm, the green arrow refers to the living cells, the red arrow refers to the dead cells.
Figure 8 (A and D) shows that the level of caspase-8 gene expression in HBL-100 cells was significantly higher in cells treated with β-Carotene than in cells treated with CeO₂ NPs. In contrast, CAL-51 cells treated with CeO₂ NPs showed significantly higher caspase-8 gene expression at P≤0.05. There was no significant difference in caspase-9 and p53 gene expression in all treated cells at P≤0.05. This indicates that apoptosis was carried out via the extrinsic death domain. The two substances (CeO₂ NPs and β-Carotene) had the ability to cause cell death and DNA damage in cell lines and induced the external pathway of apoptosis [47].
Figure 8- Gene expression of caspase8, caspase9, p53 in HBL-100 and CAL-51 cells treated with the IC$_{50}$ of CeO$_2$ NPs (CeO$_2$), β-Carotene (β-C), and untreated cells (control). A- The increase of caspase 8 expression in HBL-100 cells treated with β-C compared to untreated cells and cells treated with CeO$_2$ NPs at P≤0.05. D- The increase of caspase 8 gene expression in CAL-51 cells treated with IC$_{50}$ of CeO$_2$ NPs compared to untreated cells at P≤0.05. B and E- No significant deference in caspase 9 expression in the two cell lines; C and F- No significant difference in p53 expression in the two cell lines.
In conclusion, CeO$_2$ NPs have more toxic effects on MCF-7, CAL-51, and HBL-100 cells than β-Carotene, while both substances have no selectivity on cell lines. Our findings suggest that the genotoxic effects of both materials occur through the apoptosis and necrosis pathways.

References
[1] I. Binic, L. Milanka, L. Viktor and M. Jelena, "Skin ageing: Natural weapons and strategies," Evidence-based Complementary and Alternative Medicine, pp. 1-10, 2013.
[2] S. Stankic, S. Suman, F. Haque and J. Vidic, "Pure and multi metal oxide nanoparticles synthesis, antibacterial and cytotoxic properties," J Nanobiotechnology, vol.14, no. 1, p. 73, 2016.
[3] K. B. Narayanan and H. H. Park, "Pleiotropic functions of antioxidant nanoparticles for longevity and medicine," Advances in Colloid and Interface Science; vol, 202, pp, 30–42, 2013.
[4] M. Culcasi, L. Benameur, A. Mercier, C. Lucchesi, H. Rahmouni, A. Asteian, A. Botta, H. Kovacic, and S. Pietri, "EPR spin trapping evaluation of ROS production in human fibroblasts exposed to nanoceria: evidence for NADPH oxidase and mitochondrial stimulation," International Journal of Toxicology, vol. 199, no. 3, pp, 161–176, 2012.
[5] J. Bach, J. Peremarti, B. Annangi, R. Marcos, and A. Hernandez, "Oxidative DNA damage enhances the carcinogenic potential of in vitro chronic arsenic exposures," Archives of Toxicology, vol. 90, 1893–1905, 2016.
[6] M. Pesic, A. Podolski-Renic, S. Stojkovic, B. Matovic, D. Zmejkoski, V. Kojic, G. Bogdanovic, A. Pavicevic, M. Mojovic, A. Savic, I. Milenkovic, A. Kalauzi and K. Radotic, "Anti cancer effects of cerium oxide nanoparticles and its intracellular redox activity," Chemico-Biological Interactions, vol. 232, pp. 85–93, 2015.
[7] J. Dulińska , D. Gil, J. Zagajewski, J. Hartwich, M. Bodzioch, Dembińska- A. Kiec, T. Langmann, G. Schmitz and P. Laidler, "Different effect of beta-carotene on proliferation of prostate cancer cells," Biochimica et Biophysica Acta., vol. 30, no.1740, pp.189-201, 2005.
[8] N. F. Haddad, A. J. Teodoro, F. L. de Oliveira, N. Soares and R. M. de Mattos, "Lycopene and Beta-Carotene Induce Growth Inhibition and Proapoptotic Effects on ACTH-Secreting Pituitary Adenoma Cells," PLoS One, vol.11, no. 2, 149-157, 2016.
[9] A. K. Hall, "Liarozole amplifies retinoid-induced apoptosis in human prostate cancer cells," Anti-Cancer Drugs, no. 7, 312–320, 1996.
[10] H. A. Lee and S. Park, "Effect of β-carotene on cancer cell stemness and differentiation in SK-NE-BE(2)C neuroblastoma cells," Oncology Reports, pp. 1869-1877, 2013.
[11] R.I. Freshney, "Culture of Animal cells: A manual of basic technique 6th ed," Wiley –Black Well, A John Wiley & Sons , Inc Pub. New York, 2010.
[12] A.M. Al-Shammary, W.N. AL-Esmaeel, A.A. AL-Ali, A.A. Hassan and A. A. Ahmed, "Enhancement of oncolytic activity of Newcastle disease virus through combination with Retonic acid against digestive system malignancies," molecular Therapy, Vol. 27, No.451, 2019.
[13] K. Liu, P.C. Liu, R. Liu and X. Wu, "Dual AO/EB staining to detect apoptosis in osteosarcoma cells compared with flow cytometry," Medical science monitor basic research, Vol. 21, pp. 15-20, 2015.
[14] S. Kasibhatla, G. P. Amarante-Mendes, T. Brunner and D. R. Green, "Analysis of DNA Fragmentation Using Agarose Gel Electrophoresis," Cold Spring Harb Protoc, pp. 4429-4440, 2006.
[15] N. Ould-Moussa, M. Safi, M. Guedeau-Boudeville, D. Montero, H. Con-jeaud and J. Berret, "In vitro toxicity of nanoceria effect of coating and stability in biofluids," Nanotoxicology, no. 7998, pp. 811-822, 2014.
[16] S. Khan, A. Ansari, C. Rolfo, A. Coelho, M. Abdulla, K. Al-Khayal and R. Ahmad, "Evaluation of in vitro cytotoxicity, biocompatibility, and changes in the expression of apoptosis regulatory
proteins induced by cerium oxide nanocrystals," Science and Technology of Advanced Materials, 2017.

