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RESEARCH ARTICLE

In vitro investigation of the effects of boron nitride nanotubes and curcumin on DNA damage

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Abstract

Backround Stem cells provide an opportunity to analyse the effects of xenobiotic on cell viability, differentiation and cell functions. Evaluation of the possible cytotoxic and DNA damaging effects on bone marrow $CD34^+$ stem cells is important for their ability to differentiate into blood cells, and also for bone marrow diseases therapy. Boron nitride nanotubes and curcumin are potential nanoformulation agents that can be used together in the treatment of cancer or bone marrow diseases. Therefore, it is important to evaluate their possible effects on different cell lines.

Objectives In this study, it was aimed to evaluate the cytotoxic and DNA damaging effects of boron nitride nanotubes which are commonly used in pyroelectric, piezoelectric and optical applications, but there is not enough information about its biocompatibility. Also, it was intended to research the effects of curcumin being used frequently in treatment processes for antioxidant properties.

Methods The possible cytotoxic and DNA damaging effects of boron nitride nanotubes and curcumin on $CD34^+$ cells, HeLa and V79 cells were evaluated by MTT assay and Comet assay, respectively.

Results and conclusion Boron nitride nanotubes and curcumin had cytotoxic effects and cause DNA damage on $CD34^+$ cells, HeLa and V79 cells at several concentrations, probably because of increased ROS level. However, there were not concentration - dependent effect and there were controversial toxicity results of the studied cell lines. Its mechanism needs to be enlightened by further analysis for potential targeted drug development.

Keywords Bone marrow $CD34^+$ stem cell · Boron nitride · Curcumin · Comet assay · V79 cell · HeLa cell

Introduction

Stem cells have important role in biomedical applications, in regenerative therapy and toxicity testing by virtue of their characteristics such as long-term cleavage, self-renewal, and differentiation into cells like neurons, cardiomyocytes, hepatocytes [1, 2]. Stem cell-based toxicity analyzes allow the investigation of the effects of xenobiotics on cell viability, differentiation process, and cell functions. Difficulties in adapting the knowledge obtained from in vivo and in vitro toxicity testing methods to humans have led researchers to use stem cells and 3D culture systems [3].

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Studies on bone marrow stem cells have gained speed in the twentieth century due to their usage in medicine. The microenvironment and stem cell diversity in the bone marrow are being investigated by defining cell surface antigens using microanalyses and transgenic mouse models [4]. Hematopoietic stem cells that are progenitors of blood cells and mesenchymal stem cells which have the ability to differentiate into osteocytes, chondrocytes and adipocytes are widely used in the study mentioned above. The use of hematopoietic and mesenchymal stem cells in toxicity studies are important for determining the effects of test material on the differentiation process. The examination of the genotoxic effects is necessary for the treatment and prevention of diseases such as anemia and cancer. In particular, genotoxic effects which may be permanent through bone marrow long term hematopoietic stem cells [5].

Nanomaterials are used in many fields such as medicine, textile, electronics, agriculture due to their reactive, electronic

and catalytic properties that depend on their small size (1–100 nm) and their large surface-to-volume ratio [6, 7]. Boron nitride nanotubes (BNNs), one of the many nanomaterials that differ according to their physical, chemical and biological properties, also have a wide range of use due to their advantages such as high Young's modulus, oxidation resistance and thermal conductivity [8, 9]. The nanotubes can be used as nanovector in clinical and biomedical applications because of their biological inertness. However, solubility problem of BNNs in biological fluids requires nanotubes to be subjected to processes such as polymer coating and chemical modification [10].

Nanoparticle formulations containing curcumin are used to increase the bioavailability of the phenolic compound curcumin, which is used in Indian and Chinese traditional medicine applications because of the anti-inflammatory - antioxidant -antimicrobial -anticancer effects [11, 12]. BNNs are potentially suitable formulation agent for benefiting from the biological effects of curcumin.

It is necessary to understand the mechanisms of cytotoxicity and genotoxicity of stem cells, which may be an appropriate alternative to animal experiments as an indispensable part of toxicity tests. Additionally, it is important to develop an appropriate formulation to increase the solubility of BNNs and curcumin in biological fluids.

In the scope of this study, it is aimed to evaluate the cytotoxic effects and the effects of BNNs and curcumin on DNA damage on HeLa and V79 cells by MTT and comet assays. Also, it is aimed to evaluate the effects of BNNs and curcumin on DNA damage on bone marrow $CD34^+$ cells.

Methods

Boron nitride nanotubes and curcumin dispersion preparation

BNNs were purchased from Sigma-Aldrich (802824) and dispersions were prepared in sterile distilled water. The BNNs had typical diameters of 5 nm on the average and surface area were more than 100 m^2/g. The nanotubes were composed of H-BN and BNH (0–25%), BNN (50%) and elemental B (<25%). BNNs dispersions were prepared as 1 mg/ml and 2 mg/ml samples. The ultrasonic probe and the ultrasonic bath were used to provide a homogeneous distribution of BNNs stock solutions.

Curcumin was purchased from Sigma-Aldrich (C1386). Curcumin was prepared in 1 and 1,5 mg/ml stock solutions in dimethyl sulfoxide (DMSO, Sigma) and diluted with sterile distilled water (final DMSO concentration was 1% (*v*/v)) for experiments.

Boron nitride nanotubes characterization

The morphological characteristics of BNNs dispersion in sterile distilled water which prepared in the same way described before were analyzed using Transmission Electron Microscope (TEM) technique. The analyses were performed at the National Nanotechnology Research Center (UNAM) at Bilkent University using Tecnai G2 F30-FEI model transmission electron microscope with an acceleration potential of 300 kV.

The zeta potential of BNNs dispersion in sterile distilled water was determined by Laser Doppler Anemometry in Zetasizer equipment (Zetasizer-Nanoseries ZS, Malvern Instruments) at UNAM.

Cell culture

HeLa cells (American Type Culture Collection (ATCC) CCL-2, human cervix carcinoma) and V79 cells (ATCC CCL-93, Chinese hamster lung fibroblasts) were purchased from ATCC. The human bone marrow $CD34^+$ cells were purchased from Lonza.

HeLa cells were cultured in RPMI (Biological Industries) and DMEM (Sigma) containing 1% penicillin-streptomycin (Biological Industries) and 10% fetal bovine serum (Sigma). V79 cells were cultured in DMEM (Sigma) containing 1% penicillin-streptomycin and 10% fetal bovine serum.

The bone marrow $CD34^+$ cells were thawed using IMDM containing 10% FBS and 20 U/ml Deoxyribonuclease I (Sigma) and grown in Iscove's Modified Dulbecco's Medium (IMDM, Lonza) supplemented with 15% fetal bovine serum, 1% penicillin-streptomycin, 10 μg (25 ng/ml) stem cell factor (Sigma), 4 μg (10 ng/ml) In-6 and In-3 (Sigma). The cells were cultured in a 5% carbon dioxide-humidified atmosphere at 37 °C.

MTT assay

The methyl thiazol tetrazolium bromide (MTT, Sigma) assay was used for assessing cell viability, as described by Mosmann et al. [13] and Ferrari et al. previously [14]. Briefly, HeLa cells with V79 cells were cultured and 10.000 cells were seeded in per chambers of 96 well-plate and allowed to attach for 24 h. After incubation, the cells were exposed to different concentrations of BNNs (10–300 μg/ml) [15], curcumin (10–300 μg/ml) [16] and combination of equal concentrations of BNNs and curcumin (10–300 μg/ml) in medium for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. When the exposure time ended, cells medium was aspirated and 10 μl of MTT solution (5 mg/ml in PBS) was added to each well to evaluate cell survival. After 4 h incubation, the cell media was replaced with 100 μl DMSO and the plates were shaken for 5 min. The absorbance

was determined at 570 nm by microplate reader and IC_{50} values were calculated using concentration-response curves to express the effects of test materials on cell viability.

