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Induction of Apoptosis in Ehrlich Ascites Carcinoma Cells Through an Intrinsic Pathway by Ni(II)-benzoin Thiosemicarbazone Complex [$Ni(BTSC)_2$]

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Abstract Cancer is one of the leading causes of morbidity and mortality through worldwide. Globally cancer recognized as the second leading cause of death. Therefore, the discovery and development of new potent and selective anticancer drugs are of high importance in modern cancer research. The objective of this study was to find out the mechanism through which Ni(II)-benzoin thiosemicarbazone complex exerts its antitumor activity against Ehrlich Ascites Carcinoma (EAC) cells bearing swiss albino mice. Induction of apoptosis in EAC cells was confirmed by observation of nuclear morphology and DNA fragmentation assay. The mRNA expression of several apoptotic genes like B-cell lymphoma 2 (bcl-2), B-cell lymphoma extra-large (bcl-xL) caspase-8, and proapoptotic genes p53 or tumor protein, bcl-2 associated X protein (bax), caspase-9, caspase-3 and poly-ADP ribose polymerase (PARP-1) reveal the induction of apoptosis by $Ni(BTSC)_2$ in EAC cell. Inhibition of $Ni(BTSC)_2$ induced apoptosis by Caspase 3 inhibitor treatment affirmed that the induction of intrinsic apoptosis pathway on EAC cells. Reactive Oxygen Species (ROS) generation after $Ni(BTSC)_2$ treatment confirmed that the induction of apoptosis by $Ni(BTSC)_2$ occurred through an ROS-dependent mitochondria-mediated intrinsic pathway rather than an extrinsic pathway. Thus, this study provides evidence to carry out further researches in a way to formulate novel anticancer drugs.

Keywords: *EAC cells, nickel benzoin thiosemicarbazone complex, intrinsic pathway, ROS, caspase inhibitor*

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1. Introduction

Cancer is a class of diseases in which a group of cells display uncontrolled growth, invasion (intrusion on and destruction of adjacent tissues) and sometimes metastasis (spread to other locations in the body via lymph or blood). The inactivation of programmed cell death, or apoptosis, is central to the development of cancer. Apoptosis, or programmed cell death, evolved as a rapid and irreversible process to efficiently eliminate dysfunctional cells [1]. Apoptosis can be initiated through one of two pathways, one is intrinsic pathway and other is the extrinsic pathway [2]. The intrinsic (mitochondrial) pathway is induced by cellular stress, infection and DNA damage and results in the activation of pro-apoptotic bcl-2 associated X (Bax) or bcl-2 homologous killer (bak) proteins oligomerize to creat holes or pores on the mitochondrial outer membrane and release cytochrome c to the cytosol [3]. The another pathway is extrinsic (death receptor) pathway initiated by death ligand such as tumor necrosis factor α (TNF α) or TNF-related apoptosis inducing ligand (TRAIL) binding to cell surface receptors and activate caspase-8, which then activate downstream caspase-3 directly which play essential role in programmed cell death [4]. These two pathways are executed mainly by caspases a family of endoproteases that provide critical links in cell regulatory networks controlling inflammation and cell death with caspase-8 and -9 engaging in the extrinsic and intrinsic pathways, respectively [5,6]. In addition, reactive oxygen species (ROS), a series of oxygen metabolism byproducts, have a role in cell signaling, including; apoptosis; gene expression; and the activation of cell signaling cascades [7]. In our previous work, we have confirmed the anticancer properties of $Ni(BTSC)_2$ by measuring *in vivo* tumor cell growth inhibition, tumor weight measurement, survival time of tumor bearing swiss albino mice and also hematological parameters against EAC cells [8]. The present work is the continuation of our previous work.

Here we have reported the apoptotic pathway of nickel (II) benzoin thiosemicarbazone complex against EAC cells in swiss albino mice.

2. Materials and Methods

2.1. Chemicals

All chemicals and reagents used to carry out the research work were of reagent grade.