[17] R. Prasad, H. Wallace, T. Tennant, D. Kitchin and C. Kligerman, "The Genotoxicity of Titanium Dioxide and Nanoceria in Vitro, Presented at Genetics & Environmental Mutagenesis Society," Chapel Hill, North Carolina, 2010.

[18] S. Balaji, B. Mandal and D. Sen, "Biogenic Ceria Nanoparticles (CeO₂ NPs) for Effective Photocatalytic and Cytotoxic Activity," Bioengineering, pp. 7-26 2020.

[19] S. Mittal and A.K. Pandey, "Cerium oxide nanoparticles induced toxicity in human lung cells: Role of ROS mediated DNA damage and apoptosis," BioMed Research International, pp.891-934, 2014.

[20] J. M. Perez, A. Asati, S. Nath and C. Kaittanius, "Synthesis of biocompatible dextran-coated nanoceria with pH-dependent antioxidant properties," Small, vol. 4, no.5, pp. 552–556. 2008.

[21] B.C. Nelson, M.E. Johnson, M.L. Walker, K.R. Riley and C.M. Sims, "Antioxidant cerium oxide nanoparticles in biology and medicine," Antioxidants (Basel), pp. 5-15, 2016.

[22] E.G. Heckert, A.S. Karakoti, S. Seal and W.T. Self., The role of cerium redox state in the SOD mimetic activity of nanoceria. Biomaterials; vol. 29, no.18, pp. 2705–2709, 2008.

[23] A. A. Dayem, M. K. Hossain, S. B. Lee, K. Kim, S. K. Saha, G.M. Yang, H. Y. Choi, and S. G. Cho. "The Role of Reactive Oxygen Species (ROS) in the Biological Activities of Metallic Nanoparticles," International Journal Molecular Science, vol.18, no. 120, 2017.

[24] Z.; Diaconescu, D.; Rugină, C.; Coman, C.; Socaciu, L.; Leopold and A. Vulpoi, "New insights regarding the selectivity and the uptake potential of nanoceria by human cells," Article in Colloids and Surfaces A Physicochemical and Engineering Aspects, 2017.

[25] A. Avalos, A. I. Hasa, D. Mateo and P. Morales, 2013. Cytotoxicity and ROS of manufactured silver nanoparticles of different sizes in hepatoma and leukemia cells. Journal of Applied Toxicology 10,1002.

[26] P. Palozza, S. Serini, N. Maggiano, M. Angelini, A. Boninsegna and F. Di Nicuolo," Induction of cell cycle arrest and apoptosis in human colon adenocarcinoma cell lines by β-carotene through down-regulation of cyclin A and Bcl-2 family proteins" Carcinogenesis. vol.23, no.1, pp.11–18, 2002.

[27] X. Dai, H. Cheng1, Z. Bai and J. Li, "Breast Cancer Cell Line Classification and Its Relevance with Breast Tumor Subtyping" Journal of Cancer, Vol. 8. 2017.

[28] M.D. Cuence-Lopez, C.J. Montero, A. Part, A. Pandiella and A. Ocana, "Phospho-kinase profile of triple negative breast cancer and androgen receptor signaling," BMC cancer, vol. 14, no. 302, 2014.

[29] E. Grulke, K. Reed, M. Beck, X. Huang, A. Cormack and S. Seal, "Nanoceria: Factors affecting its pro- and anti-oxidant properties" Environment Science Nano no.1, pp. 429–444, 2014.

[30] C.C. Yu, F.Y. Ko, C.S. Yu, C.C. Lin, Y.P. Huang and J.S. Yang, "Norcantharidin triggers cell death and DNA damage through S-phase arrest and ROS-modulated apoptotic pathways in TSGH 8301 human urinary bladder carcinoma cells," International Journal Oncology, vol. 41, no. 10, pp. 50-60, 2012.