Comet assay

The basic alkaline comet assay was performed essentially as described by Singh et al. [17] with modifications of Hartmann et al. [18]. Briefly, regular cell slides were coated with 1.25% normal melting point agarose (NMPA) and allowed to dry. For the cell exposure to test compounds, 2500 human bone marrow $CD34^+$ hematopoietic progenitor cells were seeded in 96 well plates and allowed to attach for 24 h. After this period, the cells were exposed to different concentrations of BNNs, curcumin and, the combination of BNNs and curcumin (0.5–100 μg/ml) for 30 min and 24 h. 1% DMSO was used as a negative control and 50 μM H_2O_2 was used as positive control. At the end of exposure time, the cells were centrifuged at 200 g for 15 min and the supernatant was discarded to leave 50 μl cell suspension at the bottom of the plate well. The cells were resuspended in 1.25% low melting point agarose (LMPA) and this suspension was spread on a pre-coated slides. After removing the coverslip, the slides were submerged into lysing solution (2.5 M NaCl, 100 mM EDTA, 100 mM Tris, 1% sodium sarcosinate, 1% Triton-X 100, 10% DMSO, pH 10; 4 °C) for 24 h. After lysis, the slides were left in electrophoresis solution (300 mM NaOH and 1 mM sodium EDTA, pH 13) for 20 min at 4 °C and electrophoresis were performed for 20 min at 4 °C by applying electric current of 300 mA and 24 V. Following, the slides were washed in neutralisation solution (0,4 M Tris-HCl, pH 7.5) for 15 min and then they were incubated in 50%, 75%, 98% of ethyl alcohol for 5 min each. The dried slides were stained with EtBr (20 μg/ml in distilled water, 60 μg/slide) and examined by Leica® fluorescence microscope. Computer-based analysis system (Comet Analysis Software, version 3.0, Kinetic Imaging Ltd., Liverpool, UK) was used to measure the DNA damage. In order to visualize DNA damage, 100 nuclei per slide were examined at 40× magnification. Results were expressed as the fraction of total DNA in the tail ("tail moment") and percent of DNA in tail ("tail intensity").

Also, 30.000 HeLa and V79 cells were seeded in 24 well-plate and were allowed to attach for 24 h. Following this period, the cells were treated with different concentrations of BNNs, curcumin and combination of BNNs and curcumin (0.5–100 μg/ml) in the medium for 30 min and 24 h at 37 °C and 5% CO_2. At the end of the treatment, the cells were washed with PBS and trypsinized with trypsin/EDTA and resuspensions of the cells were centrifuged in Eppendorf tubes at 1200 rpm for 5 min. The supernatant was discarded to leave 50 μl cell suspension at the bottom of the tubes. The cells were suspended in 0.5% LMPA to spread on slides which pre-coated with 1% NMPA, the comet assay protocol was followed as described before.

Statistical analysis

The MTT and comet experiments were repeated 3 times and the results were given in mean ± standard deviation format. For MTT and comet assays, the statistical analysis was performed by SPSS for Windows 10.5 computer program. Differences between the means of data were compared by the one-way analysis of variance (ANOVA) test and post hoc analysis of group differences by least significant difference (LSD) test. Also, the p value of less than 0.05 was considered as statistically significant.

Results

BNNs characterization with TEM and zeta potential analysis

According to the TEM images of BNNs dispersion (Fig. 1), it seems BNNs have a straight and multi-walled tubular structure with a diameter of approximately 8 nm and a length of up to several micrometers. Also, it is possible to see particles with diameter of about 7 nm that are probably composed of BNH, H-BN and elemental B.

Zeta potential analysis is important to determine the surface charge of nanoparticles and to predict in vivo fate of nanoparticles because of their cellular interaction related to activation, agglutination, adhesion and for colloidal nanoparticle stability which is described as highly unstable (±0–10 mV), relatively stable (±10–20 mV), moderately stable (±20–30 mV) and highly stable (±30 mV), respectively [19–21]. The zeta potential of BNNTs dispersion was detected as −50,9 mV (Fig. 2). It demonstrates the presence of negative charge on BNNTs and may indicate that BNNTs samples can form stable suspensions on these conditions.

Cytotoxicity of boron nitride nanotubes and curcumin in HeLa and V79 cells

The cytotoxic effects of boron nitride nanotubes and curcumin were examined by MTT assay on HeLa cells and V79 cells for 24 h incubation period (Fig. 3a-c). Boron nitride nanotubes and curcumin were treated alone and in the combination of two at a dose range of 10–300 μg/ml.

In HeLa cells, it was observed that the cell viability was not concentration-dependent in boron nitride nanotubes treated cells. The viability of HeLa cells increased at concentrations of 10 μg/ml, 20 μg/ml, 100 μg/ml and 200 μg/ml when compared to the negative controls. BNNs showed the highest

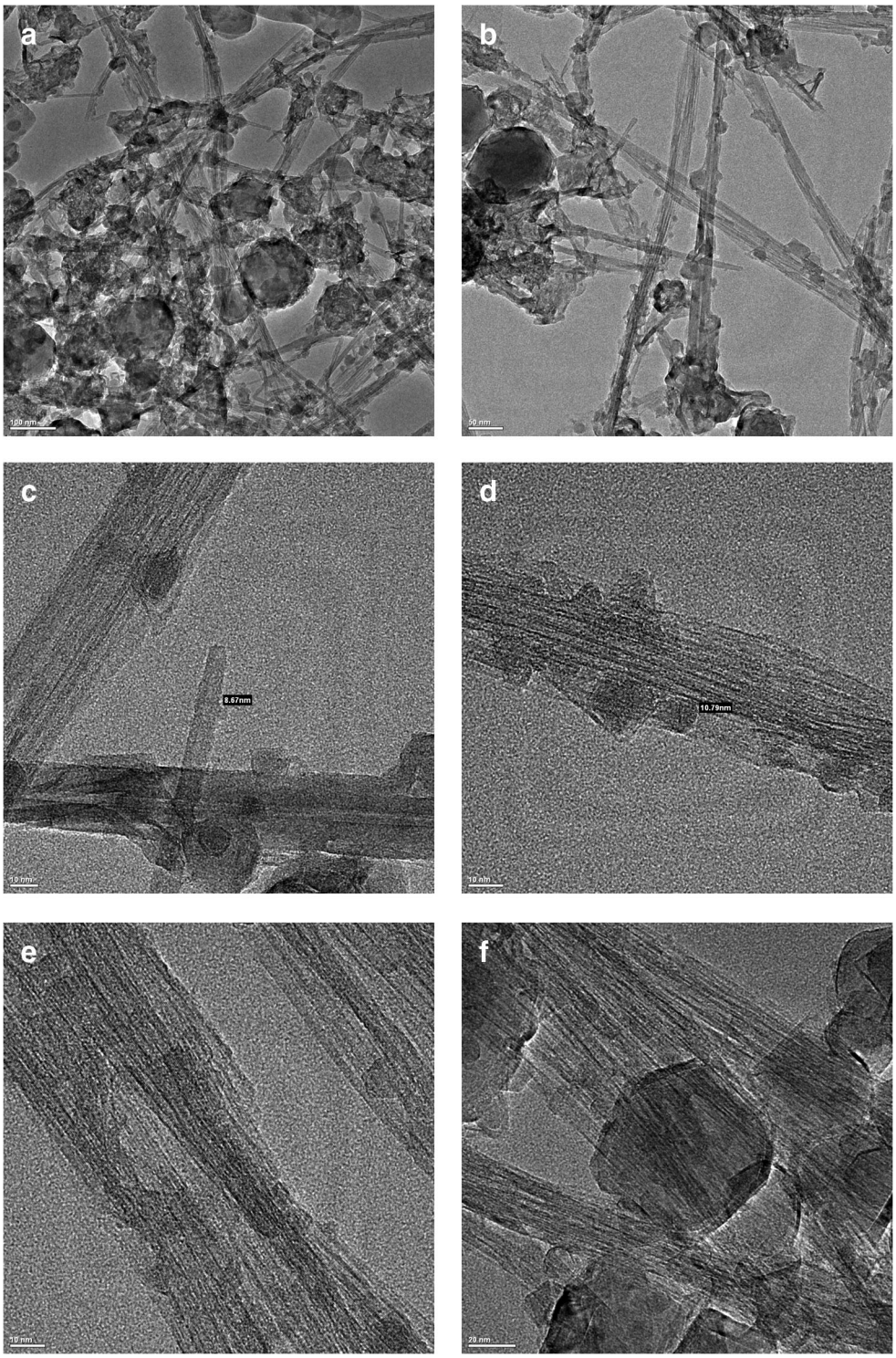

Fig. 1 TEM images of BNNs dispersion at (**a**) 100 nm, (**b**) 50 nm, (**c**) 10 nm, (**d**) 10 nm, (**e**) 10 nm and (**f**) 20 nm

cytotoxic effect on HeLa cells at a concentration of 50 μg/ml and the cell viability decreased to 66%. The IC_{50} value of the boron nitride nanotubes could not be determined in the studied concentration range. Furthermore, the cell viabilities of all studied concentrations were significantly more than positive control (Fig. 3a). In the boron nitride nanotubes and curcumin co-treated cells, the cell viability decreased at a concentration range of 10–100 μg/ml, whereas the cell viability increased at a concentration range of 100–300 μg/ml. Also, there was decreased cell viability at the concentration of 100 μg/ml when compared to positive control (Fig. 3b). In the curcumin-treated cells, there were decreases in cell viability, except for the

