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Article

Development of anti-cancer bionanoparticles isolated from corn for bone cancer treatment

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SUMMARY

Nanoparticles (NPs) are particles ranging from 1 to 100 nm in size. NPs can be derived from various sources such as copper, gold, and even plants such as kiwis and corn. Previous studies have shown that nanoparticles derived from corn (cNPs) displayed anticancer properties, but their effects were not tested on bone cancer samples. Current bone cancer treatments are limited as anticancer drugs often either cannot reach the tumor or have little effect on it. Therefore, in this study, we hypothesize that cNPs may have anti-proliferative effects on bone cancer and metastasized bone cancer. First, we found that SAOS-2 (human osteosarcoma) cell viability decreased to 0% when treated with a 20% cNP concentration, and NCI-H526 (human lung carcinoma metastasized to bone marrow) cell viability decreased to 0% when treated with only a 15% cNP concentration. These results indicate that NCI-H526 is more sensitive to cNPs than SAOS-2. Secondly, we found that cNP treatment reduced the expression of BCL2, a gene that prevents apoptosis, suggesting that the cNP treatment induced BCL2-dependent apoptosis. Overall, these results indicate that cNPs have anti-proliferative effects on bone cancer cells and cancer cells that metastasize to the bone. Therefore, our study suggests that cNPs may be applied to the development of bone cancer treatment.

INTRODUCTION

Nanoparticles (NPs) are defined as particles with sizes ranging from 1 to 100 nm (1). NPs display intriguing properties depending on their sizes, and these properties may not be displayed by their macroscopic counterparts (1). For example, properties such as malleability, hardness, and melting point are different in copper nanoparticles and macroscopic copper (2). These particles can be produced naturally, or they can be artificially engineered to have specific characteristics (2). The unique properties of NPs, in addition to their relative ease of production, have led to their use in medicine, as well as cosmetics, sports, self-cleaning surfaces, aerospace engineering, and more (2). In the medical field, NPs have been used as a method of drug delivery as their small size allows them to penetrate tissue and effectively deliver chemotherapy drugs to cancer cells (3).

Edible nanoparticles (ENPs) are nano-sized vesicles derived from edible plants (4). ENPs have been isolated from many plant species such as ginger, grapes, lemon, tomato, broccoli, sunflower, orange, kiwi, pear, soybean, grapefruit, and coconut (4). These ENPs are loaded with plant-derived microRNAs, proteins, lipids, and phytochemicals (4). Recently, corn-derived NPs (cNPs), although not studied intensely, have shown promise as a novel cancer treatment (5). One study demonstrated the anticancer effects of cNPs on colon cancer and leukemia in mice (5). The study described the average size of the cNPs as 80 nm with a density of 35.6 ± 1.9×10^{11} particles/mL (5). The anticancer properties of cNPs are likely due to corn's vitamins, minerals, and xanthophylls (5). Xanthophylls have been shown to have strong anticancer properties (6). Xanthophylls have been shown to inhibit cancer cell proliferation, protect against DNA damage, and induce cancer cell death (5). The mechanisms by which xanthophylls exert their anticancer effects are not very well understood. In addition, cNPs are also a promising cancer treatment method due to their ease of production (5). Corn is a very high-yield crop compared to alternatives, such as wheat and rice, and can be quickly grown in substantial amounts (7). This makes cNPs an effective and cost-efficient option for cancer-treating NPs (5).

Bone cancer is a rare cancer with approximately 1 in 100,000 people diagnosed each year in the United States (8). There are three types of bone cancer: osteosarcoma, Ewing sarcoma, and chondrosarcoma (9). Osteosarcoma and Ewing sarcoma are most common in people under 20 years old, while chondrosarcoma mostly affects those over 40 years old (10). Although studies have been performed studying the effects of NPs on bone cancer, no studies have been performed to determine the effects of cNPs on bone cancer. Current bone cancer treatments are limited because therapeutic drugs fail to target the cancer sites. Drugs can often have trouble penetrating bone tissues' dense extracellular matrix, and multi-drug resistance has caused bone cancers to resist the effects of common anti-cancer drugs (11). Nanoparticle treatments for cancers are in their pilot stages of development, and some NP-based cancer treatments, such as gold NPs and iron oxide NPs, have entered clinical trials (12).