2.2. Experimental Animal

Adult male Swiss albino mice, 5-7 weeks old (25 ± 4 g body weight), were collected from animal resource branch of the International Centre for Diarrheal Disease Research (ICDDR'B), Mohakhali, Dhaka, Bangladesh. Animals were housed in iron cages containing sterile paddy husk and saw dust as bedding material under hygienic conditions with a maximum of six animals in a cage. They were maintained under controlled conditions (12:12 h light–dark with temperature 22 ± 5 °C). Standard mouse diet (recommended and prepared by ICDDR'B and water were given in adequate. Ethic approval was obtained from institutional animal, medical ethics, biosafety and biosecurity committee for experimentations on animal, human, microbes, and living natural sources from Institute of Biological Sciences, University of Rajshahi, Bangladesh (225/320-IAMEBBC/IBSc).

2.3. Synthesis of the compound

2.3.1. Synthesis of Ni (II)-benzoin thiosemicarbazone complex:

The compound was synthesized according to the method as described in the literature [9,10]. For benzoin thiosemicarbazone (BTSC), benzoin and thiosemicarbazide (1:1 molar ratio) were mixed together and refluxed for a period of 3-4 hours and then distilled to half of the total volume. A saturated solution of nickel (II) acetate in ethanol was added to the condensed solution. Within a few minutes black crystals of Ni(II)-benzoin thiosemicarbazone were obtained. The crystals were then recrystallized twice, dried in an oven at 50°C and stored in a desiccator.

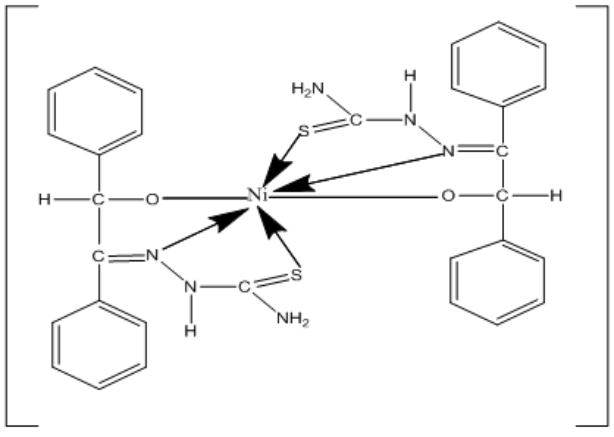


Figure 1. Structure of $Ni(BTSC)_2$ complex

2.3.2. Characterization of the schiff base complex

The synthesized compound was characterized by taking melting point and IR spectra as we reported previously [8].

2.4. Cell Lines

Ehrlich Ascites Carcinoma (EAC) cells were obtained by the courtesy of Indian Institute of Chemical Biology (IICB), Kolkata, India. The cells were maintained as mammary gland cancer cell in ascites in Swiss albino mice by intraperitoneal inoculation (biweekly) of 2×10^6 cells/mouse. For in vitro study, EAC cells were cultured in RPMI-1640 medium having glucose, 2 mM L-glutamine in presence of 10% fetal calf serum, and 1% (v/v) penicillin-streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

2.5. *In vitro* Cell Growth Inhibition

In vitro effect of $Ni(BTSC)_2$ against EAC cell growth inhibition was assayed by MTT colorimetric assay. NAD(P)H-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to its insoluble formazan, which has a purple color. For this experiment, 4×10^6 EAC cells in 200 µL RPMI-1640 media were plated in the 96-well flat bottom culture plate in presence of six different concentrations (5 µg, 10 µg, 20 µg, 40 µg, 80 µg and 120 µg/mL) of $Ni(BTSC)_2$ and incubated for 24 h at 37 °C in CO_2 incubator. EAC cells treated with dimethyl sulfoxide (DMSO), was used as control. After the incubation period, aliquot were removed carefully, and 180 µL of phosphate buffered saline (PBS) and 20 µL of MTT were added to each well and further incubated for 8 h at 37 °C. The aliquot was removed again and 200 µL of acidic isopropanol was added and incubated again at 37 °C for 1 h. Subsequently, absorbance was taken at 570 nm in microtiter plate reader (Optica Microplate Reader, Mikura Ltd., Horsham, UK). The following equation was used to calculate the cell proliferation inhibition ratio:

$$\text{Proliferation inhibition ratio } (\%) = \frac{(A-B)\times 100}{A}$$

Where A is the OD 570 nm of the cellular homogenate (control) without experimental compounds and B is the OD 570 nm of the cellular homogenate with experimental compounds.