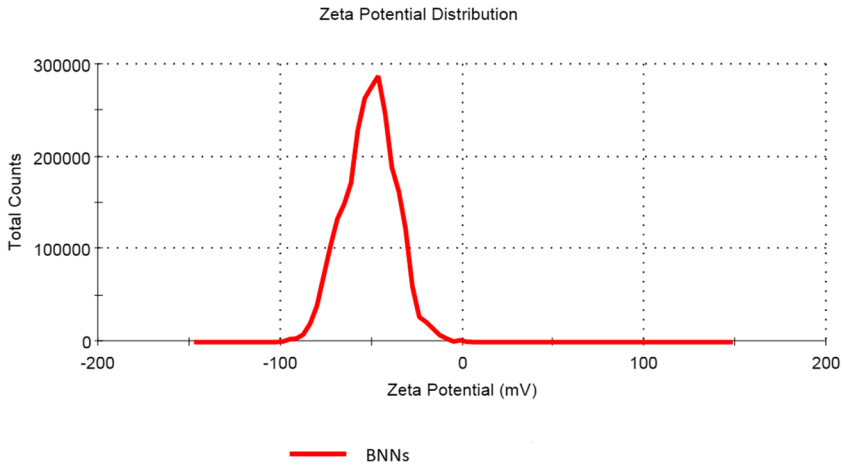

Fig. 2 Zeta Potential of BNNs dispersion in water

300 μg/ml concentration, when compared to the negative control (1% DMSO). The changes in cell viability were not in a concentration-dependent manner and there was a general reduction in the range of 10–100 μg/ml. However, the increase of cell viability was concentration-dependent in the range of 100–300 μg/ml, which is the same as in the BNNs

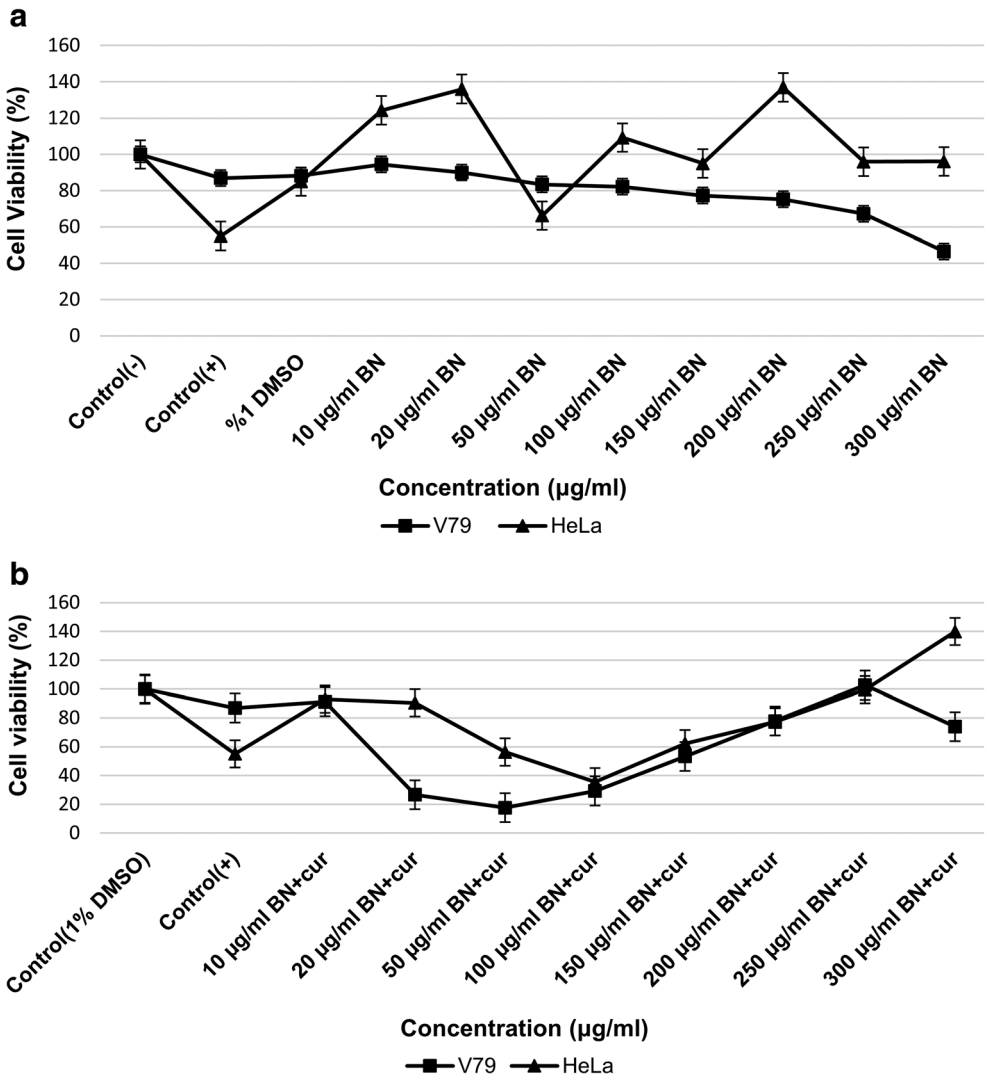

Fig. 3 **a** MTT assay results of HeLa and V79 cells incubated with boron nitride nanotubes (BN) for 24 h. **b** MTT assay results of HeLa and V79 cells incubated with boron nitride nanotubes (BN) – curcumin (cur) for 24 h. **c** MTT assay results of HeLa and V79 cells incubated with curcumin (cur) for 24 h. **d** The image of MTT result for HeLa cells

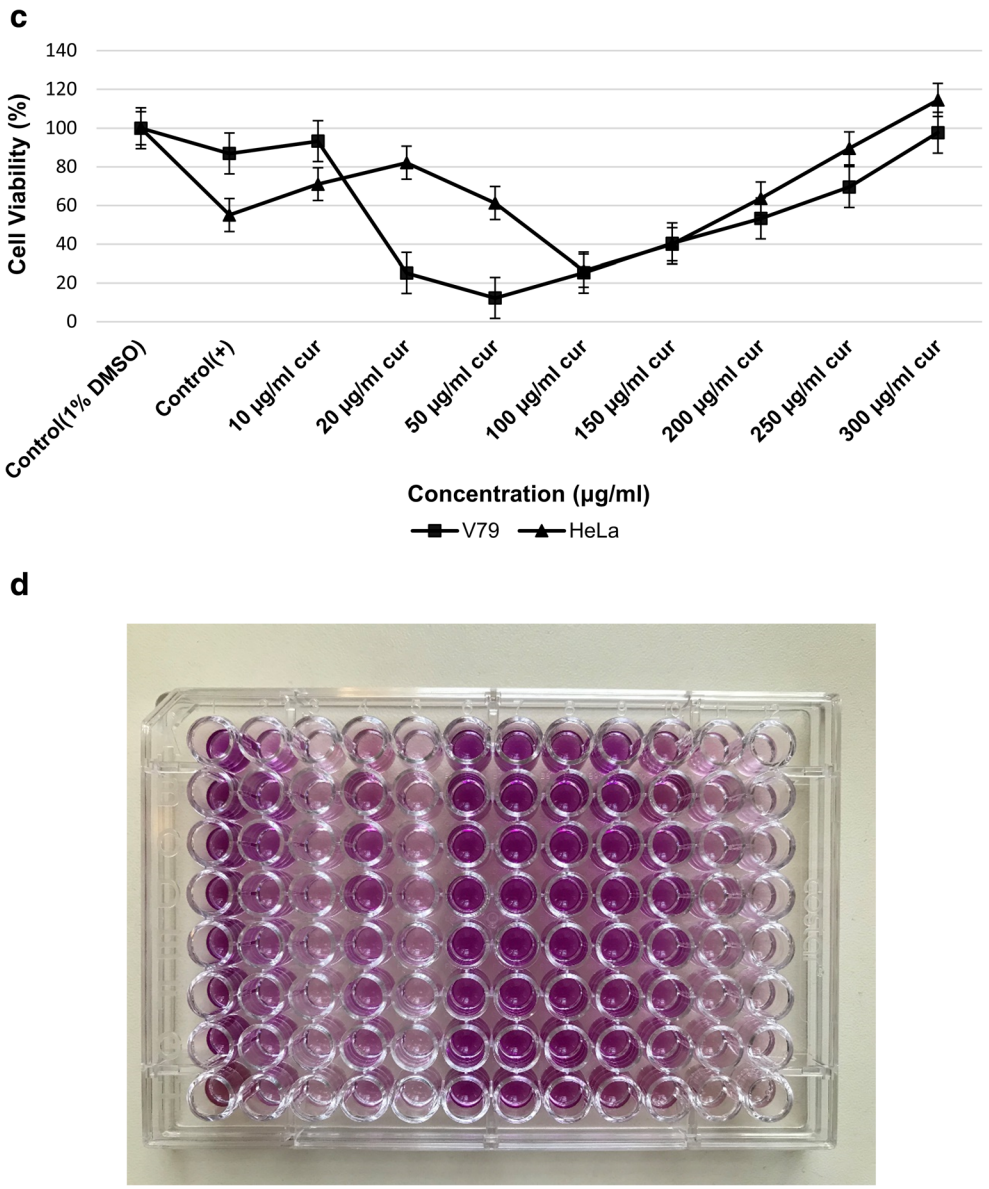

Fig. 3 continued.

and curcumin co-treated cells. Curcumin showed a significant cytotoxic effect at the concentration of 100 µg/ml, which reducing cell viability to 26%. IC_{50} value was not obtained because of cell viability. Additionally, the cell viabilities decreased at the concentrations of 100 µg/ml and 150 µg/ml when compared to positive control (Fig. 3c).