Therefore, we tested the anti-proliferative properties of cNPs on bone cancer in this study. The two types of bone cancer cell lines used in this study are SAOS-2, cancer cells originating from the bone, and NCI-H526, lung cancer cells that metastasize to the bone. We hypothesized that the small size of cNPs may effectively block cancer cell proliferation

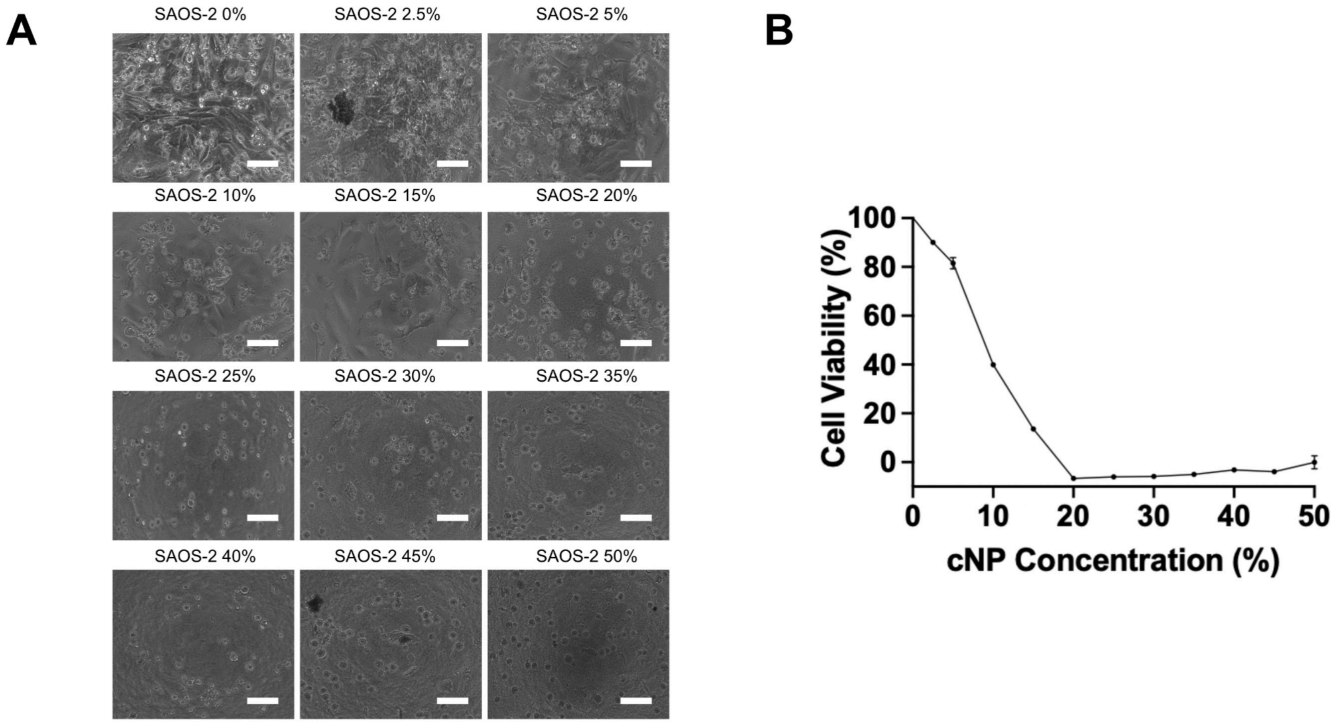

Figure 1. Corn-derived NPs (cNPs) decreased the cell viability of SAOS-2 bone cancer cells. (A) Phase contrast image of SAOS-2 cells after treatment of cNPs for seven days. The percentage indicates cNP diluted with the cell culture media (v/v). The cell images were taken after seven days of treatment. Scale bar = 50 μM. **(B)** Line graph showing mean and standard deviation of cell viability measured by Prestoblue assay after treatment of cNPs for seven days (n = 3). SAOS-2 cancer cells were treated with twelve different cNP concentrations (0, 2.5, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50%).