2.7. Cell morphologic Change and Nuclear Damage

EAC cells were collected from the treated and untreated EAC cells bearing Swiss albino mice and washed twice with phosphate buffer saline (PBS). Then the cells were stained with 4', 6-diamidino-2-phenylindole (DAPI) at 37°C for 20 min and washed twice again with PBS. After that the morphology of EAC cells were observed by using a fluorescence microscope (Olympus iX71, Seoul, Korea) [11].

2.8. DNA Fragmentation Assay

DNA fragmentation assay by agarose gel electrophoresis was determined by the method described previously [12]. EAC cells collected from mice treated with $Ni(BTSC)_2$ at the dose of 8mg/kg/mice (five consecutive days) and from control mice. The cells were washed with PBS and resuspended again in PBS. The total DNA was isolated by using a DNA extraction kit (Promega, Madison, WI, USA) and analyzed by electrophoresis on 1.5% agarose gel containing 0.1µg/mL ethidium bromide and visualized under Ultraviolet illuminator.

2.9. Effect of Caspase Inhibitors on EAC Cells

In order to confirm the involvement of caspases in the $Ni(BTSC)_2$ induced cell death, the untreated EAC cells were collected on day six after tumor inoculation and washed with PBS [13]. Then these cells were cultured in RPMI-1064 media with Z-DEVD-fmk (caspase-3 inhibition, 2µmol/mL) and Z-IETD-fmk (caspase-8 inhibitor, 2µmol/mL) and incubated in CO_2 incubator for 2h. Then the cells were treated with $Ni(BTSC)_2$ and incubated for another 24 hours at 37°C in CO_2 incubator. Finally, the cells were counted using hemocytometer and determined the effects of experimental compounds on cell growth inhibition in presence or absence of specific inhibitor.

2.10. Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted using TRIzol method from mice receiving $Ni(BTSC)_2$ at the dose of 8mg/kg/day and control EAC cells bearing mice on day six of tumor implantation. Total RNA was used as a template for reverse transcription using the following protocol: each 20µL reaction contained 125µM deoxynucleotide (dNTP), 100 pmol random hexamer, 100 pmol oligo dT_{18} primer, 50 units of MuLV reverse transcriptase (New England Bio lab, Ipawich, MA, USA), diethylpyrocarbonate (DEPC) treated water and 3µg total RNA. Briefly, RNA and oligo dT_{18} primer where incubated at 70°C for 15 minutes then immediately placed on ice, after which the other components were added and incubated at 42°C for 1h and then at 70°C for 15 minutes. Thus the cDNA was prepared and used as template in PCR for examined the expression of one housekeeping gene (GAPGH) and eight growth regulatory genes, namely, Bcl-2, Bax, p53, Bcl-xL, PARP-1 and Caspase-3,-8,-9. Reaction mixture were prepared containing 1X of Taq polymerase, 25pmol each of forward and reverse primer, 2.5mM of each dNTP and 0.25U of platinum Tag polymerase (Tiangen, Beijing, China), 2µL cDNA and nuclease-free water to a total volume of 25µL. Primer sequences are listed in Table 1. BioRad (Hercules CA, USA) gradient thermal cycler was used for amplifications. All the PCR products were analyzed in 1.5% agarose gel and Gene Rular 1kb DNA ladder (Fermentus, Pittsburgh, PA, USA) was used as marker. The PCR was performed under the following conditions: 3 min initial denaturation at 95°C; 35 cycles of denaturation (1 min at 95°C), annealing (1 min at 55°C for p53, Bcl-2, Caspase-3, -8, -9 genes and at 54°C for Bax, GAPDH, Bcl-xL genes), and extension (1 min at 72°C); a final extension at 72°C for 10 min.

2.11. Measurement of Reactive Oxygen Species (ROS) Generation

The ROS level of the cells were examined using 2′, 7′-dichlorodihydrofluoresce in diacetate (DCFH-DA; Invitrogen, USA). The cells were collected from control mice and treated mice on day six of tumor inoculation and washed with PBS at 1200rpm for 2minutes. Then the cells were incubated with DCFH-DA 10µL (50µM final concentration) at 37^0C for 30minutes in the dark. Again the cells were washed twice with PBS and maintained in 1mL culture medium and finally ROS generation was assessed using a fluorescence microscope at excitation and emission wavelengths of 485nm and 530nm, respectively [14].