In V79 cells, there was a general decrease in boron nitride nanotubes treated cells at the concentration of 10–300 µg/ml. There were concentration-dependent decreases at above 100 µg/ml compared to positive control. Additionally, the IC_{50} value of boron nitride nanotubes in the studied concentration range was determined to be 291.74 µg/ml (Fig. 3a). In the boron nitride nanotubes and curcumin co-treated cells, the cell viability decreased between 10 and 50 µg/ml and increased between 50 and 250 µg/ml. The IC_{50} value was not detected in the studied dose range and a significant cell viability increase was seen at 250 µg/ml when compared to negative control (1% DMSO). Also, there was a decrease of cell viability at above 10 µg/ml concentration except for 250 µg/ml (Fig. 3b). Furthermore, in the curcumin-treated cells, there was a general decrease at all concentrations compared to negative control and positive control. The cell viability showed significant decrease in the concentration range of 10–50 µg/ml when compared to negative control, whereas there was an increase above these concentrations. For this reason, the IC_{50} value was not determined for curcumin-treated cells (Fig. 3c).

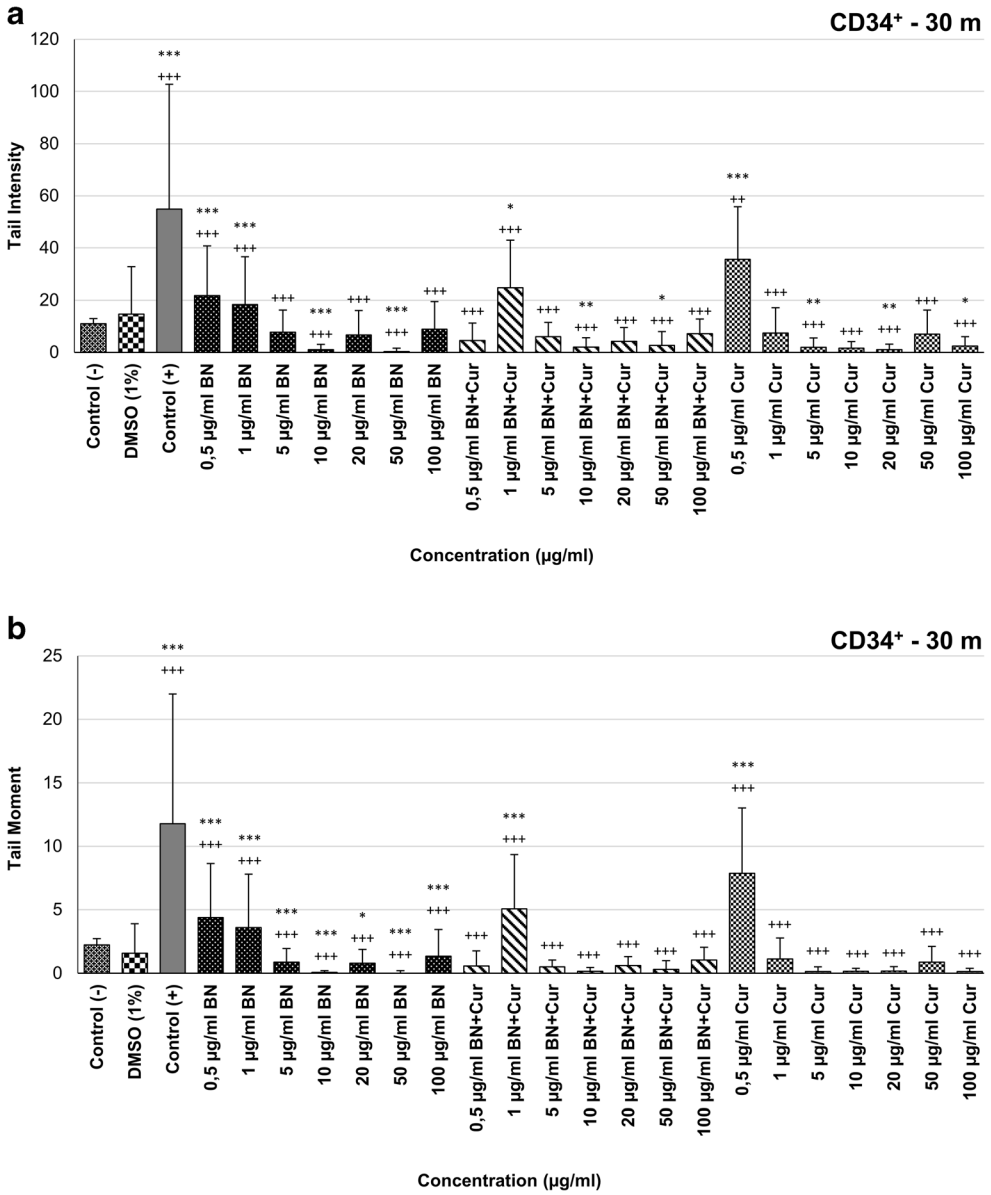

Fig. 4 **a** Tail intensity from the comet assay of bone marrow CD34$^+$ hematopoietic progenitor cells treated with boron nitride (BN), boron nitride + curcumin (BN + Cur) and curcumin (Cur) for 30 min (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significantly different from negative control. $^+p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$, significantly different from positive control). The values are expressed in mean ± standard deviation format. **b** Tail moment from the comet assay of bone marrow CD34$^+$ hematopoietic progenitor cells treated with boron nitride (BN), boron nitride + curcumin (BN + Cur) and curcumin (Cur) for 30 min (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significantly different from negative control. $^+p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$, significantly different from positive control). The values are expressed in mean ± standard deviation format. **c** Tail intensity from the comet assay of HeLa cells treated with boron nitride (BN), boron nitride + curcumin (BN + Cur) and curcumin (Cur) for 30 min (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significantly different from negative control. $^+p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$, significantly different from positive control). The values are expressed in mean ± standard deviation format. **d** Tail moment from the comet assay of HeLa cells treated with boron nitride (BN), boron nitride + curcumin (BN + Cur) and curcumin (Cur) for 30 min (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significantly different from negative control. $^+p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$, significantly different from positive control). The values are expressed in mean ± standard deviation format. **e** Tail intensity from the comet assay of V79 cells treated with boron nitride (BN), boron nitride + curcumin (BN + Cur) and curcumin (Cur) for 30 min (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significantly different from negative control. $^+p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$, significantly different from positive control). The values are expressed in mean ± standard deviation format. **f** Tail moment from the comet assay of V79 cells treated with boron nitride (BN), boron nitride + curcumin (BN + Cur) and curcumin (Cur) for 30 min (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significantly different from negative control. $^+p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$, significantly different from positive control). The values are expressed in mean ± standard deviation format