and induce cell death. Overall, our study showed that cNP treatment was effective against both SAOS-2 and NCI-H526 bone cancer cell lines. NCI-H526 was particularly sensitive to treatment with low doses of cNPs. Also, the cNP treatment decreased the expression of BCL2, a gene that prevents apoptosis. Therefore, cNPs could work as a novel bone cancer treatment. The small size of these NPs could effectively penetrate through the bone, and the cost-effectiveness of cNPs would allow for ease of mass-production.

RESULTS

In this study, after isolating the cNPs from the corn kernel, we tested whether isolated cNPs have anti-proliferative effects on SAOS-2 and NCI-H526 bone cancer cells and investigated the minimum and maximum cNP concentrations that would induce bone cancer cell death. A previous study confirmed that cNPs have anti-proliferative effects in some cancer cells, including colon26 tumor cells (cancer cells derived from mice), RAW264.7 macrophage-like cells, and NIH3T3 cells (embryonic mouse fibroblast) (5). Since cNPs had an effect on other cancer types, we hypothesized that cNPs would also have anti-proliferative effects on SAOS-2 and NCI-H526 bone cancer cells. Both cells were treated with twelve cNP concentrations: 0, 2.5, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50%. The cNP concentration indicates the percentage of cNPs in media (cNP volume / total volume including cell culture media), so the 50% concentration indicates a solution of equal volumes of cNPs and cell culture media.

SAOS-2 are adherent cells that normally grow when the cells are attached to the surface of the culture plate. However, when SAOS-2 cells were treated with cNP concentrations above 20%, no attached cells were visible, indicating that no cells were alive (**Figure 1A**). Cell viability decreased in a dose-dependent manner as cNP concentrations increased from 2.5% to 20% (**Figure 1B**). The cNP concentration was inversely proportional to the SAOS-2 cell viability, and cell viability was reduced to 0% at cNP concentrations above 20% (**Figure 1B**). The results of this study show that cNPs promote cell death in SAOS-2 bone cancer cells, cNP concentrations greater than or equal to 2.5% reduced SAOS-2 viability, and cNP concentrations greater than 20% caused cell viability to decrease to 0%.

NCI-H526 cells are far less resistant to the anti-proliferative effects of cNPs than SAOS-2 cells, as cell viability dropped below 10% when treated with only a 2.5% cNP concentration, and all cell viability was reduced to 0% when treated with cNP concentrations of 15% or higher (**Figure 2A**). The results of this study show that cNPs promote cell death in NCI-H526 bone cancer cells, cNP concentrations of 2.5% and above reduced NCI-H526 viability, and cNP concentrations of 15% and above caused cell viability to decrease to 0% (**Figure 2B**).