2.12. Statistical Analysis

The experimental results have been expressed as the mean ± S.E.M. Data have been calculated by one was ANOVA followed by Dunnett "t" test for the determination of statistical significance using SPSS software of 20 version. The difference was considered to be statistically significant when $p < 0.05$.

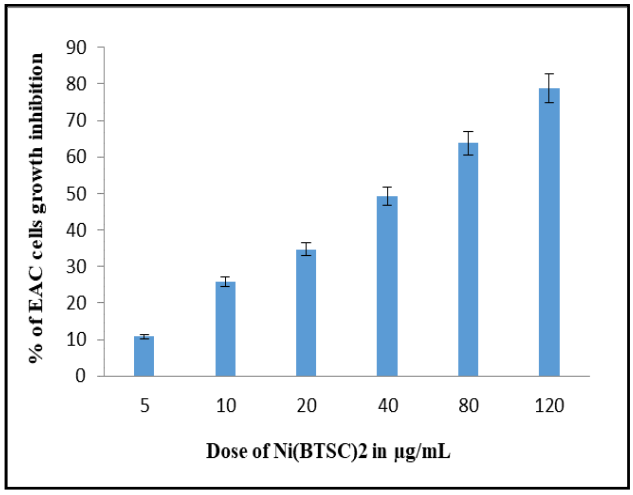
Table 1. Primer sequences and PCR protocol used for the RT-PCR assay

Sl. No.	Target genes	Primers	Amplification (kb)
1	Bcl-2 F	5′-GTGGAGGAGCTCTTCAGGGA-3′	0.200
	Bcl-2 R	5′-AGGCACCCAGGGTGATGCAA-3′	
2	BAX F	5′-CGCCCACCAGCTCTGAGCAGA-3′	0.500
	BAX R	5′-GCCACGTGGGCGTCCCAAAGT-3′	
3	P^{53} F	5′-GCGTCTTAGAGACAGTTGACT-3′	0.550
	P^{53} R	5′-GGATAGGTCGGCGGTTCATGC-3′	
4	Bcl-xL F	5′-TTGGACAATGGACTGGTTGA-3′	0.700
	Bcl-xL R	5′-GTAGAGTGGATGGTCAGTG-3′	
5	Caspase-3 F	5′-TTAATAAAGGTATCCATGGAGAACACT-3′	0.300
	Caspase-3 R	5′-TTAGTGATAAAAATAGAGTTCTTTTGT-3′	
6	Caspase-8 F	5′-CTGCTGGGGATGGCCACTGTG-3′	0.450
	Caspase-8 R	5′-TCGCCTCGAGGACATCGCTCTC-3′	
7	Caspase-9 F	5′-ATGGACGAAGCGGATCGG-3′	0.400
	Caspase-9 R	5′-CCCTGGCCTTATGATGTT-3′	
8	PARP-1 F	5′-AGGCCCTAAAGGCTCAGAAT-3′	0.270
	PARP-1 R	5′-CTAGGTTTCTGTGTCTTGAC-3′	
9	GAPDH F	5′- GTGGAAGGACTCATGACCACAG-3′	0.350
	GAPDH R	5′- CTGGTGCTCAGTGTAGCCCAG-3′	

2. Results

2.1. In vitro EAC Cell Growth Inhibition with $Ni(BTSC)_2$

In vitro effect of $Ni(BTSC)_2$ on EAC cell growth inhibition have been investigated by MTT assay. The $Ni(BTSC)_2$ induced EAC cell death is a dose dependent manner (Figure 2). The $Ni(BTSC)_2$ showed 10.8%, 25.88%, 34.7%, 49.25, 63.74% and 78.89% cell growth inhibition at the concentration of 5,10,20,40,80 and 120 µg/mL respectively. The effect decreased with the reduction in concentration of $Ni(BTSC)_2$.



Data are expressed as the mean of results in 3 repeated experiments. Error bar represent standard error of mean.