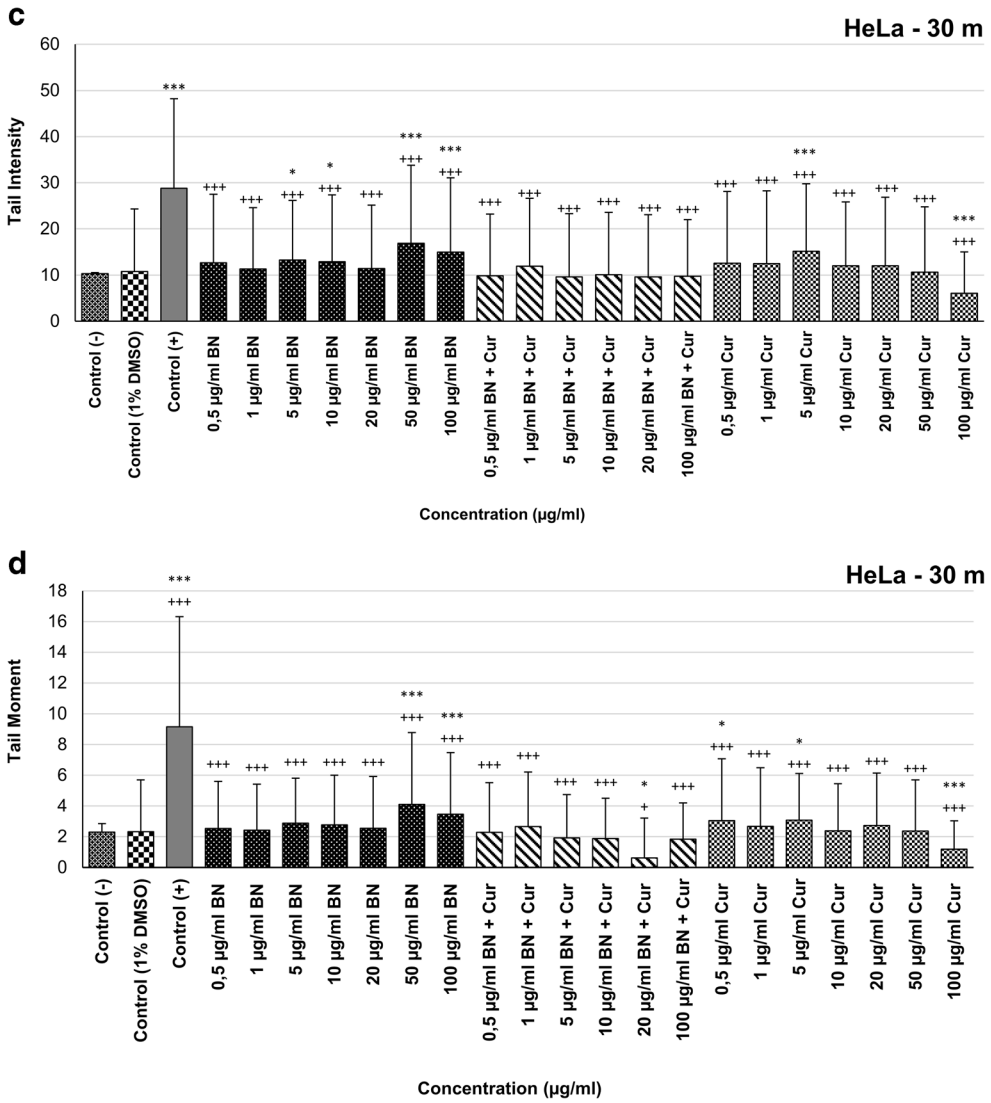

Fig. 4 continued.

The effects of boron nitride nanotubes and curcumin on DNA damage on human bone marrow CD34$^+$ hematopoietic progenitor cells, HeLa and V79 cells

Short incubation time (30 min)

The DNA damage was expressed as tail intensity and tail moment in human bone marrow CD34$^+$ hematopoietic progenitor cells, HeLa cells, and V79 cells treated with boron nitride nanotubes, boron nitride nanotubes + curcumin and curcumin at a wide range of concentration (0.5–100 μg/ml) for 30 min (Fig. 4a-f).

According to the data of bone marrow CD34$^+$ cells, boron nitride nanotubes caused to increase in DNA damage at the concentrations of 0.5 μg/ml and 1 μg/ml, when compared to negative control. In BNNs and curcumin co-treated cells, an increase in DNA damage was observed at 1 μg/ml. There was a significant increase in DNA damage at 0.5 μg/ml concentration in curcumin-treated cells. Also, there was a decrease in the DNA damage at all studied concentrations when compared to positive control (Fig. 4a, b).

In HeLa cells, BNNs increased the DNA damage at all studied concentrations (0.5–100 μg/ml). Additionally, the DNA damage of BNNs and curcumin co-treated cells increased at the concentrations of 1 μg/ml and 50 μg/ml when compared to negative control (1% DMSO). In curcumin-treated cells, there were increases of the DNA damage at all studied concentrations except for 50 μg/ml and 100 μg/ml (Fig. 4c-d).

In BNNs treated V79 cells, there were increases in DNA damage at the concentrations of 5 μg/ml, 20 μg/ml and 100 μg/ml. There was a significant decrease in the DNA damage of BNNs treated cells at the concentrations of 0.5 μg/ml, 20 μg/ml and 100 μg/ml, when compared to positive control.

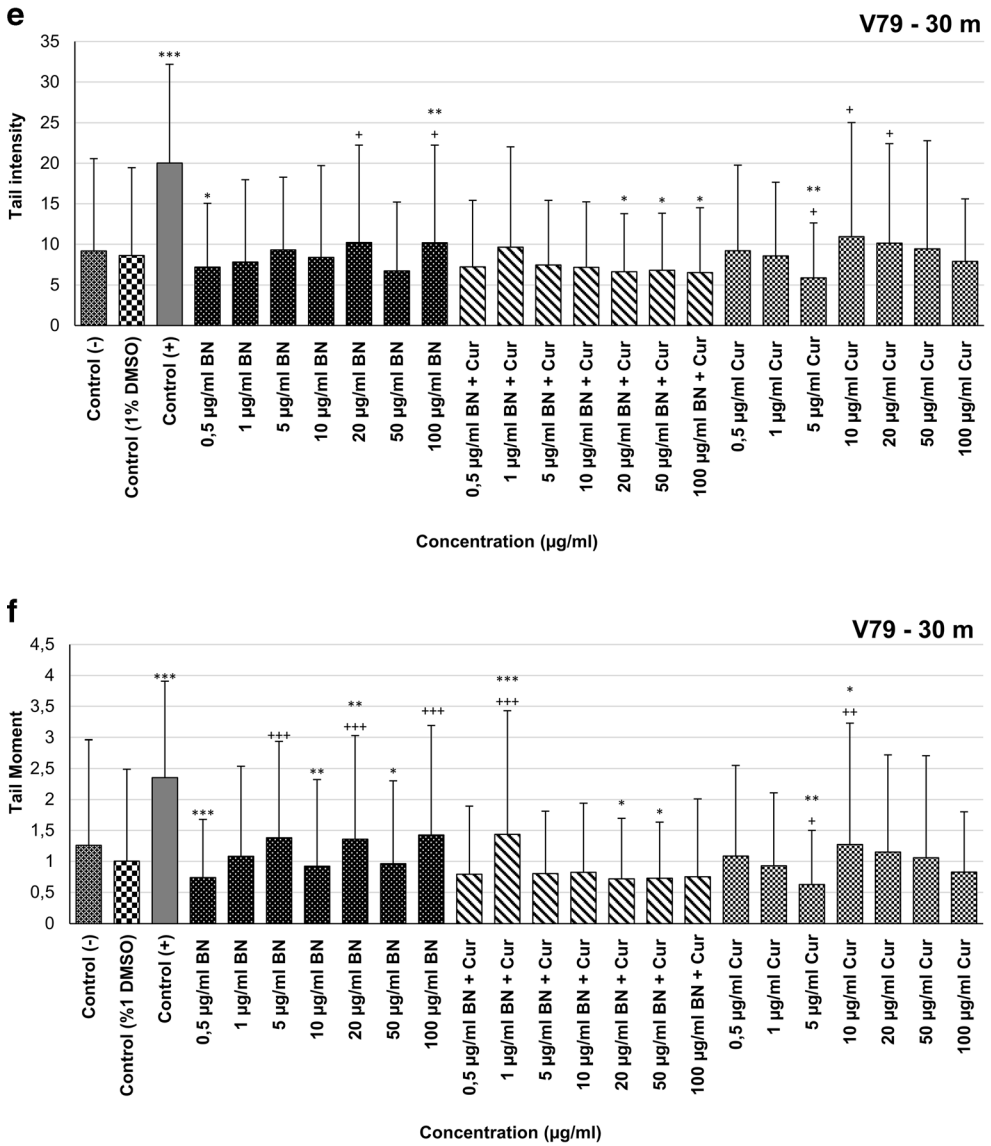

Fig. 4 continued.

Additionally, the DNA damage increase was observed at the concentration of 1 µg/ml in BNNs and curcumin co-treated cells. However, there were slight increases at the concentrations of 0.5 µg/ml, 10 µg/ml, 20 µg/ml and 50 µg/ml in curcumin treated cells, when compared to negative control (1% DMSO) (Fig. 4e, f).

Long incubation time (24 h)

The DNA damage was expressed as tail intensity and tail moment in human bone marrow $CD34^+$ hematopoietic progenitor cells, HeLa cells and V79 cells treated with BNNs, BNNs + curcumin and curcumin at a wide range of concentration (0.5–100 µg/ml) for 24 h (Fig. 5a-f).

In boron nitride nanotubes treated $CD34^+$ cells, the DNA damage increased at the concentrations of 1 µg/ml, 10 µg/ml and 100 µg/ml, when compared to negative control. There were increases of DNA damage at the concentrations of 0.5 µg/ml, 1 µg/ml and 10 µl/ml in BNNs and curcumin co-treated cells. Additionally, curcumin caused to increase of the DNA damage at all studied concentrations except for 50 µg/ml (Fig. 5a, b).