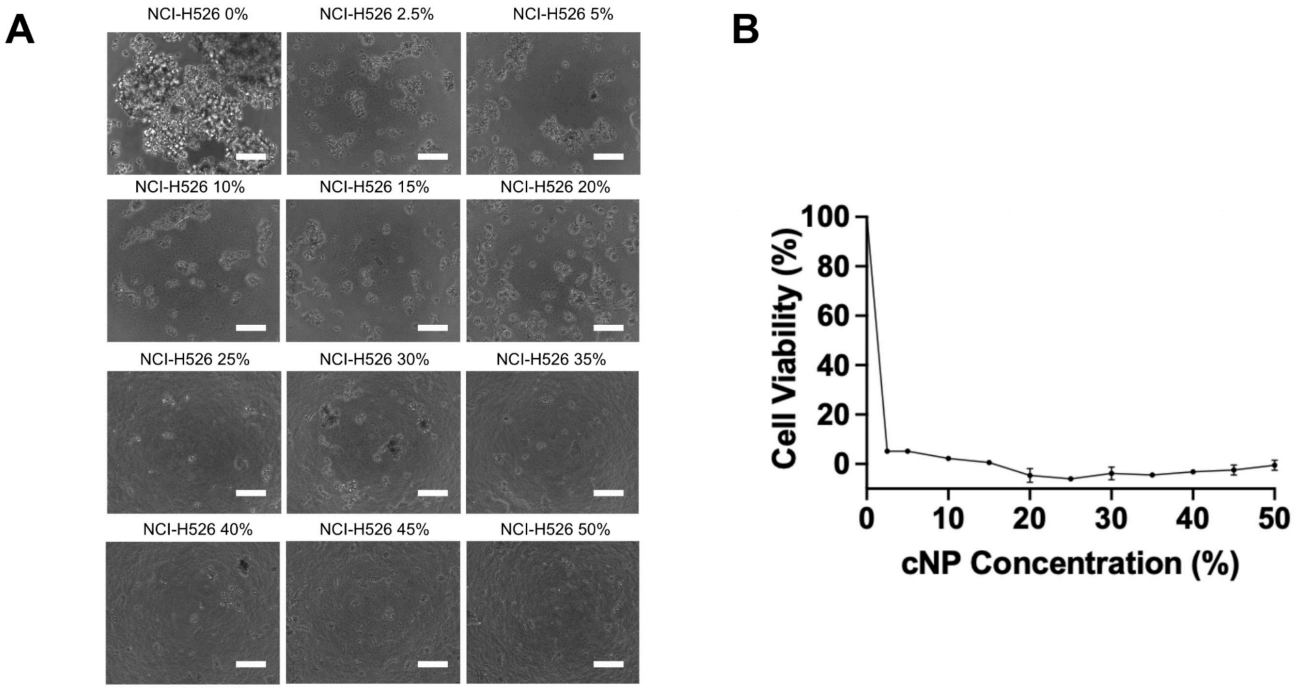

Figure 2. Corn-derived NPs (cNPs) decreased the cell viability of NCI-H526 cancer cells metastasized to bone. (A) Phase contrast image of NCI-H526 cells after treatment of cNPs for seven days. The percentage indicates cNP diluted with the cell culture media (v/v). The cell images were taken after seven days of treatment. Scale bar = 50 μM. **(B)** Line graph showing mean and standard deviation of cell viability measured by Prestoblue assay after treatment of cNPs for seven days (n = 3). NCI-H526 cells were treated with twelve different cNP concentrations (0, 2.5, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50%).

Next, we aimed to verify the effect of cNPs on inducing cell death. The expression level of BCL2, BAX, and GAPDH genes associated with apoptosis were analyzed in NCI-H526 and SAOS-2 cells. Since a previous study indicated that cNPs have anticancer effects, we hypothesized that cNPs may have induced cell death through apoptosis (5). Therefore, we expected that cNP-treated cells might decrease the expression of BCL2 (anti-apoptotic gene) and increase the expression of BAX (pro-apoptotic gene).

We used a 5% cNP concentration to analyze the effect of cNPs on gene expression because a 5% cNP concentration efficiently decreased cell viability. We analyzed BCL-2/BAX gene expression using RT-PCR because they regulate both caspase-dependent and caspase-independent apoptosis (13). When cancer cells were treated with cNPs, the expression of BCL2 decreased in NCI-H526 (**Figure 3A**). Although there is no statistical difference in BAX expression, there appears to be a trend in the increasing direction in NCI-H526 (p = 0.622) (**Figure 3A**). When the gene expression was quantified and normalized by GAPDH, BCL2 was significantly decreased in 5% cNP-treated NCI-H526 compared to the 0% cNP control sample (p = 0.0059) (**Figure 3B**). However, there was no significant change in the expression of BAX in 5% cNP-treated NCI-H526 compared to the 0% cNP control sample (p = 0.622) (**Figure 3B**). When the expression of these genes was quantified in the SAOS-2 samples, no significant change was found in either gene (**Figure 3B**). Overall, our result indicates that a 5% cNP concentration causes increased expression of the apoptotic gene BCL-2 in NCI-H526 cells, suggesting that cNPs may function to induce apoptosis in cancer cells.