Figure 2. *In vitro* effect of $Ni(BTSC)_2$ on EAC cell growth inhibition

2.2. $Ni(BTSC)_2$-induced Morphological Changes and Nuclear Damage

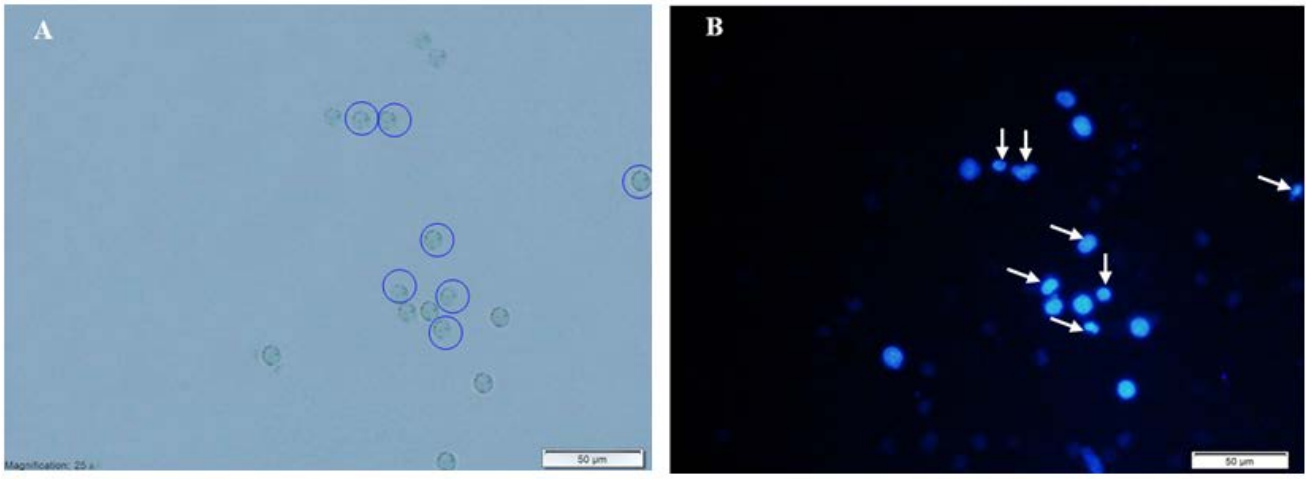
Morphological changes of EAC cells in absence and presence of $Ni(BTSC)_2$ at the dose of 8 mg/kg/day, were studied using a fluorescence microscope (Olympus iX71, Seoul, Korea) and confirmed by 4', 6-diamidino-2-phenylindole (DAPI) staining. Round and homogeneously stained nuclei were observed in the control group (Figure 3A) and the cells of treated group exhibited manifest fragmented DNA in nuclei (Figure 3B). Apoptotic morphologic alterations (e.g. fragmentation, cell membrane blebbing and nuclear condensation) were also observed clearly by optical microscopy and these results suggested that $Ni(BTSC)_2$ could induce apoptosis of EAC cells.

2.3. DNA Fragmentation Assay

Apoptotic DNA fragmentation is a key feature of apoptosis, which is characterized by the activation of endogenous endonucleases, particularly the caspase-3 activated DNase (CAD), with subsequent cleavage of nuclear DNA into internucleosomal fragments of roughly 180 base pairs (bp) and multiples there of (360, 540 etc.) [15]. The activation of the endogenous Ca^{2+}/Mg^{2+} dependent endonuclease is the most distinctive biochemical hallmark of apoptosis. This activated endonuclease mediated the cleavage of internucleosomes and generates oligonucleotide fragments of about 180-200 base pairs length or their polymers. Apoptotic DNA fragmentation was obtained in agarose gel electrophoresis of DNA preparation from $Ni(BTSC)_2$ treated EAC cells which is characteristic feature of apoptosis induction. On the other hand, control group shown smear-like DNA degradation. Result was shown in Figure 4.