When the DNA damage of HeLa cells was evaluated, boron nitride increased the DNA damage at the concentrations of 1 and 100 µg/ml. However, in BNNs and curcumin co-treated cells there were more increased DNA damage concentrations (0.5 µg/ml, 20 µg/ml, 50 µg/ml and 100 µg/ml). Furthermore, increases in DNA damage were observed at all studied concentrations except for 1 µg/ml and 10 µg/ml in curcumin-treated cells (Fig. 5c, d).

The DNA damage was determined at all studied concentrations except for 10 µg/ml and 100 µg/ml in boron nitride

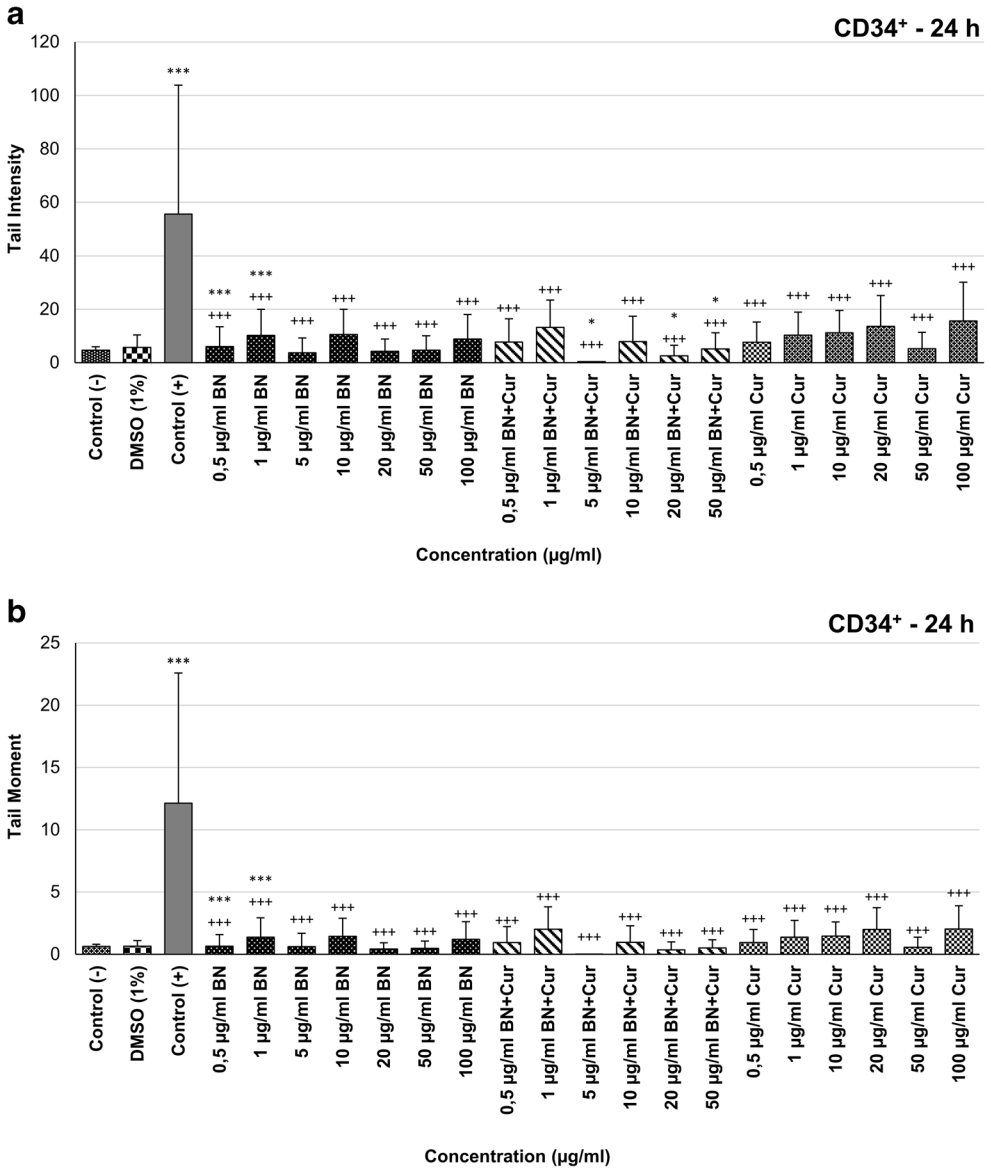

Fig. 5 **a** Tail intensity from the comet assay of bone marrow CD34$^+$ hematopoietic progenitor cells treated with boron nitride (BN), boron nitride + curcumin (BN + Cur) and curcumin (Cur) for 24 h (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significantly different from negative control. $^+p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$, significantly different from positive control). The values are expressed in mean ± standard deviation format. **b** Tail moment from the comet assay of bone marrow CD34$^+$ hematopoietic progenitor cells treated with boron nitride (BN), boron nitride + curcumin (BN + Cur) and curcumin (Cur) for 24 h (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significantly different from negative control. $^+p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$, significantly different from positive control). The values are expressed in mean ± standard deviation format. **c** Tail intensity from the comet assay of HeLa cells treated with boron nitride (BN), boron nitride + curcumin (BN + Cur) and curcumin (Cur) for 24 h (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significantly different from negative control. $^+p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$, significantly different from positive control). The values are expressed in mean ± standard deviation format. **d** Tail moment from the comet assay of HeLa cells treated with boron nitride (BN), boron nitride + curcumin (BN + Cur) and curcumin (Cur) for 24 h (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significantly different from negative control. $^+p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$, significantly different from positive control). The values are expressed in mean ± standard deviation format. **e** Tail intensity from the comet assay of V79 cells treated with boron nitride (BN), boron nitride + curcumin (BN + Cur) and curcumin (Cur) for 24 h (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significantly different from negative control. $^+p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$, significantly different from positive control). The values are expressed in mean ± standard deviation format. **f** Tail moment from the comet assay of V79 cells treated with boron nitride (BN), boron nitride + curcumin (BN + Cur) and curcumin (Cur) for 24 h (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significantly different from negative control. $^+p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$, significantly different from positive control). The values are expressed in mean ± standard deviation format

treated V79 cells. Also, there were increases of the DNA damage at concentrations of 5 µg/ml, 20 µg/ml and 100 µg/ml in boron nitride nanotubes and curcumin co-treated cells. When curcumin-treated cells were evaluated, there were increases of the DNA damage at the concentrations of 0.5 µg/ml, 20 µg/ml, 50 µg/ml, 100 µg/ml (Fig. 5e, f). Furthermore, there