DISCUSSION

Our results show that NCI-H526 is more sensitive to the anti-proliferative effects of cNPs than SAOS-2 is. Also, the 5% cNP treatment significantly decreased BCL2 expression only in NCI-H526 cells, suggesting that BCL2-dependent apoptosis occurred in NCI-H526 cells. Overall, these results indicate that cNPs have anti-proliferative effects on bone cancer cells.

The validity of the results of this experiment is limited as the experiment was only performed once. Therefore, the experiment should be reproduced to verify the results. Similar experiments with cNPs should also be done on cell migration, invasion, and angiogenesis to determine the effects of cNPs on cancer progression. For cell migration, a wound healing assay should be performed to analyze the cancer cell migration rate. For cell invasion, the amount of cancer cells penetrating through the extracellular matrix should be analyzed. For angiogenesis, the growth of endothelial cells should be measured after cNP treatment. Xanthophylls, a yellow pigment in cNPs, have previously been shown to have anti-tumor and anti-inflammatory effects. Future studies could therefore test the effects of isolated xanthophylls from cNP on cancer cell cultures. This experiment was also limited

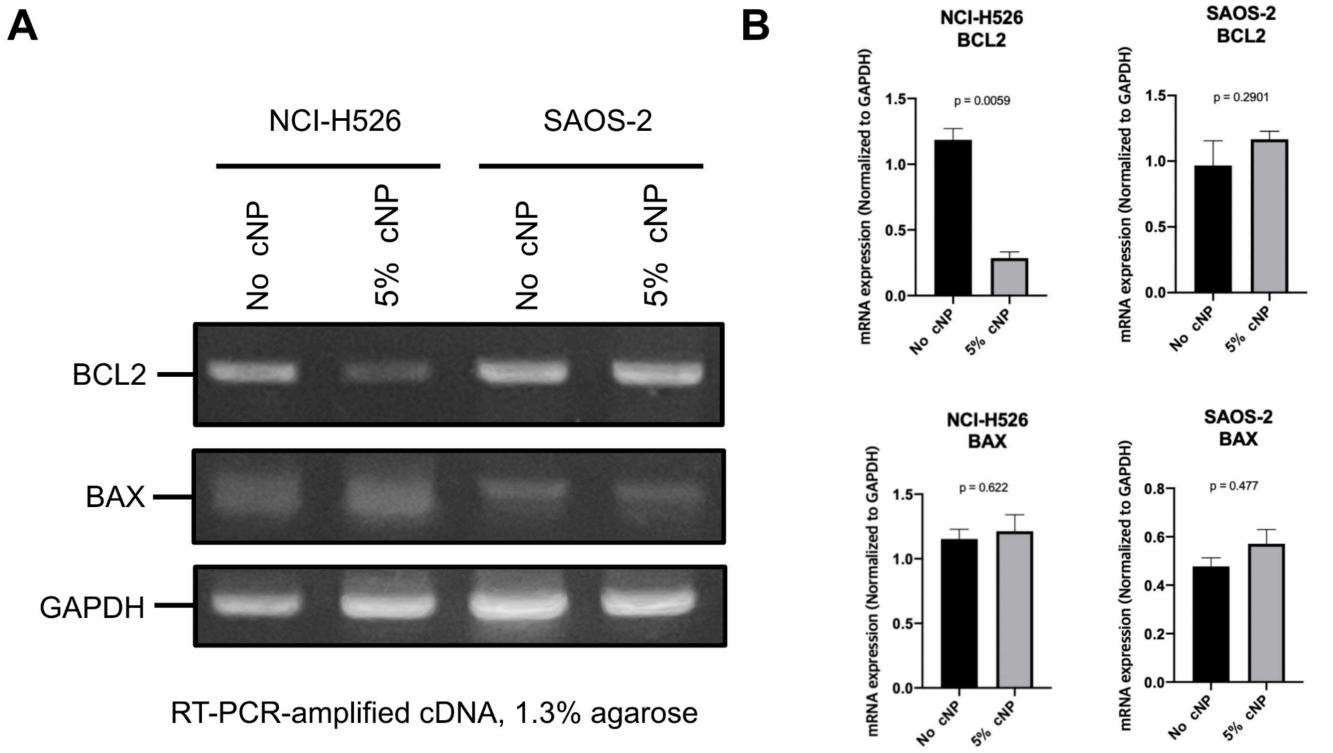

Figure 3. cNP decreased BCL2 expression level in NCI-H526. (A) Agarose gel image showing amplified DNA from BCL2, BAX, and GAPDH after RT-PCR. **(B)** Bar graph showing mean and standard deviation of normalized BCL2 and BAX expression level. GAPDH gene expression was used to normalize BCL2 and BAX expression levels (n = 2). An unpaired t-test was used to calculate the *p*-value.

to *in vitro* models. In previous studies, cNPs were taken in by both healthy cells and cancer cells, but cell proliferation was only prevented in cancer cells (5). Therefore, *in vivo* models should be tested to validate the anticancer effects of cNPs further and to test the effects of cNP treatment on living animals and healthy normal cells. *In vivo* models or 3D cell cultures could also test the ability of cNPs to penetrate bones and tissue. Since there are several types of cell death other than apoptosis, the effect of cNPs on ferroptosis and necroptosis should be analyzed further. It could be possible that the observed decrease in cell viability was attributed to cNPs preventing the uptake of nutrients by the cancer cells. Studies could be done to test the effects of cNPs on cell nutrient uptake.