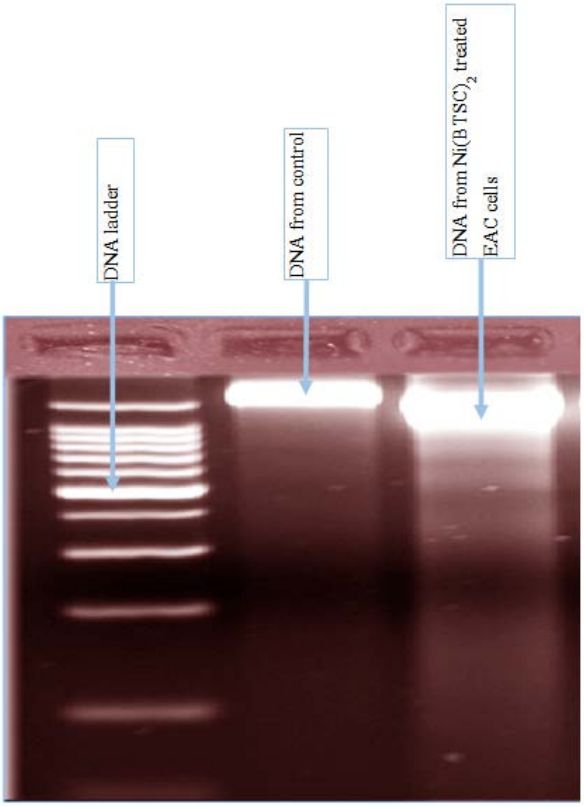
2.4. Effect of Caspases on $Ni(BTSC)_2$-induced Cytotoxicity in EAC Cells

Caspase inhibitors Z-DEVD-fmk (caspase-3 inhibitor) and Z-IETD-fmk (caspase-8 inhibitor) were used to detect the involvement of specific caspases in the apoptotic cell death of EAC cells induced by the treatment of $Ni(BTSC)_2$. In the presence of $Ni(BTSC)_2$ growth inhibition of EAC cells was found 78.89%, in where 3.88% cell growth inhibition was found in presence of Z-DEVD-fmk (caspase-3 inhibitor) and 75.01% cell growth inhibition was found in presence of Z-IETD-fmk (caspase-8 inhibitor) in the culture medium (Figure 5).



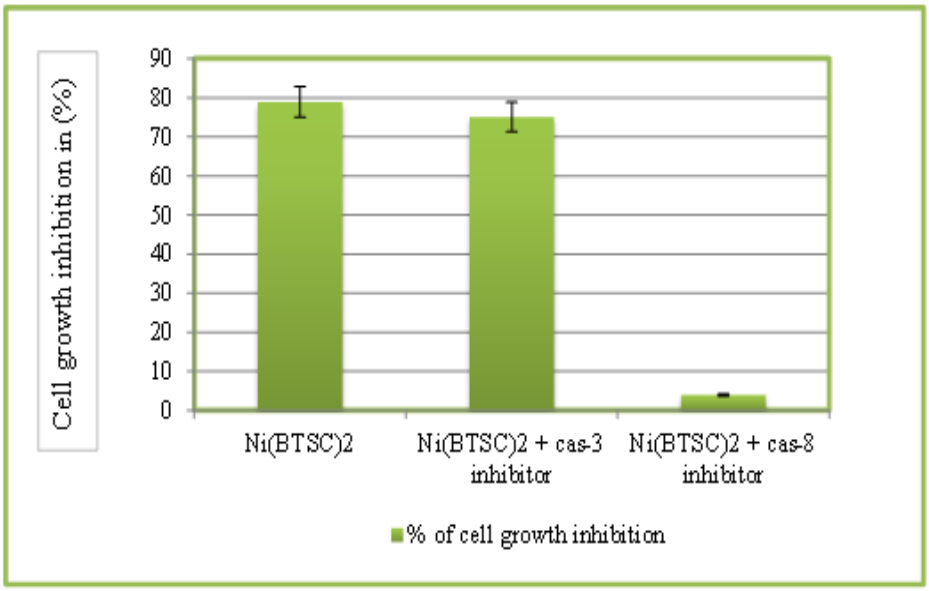
Cells were collected from untreated EAC bearing mice A (optical) and $Ni(BTSC)_2$ treated EAC bearing mice B (fluorescence). Arrows indicate apoptotic features (condensed chromatin and nuclear fragmentation).