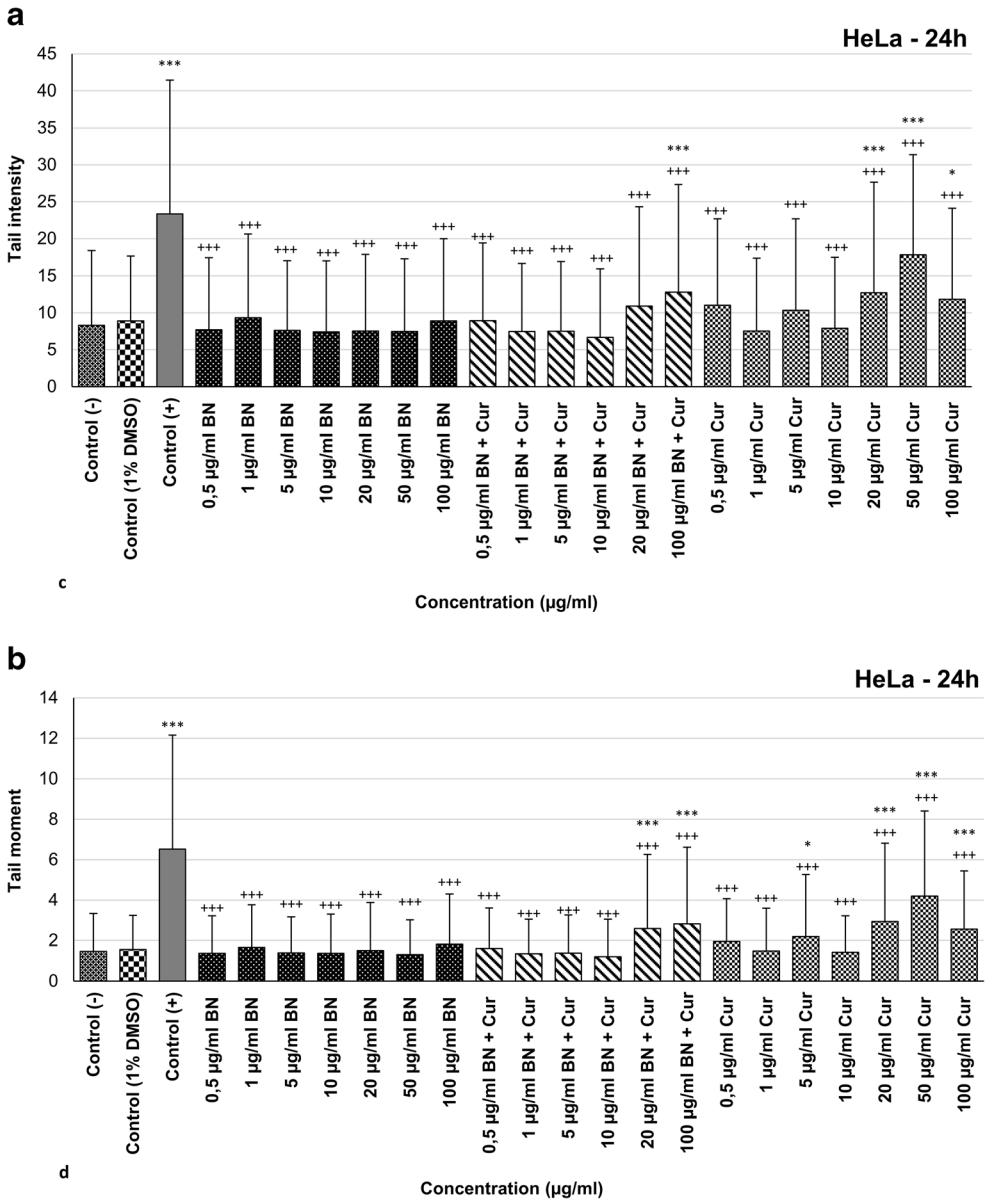

Fig. 5 continued.

were decreases of the DNA damage at all studied concentrations in $CD34^+$ cells, HeLa and V79 cells when compared to positive control.

The comet images of $CD34^+$, HeLa and V79 cells were given in Fig. 6.

Discussion

Stem cells are used for a variety of purposes in areas such as regenerative therapy (chemotherapy, tissue repair, dentistry, aesthetic surgery), drug development studies, toxicity tests, stem cell-mediated diseases and regulation of cell functions due to their self-renewal and differentiation properties [3, 22].

The stem cell studies are potential field for an alternative to the widely used animal models in assessing possible toxic effects of xenobiotics, which have an important place in human life, and for a detailed examination of toxicity mechanisms. Stem cell-based toxicology studies allow to determine the effects of toxins on the differentiation process and differentiated (mature) cell functions [1, 2]. It also removes toxicity changes arising from differences between species [23].

Bone marrow hematopoietic stem cells are responsible for hematopoiesis and are able to differentiate into various cell lines that are important for the therapeutic use and for the development of toxicity test models [24, 25]. The effects of genotoxic agents on bone marrow stem cells can provoke cancer, anemia and cell senescence. Also, this genotoxic effect can be sustained through long-lived hematopoietic stem cells.

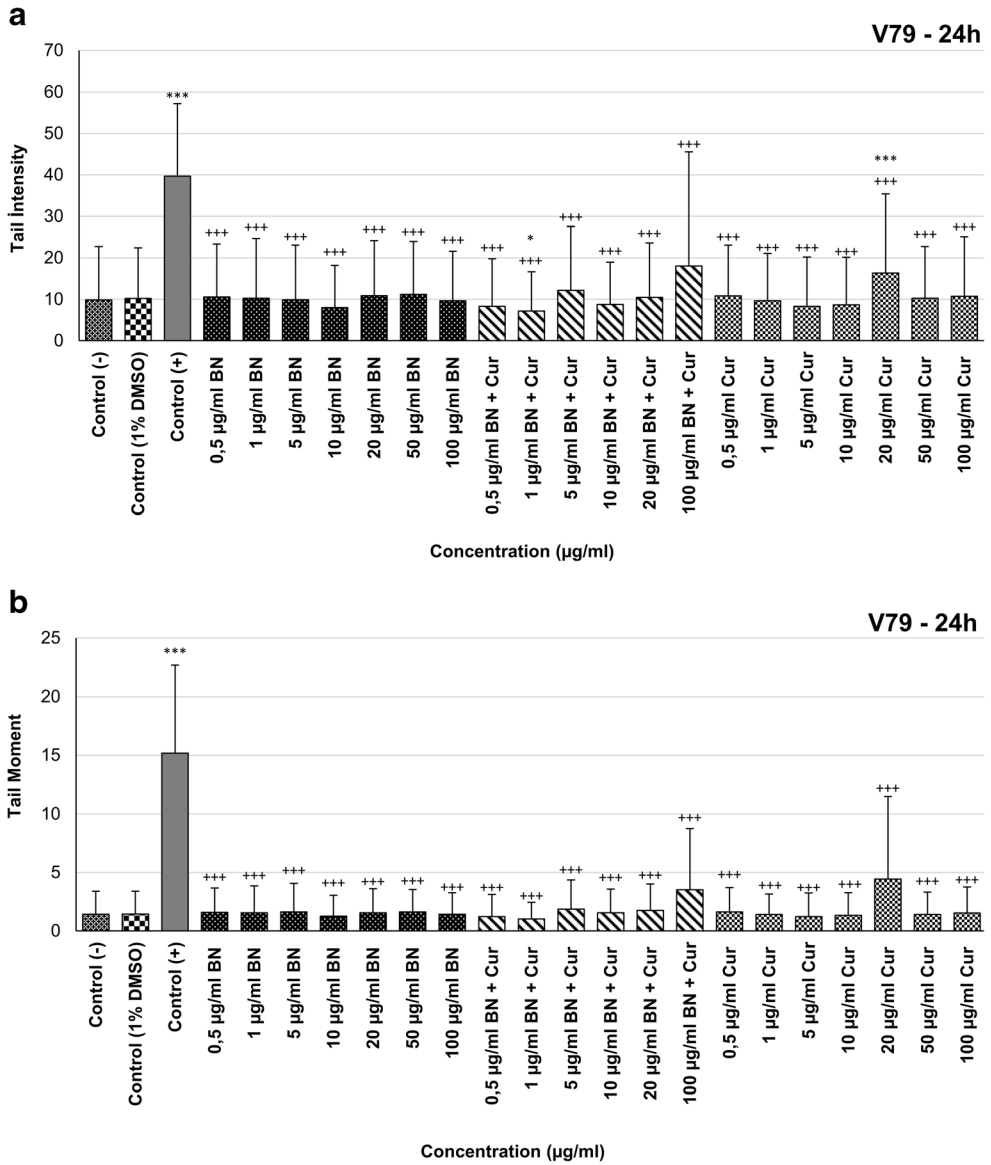

Fig. 5 continued.

Thus, hematopoietic stem cells should be evaluated for cytotoxicity and genotoxicity [5].

BNNs have potency to be used as nanovector in many field and clinical applications due to their resistance to undesirable conditions, their biological inertness, their magnetic and piezoelectric properties [10, 26].

Various formulations (nanoparticle, mucoadhesive, liposome) are used to increase the bioavailability of curcumin, a phenolic compound that has been used in traditional medical applications for a long time and has anticancer-antioxidant - anti-inflammatory effects [27–29]. In this respect, boron nitride nanotubes may be a suitable formulation material as nanovector for curcumin formulations [30–33]. However, there are different findings of BNNs and curcumin toxicity.

The aim of this study is to examine the cytotoxic effects and DNA damaging effects of BNNs and curcumin on bone marrow $CD34^+$ stem cells, HeLa and V79 cells for possible anticancer nanoformulation, drug development and risk assessment of potential exposure effects (e.g. boron nitride nanotubes are potential delivery agent for boron neutron capture therapy which contains ^{10}B isotopes and magnetic drug targeting) [34]. Therefore, this study may provide preliminary results for further in vitro and in vivo studies.