As previous studies on the effects of cNPs on cancer cells focused on colon cancer, this is the first cNP experiment to have been performed on bone cancer cells (5). Both SAOS-2 and NCI-H526 cancer cells were tested, and cNPs were found to have a significant impact on the viability of NCI-H526 cells by BCL2-dependent apoptosis. The results of this experiment suggest that cNPs may work as a novel bone cancer treatment. In addition to their effectiveness in *in vitro* cancer models, cNPs' small size may allow them to penetrate biological barriers, such as the small size of bone pores (20–1,500 μm), to achieve high therapeutic efficiency.

Common treatments such as chemotherapy and radiotherapy are often not remarkably effective in treating bone cancers. This is because the anticancer drugs' lack of targeting causes these drugs not to accumulate well at the tumor site, and the drugs are not always able to penetrate the dense extracellular matrix of bone tissues. In addition, multi-drug resistance results in bone tumors being insensitive to anticancer drugs. The cNP treatment addresses most of these problems. Due to the small size of cNPs, they may have less of a problem penetrating the bone tissues' extracellular matrix. As a new therapeutic method, cNPs may provide an alternative solution for overcoming drug resistance.

This study shows the anti-proliferative effects of cNPs on bone cancers and that metastasized bone cancers are more sensitive to this treatment than cancers originating in the bones. This suggests that the cNP treatment could be more effective against more dangerous types of cancer. Current bone cancer treatments are limited in effectiveness and are often too expensive for bone cancer patients. cNPs, as a novel bone cancer treatment, could overcome these limitations as they have been shown to be effective against bone cancers and very cost-effective.

MATERIALS AND METHODS

Preparation of cNPs

The corn was purchased from a market and washed three times with distilled water. The kernels of corn (100 g)

were mixed with 100 mL of distilled water and homogenized by a food processor for 5 min. The homogenized corn juice was centrifuged in row at 2000×g for 30 min, 5000×g for 40 min, and 10,000×g for 1 h at 4 °C. The supernatant was filtered through a 0.45 µm-pore size syringe filter (Millipore). 60% sucrose solution (2 mL) was added to filtrates (38 mL) in a 50 mL ultra-centrifuge tube and then ultra-centrifuged at 100,000×g for 160 min at 4 °C using Beckman Optima XL-100 K with an SW28 centrifuge rotor (Beckman Coulter). The top supernatant was removed with a syringe. The yellow-colored cNP layer that was present above the clear 60% sucrose solution was collected carefully with a syringe. The isolated cNPs were stored at -80 °C. The isolated cNPs from 100 g of corn were indicated as 100% concentration.