Figure 3. Effect of $Ni(BTSC)_2$ [8 mg/kg/day (*i.p.*)] on morphological changes of EAC cell



Apoptotic DNA fragmentation (right) from $Ni(BTSC)_2$ treated EAC cells bearing mice, Control DNA (middle) and a 1kb DNA marker (left)

Figure 4. DNA fragmentation assay in $Ni(BTSC)_2$-treated mice



Numbers of mice in each experiment were 6. The results are shown in mean±SEM. Significant value is, **p<0.01.

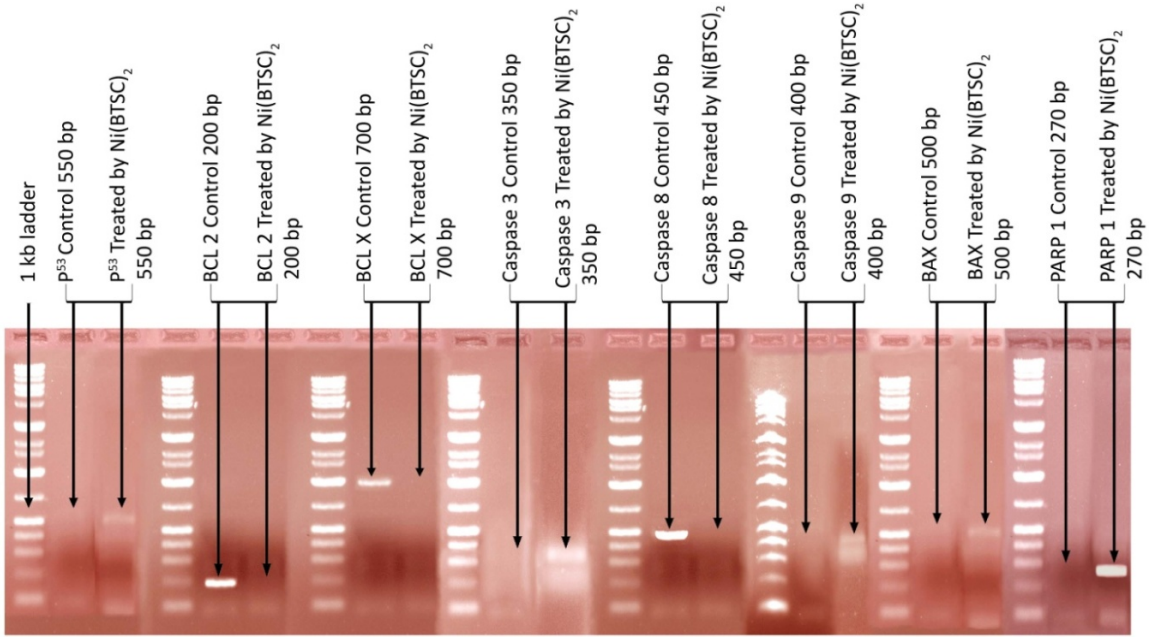
Figure 5. Effect of caspases on $Ni(BTSC)_2$-induced cytotoxicity in EAC cells

2.5. Altered Expressions of Cancer-related Genes in $Ni(BTSC)_2$ Treated EAC Cells

Reverse transcription PCR was used to study the mRNA expression levels of several tumor-related antiapoptotic genes (Bcl-2, Bcl-xL and caspase-8) and proapoptotic genes (p53, Bax, PARP-1, caspase-3 and caspase-9) in control and $Ni(BTSC)_2$-treated EAC cells (Figure 6). GAPDH primers were used for an amplification reaction to confirm the suitability of the purified RNAs and the samples were found to be suitable for RT-PCR. The control cells showed high expressions of BCL-2, BCL-xL and caspase-8 genes, whereas EAC cells treated with $Ni(BTSC)_2$ showed reduced BCL-2, BCL-xL and caspase-8 mRNA expressions. In addition, the p53, BAX, PARP-1, caspase-3 and caspase-9 genes showed increased expressions in treated EAC cells. On the other hand, no expression of these genes was found in control mice.