In our study, it was found that the cytotoxic effects of boron nitride nanotubes in HeLa cells were not concentration-dependent and showed a significant reduction in cell viability at 50 µg/ml as compared to the negative control ($p < 0.01$), while there were increased cell viabilities at several concentrations. It was found that BNNs are not cytotoxic up to 50 µg/ml concentration of HeLa cells for 1 and 3 days in the literature [35]. Also, our results showed U shaped effects between 20 and 100 µg/ml concentrations similar to the results

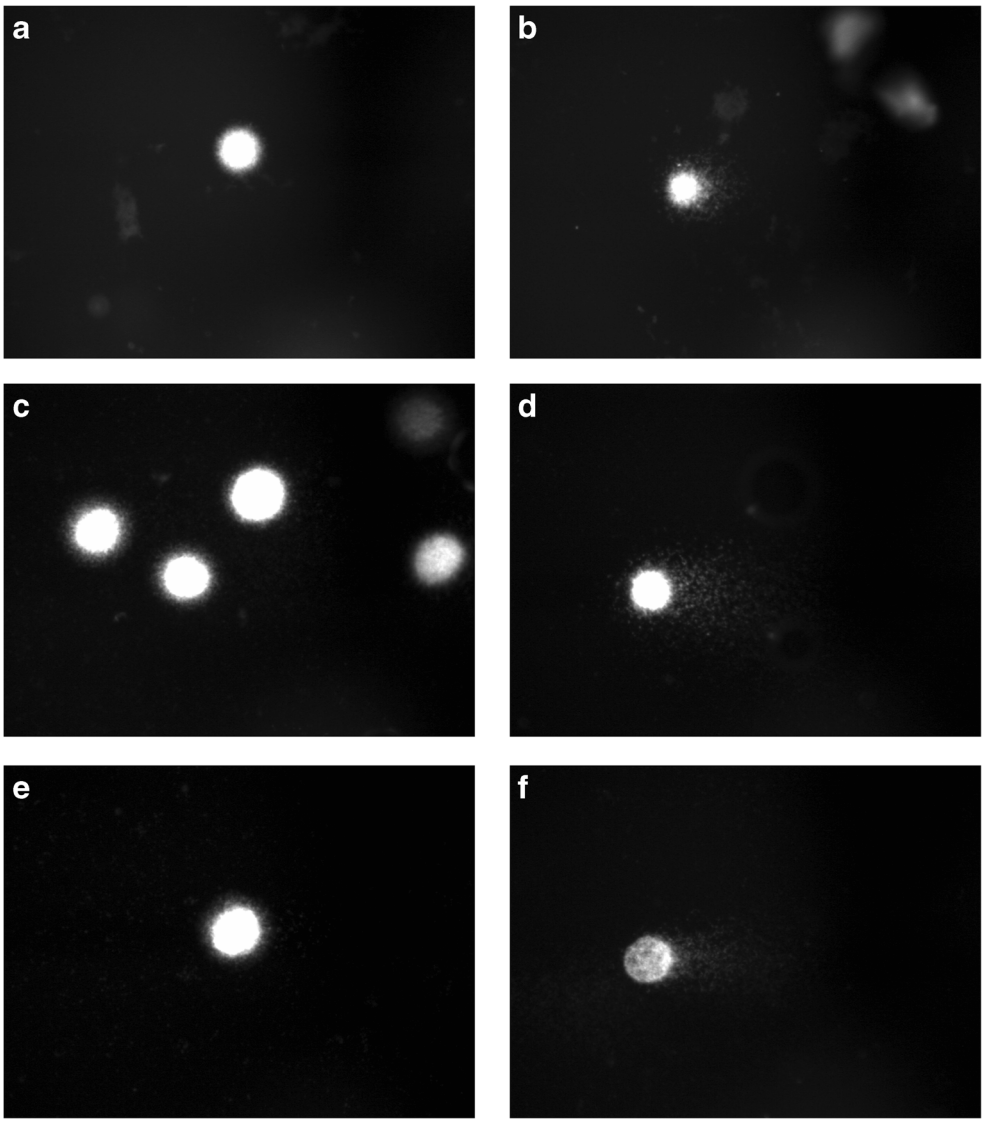

Fig. 6 The comet images of $CD34^+$ stem cells (**a**, **b**), HeLa cells (**c**, **d**) and V79 cells (**e**, **f**)

of boron [36]. In NIH/3 T3 and A549 cells, the boron nitride nanotubes did not adversely affect cell viability up to 62.5 μg/ml (IC_{50} = 62.5–125 μg/ml) [37]. Also, in another study, it was shown that BNNs were not cytotoxic for human osteoblast and mouse macrophage cells also did not affect the morphological and viability properties of human glioblastoma, human gingival fibroblast cells and human vascular endothelial cells [38–42] and these studies support our results. In a similar study, BNNs increased osteoblastic differentiation of mesenchymal stem cells by increasing alkaline phosphatase levels (2 μg/ml BNNs) at the concentration range of 0–25 μg/ml and increased the stem cell proliferation up to a concentration of 5 μg/ml BNNs [43]. The cell viability increased in HeLa cells may be explained with the possible inductive effect of BNNs. However, concentration-dependent cytotoxic effects on HeLa cells were found between 0 and 2 mg/ml for 24 and 48 h similar to V79 cells in our study [44]. Therefore, low doses of boron nitride nanotubes may be used for biological applications.

In another study, nanotubes of hydroxylated (h-BNNs) and carbohydrate bound (m-BNNs) boron nitride did not affect human dermal fibroblast (HDF) cell viability at doses of 5–200 μg/ml. On the other hand, it reduced the viability of A549 cells by 40–60% at doses of 100–200 μg/ml [45]. The transferrin-bound BNNs decreased cell viability of human umbilical vein endothelial cells and NIH/3 T3 fibroblast cells by 15% and 16% at the dose level of 100 μg/ml, respectively. [46]. When studies of BNNs are evaluated, it has been found that processes such as coating or modification, the nanotube size that affects the cellular internalization facilitated by hydrophobic property of boron nitride nanotubes and the studied cell line lead to significant differences in the cytotoxic properties of boron nitride nanotubes [47]. Additionally, different cell membrane pore sizes may cause different cytotoxic effects [44].

When the cytotoxicity results of BNNs - curcumin co-treated and curcumin-treated cells were evaluated, a similar concentration-cell viability profile was obtained in HeLa cells and V79 cells. The cell viability - concentration graphics of BNNs – curcumin and curcumin-treated cells were U shaped graphics similar with other study results that were used phenolic compounds [48, 49]. The cell viability of HeLa and V79 cells were decreased at concentrations of 100 and 50 μg/ml, respectively, when compared to negative control (1% DMSO) ($p < 0.001$). According to the results of a similar study, the effects of curcumin on the viability of HeLa cells were evaluated by the MTT method, the IC_{50} value was determined to be 16.52 μM, and in another study it was determined to be 25–50 μM, and the cytotoxic effect of curcumin had dose-dependent profile [5, 50, 51]. However, there was general increase of the cell viability at above these concentrations and it may be because of the mitochondrial function enhancement effect of curcumin [30]. Also, the similarity between the cell viability profile of curcumin-treated and boron nitride-curcumin co-treated cells may be evaluated as the marker of BNNs inertness [52].

According to the results of comet analysis for 30 m and 24 h incubation, boron nitride nanotubes caused a statistically significant increase of DNA damage in bone marrow $CD34^+$ cells, in HeLa and V79 cells at several concentrations compared to negative control. In a similar study, it has been determined that BNNs did not cause chromosomal damage in lung fibroblast cells up to 50 μg/ml, but it caused cell damage due to increased ROS (reactive oxygen species) at above of this concentration [53]. Studies on human dermal fibroblast cells and alveolar basal epithelial cells showed that BNNs at the concentration range of 100–200 μg/ml caused DNA damage [54]. The increased DNA damage caused by BNNs may be the result of increased reactive oxygen species [54].

Furthermore, curcumin increased DNA damage at several concentrations in our study and in another studies showing that the genotoxic activity of curcumin, is caused by increased lipid peroxidation and oxidative stress [55–57]. Also, curcumin has been shown to be genotoxic at high doses and antigenotoxic at low doses by Cao et al. [58]. The results of this study are consistent with our findings on DNA damage in V79 and HeLa cells for 24 h incubation.

Additionally, when the comet results of 30 min and 24 h incubation time were compared, it was observed that the DNA damage was decreased depending on incubation time. According to our results, DNA damages in all cell lines were repaired at 0.5–10 μg/ml concentration range for boron nitride nanotubes and BNNs – curcumin co-treated cells.

Finally, when the comet assay results for 24 h and MTT results of HeLa and V79 cell lines were compared, it was observed that cell viability decrease of curcumin and boron nitride nanotubes-curcumin co-treated cells (20–50 μg/ml) was compatible with the DNA damage increase. Hence, the DNA damage (strand breaks) may be the reason for cell death via ROS activity [59].

Conclusion

There are studies showing that boron nitride nanotubes may be a suitable material as nanovector and curcumin is a potential agent for targeted anticancer drug development in nanoformulation. They can be functionalized with bioactive conjugates and can be used for targeted therapy. For all that, in vitro assessment of their compatibility is important before any realistic bio-applications.

In this study, the cytotoxic effects and the effects on DNA damage of boron nitride nanotubes and curcumin were evaluated to contribute to the risk assessment of boron nitride nanotubes and curcumin exposure. According to our results, boron nitride nanotubes and curcumin had cytotoxic effects and caused DNA damage on $CD34^+$, HeLa and V79 cells at several concentrations. These concentration – effect graphics of cytotoxicity and DNA damage had U shape. These effects were probably caused by ROS increase and oxidative stress. Also, the results of curcumin and BNNs - curcumin concentration groups were similar that may be the sign of BNNs inertness. However, there are controversial results about their toxicity in the literature. Our findings also demonstrated the necessity of performing many tests at different concentrations and on different cell lines to contribute to nanovector development for DNA, drugs and radioisotopes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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