Cell imaging

Initially, 1.2 x 105 cells were prepared in a 24-well culture plate for both SAOS-2 and NCI-H526 cells. Then, various concentrations (0, 2.5, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50%) of cNPs were added to each cell culture well. The total final culture volumes were the same for each concentration of cNPs. The cell cultures mixed with cNPs were incubated for seven days. To photograph the cells on the cell culture plate, an EVOS M5000 microscope (Invitrogen) was used. The cells were visualized using transmitted light and a phase objective with 10X magnification.

PrestoBlue assay

The initial cell number was 2.0 x 10^4 cells per well for both SAOS-2 and NCI-H526 cells in 96-well culture plates. Then, various concentrations (0, 2.5, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50%) of cNPs were added to each well. The cell cultures mixed with cNPs were incubated for seven days. Then, 10% of PrestoBlue reaction buffer was added directly to cells in the culture medium. The mixed solution was incubated for five hours. The absorbance of 570 nm and 600 nm (reference wavelength for normalization) was measured. 570 nm was normalized to 600 nm by dividing the 570 nm values by 600 nm. The well treated with a 0% cNP concentration was set to 100% cell viability and the well containing no cells was set to 0% cell viability to plot the normalized 570 nm absorbance value to cell viability.

Total RNA extraction from cells

The AccuPrep® Universal RNA Extraction Kit (Bioneer) was used to extract the RNA from the cells. The RNA extraction protocol provided by the kit was used to isolate RNA from the samples. A 50 µL elution solution was used to extract the RNA from each sample.

cDNA synthesis

TOPscript™ Reverse Transcriptase (Enzymatics) was used to synthesize the cDNA from the extracted RNA samples. 5 µg of total RNA was synthesized to cDNA from each sample. Oligo (dT) primer (50 µM) was used for annealing polyadenosine tails of mRNA guiding the synthesis of cDNA. The following reaction condition was used: 1) incubate at 50 °C for 60 min, 2) incubate at 95 °C for 5 min to inactivate the reaction.

RT-PCR

BAX, BCL2, and GAPDH genes were amplified from the synthesized cDNA with AccuPower® PCR PreMix (Bioneer). cDNA (1 µg), forward primer (200 nM), and reverse primer (200 nM) were used to amplify the specific transcripts from each gene. The following primer sequences were used: BAX (Forward primer: 5'-TGGCAGCTGACATGTTTTCTG-3'

Reverse primer: 5'-GCTGCCGGTGGTCACTTC-3'), BCL2 (Forward primer: 5'-CCTGGATCAGGGGTTTTGTG-3' Reverse primer: 5'- AGACAGCCAGGAGAAATCAAAC -3'), GAPDH (Forward primer: 5'-TGCACCACCAACTGCTTAGC-3' Reverse primer: 5'-GGCATGGACTGTGGTCATGAG -3'). The following PCR reaction conditions were used: 1) 95 ˚C for 3 min, 2) 95 ˚C for 30 sec, 3) 60 ˚C for 30 sec, 4) 72 ˚C for 15 sec, 5) repeat step 2 to 4 for 29 times, 6) 72 ˚C for 5 min, 7) 12 ˚C infinitely.

Agarose gel, semi-quantitative gene expression analysis

1.3% agarose gel was prepared with RedSafe™ Nucleic Acid Staining Solution (Intron). The gel was run for 20 min at 100 V. The band intensity was quantified with ImageJ program (ver. 1.53t).

Statistical analysis

We performed all statistical analyses using the Prism 8 program (Graphpad ver. 8.4.3). An unpaired t-test was performed to calculate the *p*-value.

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