[31] P. Palozza, S. Serini, N. Maggiano, M. Angelini, A. Boninsegna and F. Di Nicuolo," Induction of cell cycle arrest and apoptosis in human colon adenocarcinoma cell lines by β-carotene through down-regulation of cyclin A and Bcl-2 family proteins," Carcinogenesis, vol.23, no.1, pp.11–18, 2002.

[32] G. M. Cooper, "The Cell: A Molecular Approach, Eighth Edition," P. 813. 2019.

[33] A. R. Gliga, K. Edoff, F. Caputo and T. Kallman, "Cerium oxide nanoparticles inhibit differentiation of neural stem cells," Sciences Report, vol.7, no. 1, pp 84-92, 2017.

[34] A. Dembinska-Kiec, A. Polus and B. Kiec-Wilk, "Prognostic activity of beta-carotene is coupled with the activation of endothelial cell chemotaxis," Biochimica et Biophysica Acta, vol. 1740, pp. 223-239, 2005.

[35] Z. Hongmei, "Extrinsic and Intrinsnic Apoptosis Signal Pathway Review," Chapter from the book Apoptosis and Medicine, 2012.

[36] L. Peng, X. He, P. Zhang, J. Zhang, Y. Li, "Comparative Pulmonary Toxicity of Two Ceria Nanoparticles with the Same Primary Size," International Journal Molecular Science, p.15, 2014.
[37] A. Stevens, J. Lowe, I. Scott, and I. Damjanov, "Core pathology. 3\textsuperscript{rd} ed.," \textit{Mosby, Elsevier}, P. 632, 2009.

[38] L. Sadeghi, F. Tanwir, V. Babodi, "In vitro toxicity of iron oxide nanoparticle: Oxidative damages on Hep G2 cells," \textit{Exp toxical pathol}, no. 67, pp. 197-203, 2015.

[39] A. Nalbantsoy, N.U. Karabay-Yavasoglu, F. Sayim, G. Deliloglu, B. Gocmen, H. Arikan and M.Z. Yildiz, "Determination of in vivo cytotoxicity of venom from the Cypriot blunt-nosed viper \textit{Macrovipera lebetina lebetina} and antivenom production," \textit{The journal of Venomous Animals and toxins including Tropical Diseases}, vol. 18, no. 2, pp. 208-216, 2012.

[40] I. Rodea-Palomares, A. L. Petre, K. Boltes, s. F. Legidgov, J. A. Perdigov, M. Rosal and F. Fernandegov-Pinasov, "Application of the combination index (CI)-isobologram equation to study the toxicological interactions of lipidregulators in two aquatic bioluminescent organisms," \textit{water research}, no. 44, pp. 427 – 438, 2010.

[41] X. Liu, B. Zhang, Y. Guo, Q. Liang, L. Wu, K. Tao, G. Wang and J. Chen, "Down-Regulation of Ap-4 Inhibits Proliferation, induces cell cycle Arrest and Promotes Apoptosis in Human Gastric Cancer Cells," \textit{PLoS One}, vol. 7, no. 5, 2012.

[42] I. Koyuncov, A. Gonelov, A. Kocyigitov, E. Temizov, M. Durgunov and C. T. Supuranov, "Selective inhibition of carbonic anhydrase-IX by sulphonamide derivative induces pH and reactive oxygen species-mediated apoptosis in cervical cancer Hela cells," \textit{J Enzyme Inhib Med Chem}, vol. 33, no. 1, pp. 1137-1149, 2018.

[43] J. Liu, Q. Yang, H. Sun, X. Wang, H. Saiyin and H. Zhang, "The circ-AMOTL1/ENO1 Axis Implicated in the Tumorigensis of OLP-Associated Oral Squamous Cell Carcinoma," \textit{Cancer Management and Research}, no. 12, pp. 7219-7230, 2020.

[44] L.C. Lai, "Role of steroid hormones and growth factors in breast cancer. Clin Chem," \textit{Lab Med}, vol. 40, no. 10, pp. 969-974, 2002.

[45] T. Ariizumiv, A. Ogose, H. Kawashimav, T. Hottav, H. Umezuv and N. Endov, "Multinucleation followed by an acytokinetic cell division in myxofibrosarcoma with giant cell proliferation," \textit{Journal of Experimental & Clinical Cancer Research}, vol. 28, no. 44, pp. 1-6, 2009.

[46] D. Ribble, N. B. Goldstein, D. A. Norris and Y. G. Shellman, "A simple technique for quantifying apoptosis in 96-well plates," \textit{BMC Biotechnology}, 2005.

[47] A. Datta, S. Mishra, K. Manna, D. K. Saha, S. Mukherjee and S. Roy, "Pro-Oxidant Therapeutic Activities of Cerium Oxide Nanoparticles in Colorectal Carcinoma Cells," \textit{ACS Omega}, no. 5, pp. 9714–9720, 2020.