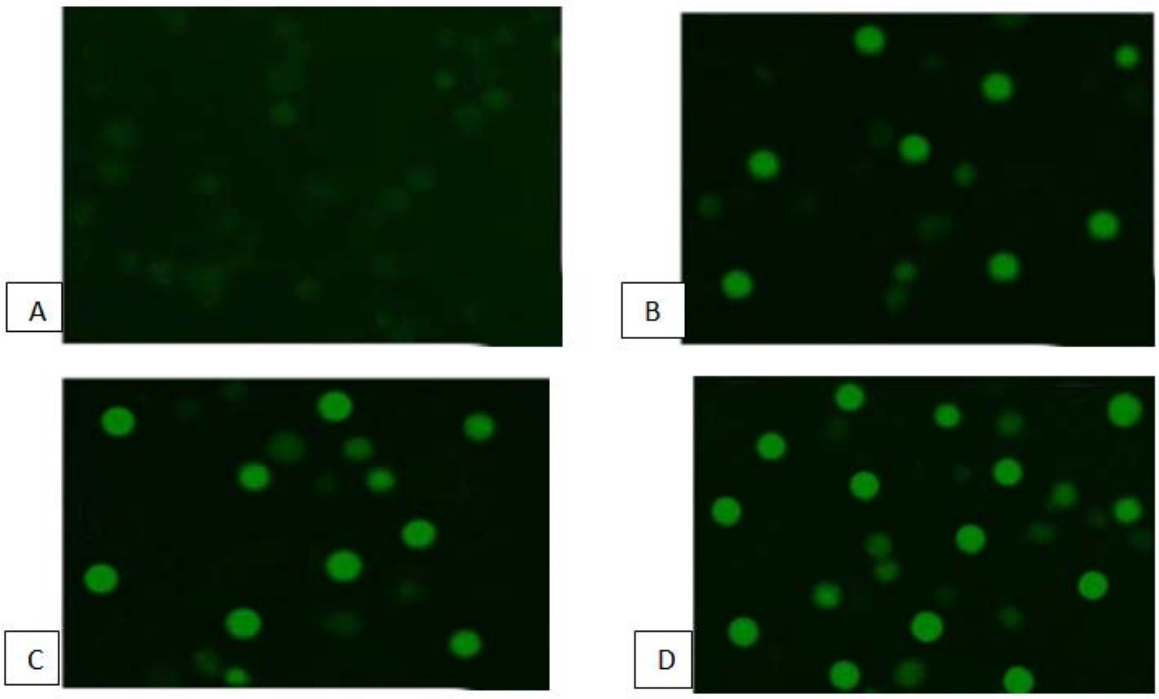
2.6. Determination of Intracellular Reactive Oxygen System in EAC Cells

2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA) is a common fluorescent probe that can be used for detecting ROS in biological media. The DCFH-DA first hydrolyzed to DCFH by intracellular esterase, which is then oxidized by reactive species and originates a fluorescent compound 2, 7- dichlorofluorescein (DCF), whose fluorescence intensity is proportional to the levels of ROS. Here, DCFH-DA was used to assess the effect of $Ni(BTSC)_2$ on ROS generation in EAC cells after 24 h treatment. As shown in Figure 7, the untreated control cells displayed little green fluorescence, while increasing concentrations of $Ni(BTSC)_2$ resulted in much stronger signals, with 8 mg/kg (*i. p.*) the compounds producing the strongest intensity.



RNA was extracted from the experimental mice on day 6 and level of mRNA expression of antiapoptotic genes (Bcl-2, Bcl-xL and caspase-8) and proapoptotic genes (p53, Bax, Caspase-3, -9 and PARP-1) were studied. In $Ni(BTSC)_2$ treated mice, all the proapoptotic genes expression increased remarkably, and the expression of antiapoptotic genes were not observed. In control group, opposite results were observed.

Figure 6. Expressions of different cancer-related genes in $Ni(BTSC)_2$-treated EAC cells



Effects of $Ni(BTSC)_2$ on the generation of ROS in EAC cells after 24 h treatment. Observation of cells by fluorescence microscopy (200× magnification) after incubation with DCFH-DA. (A) Control cells, (B) 2 mg/kg (*i.p*), (C) 4 mg/kg (*i.p.*) and (D) 8 mg/kg (*i.p.*).

Figure 7. Effect of $Ni(BTSC)_2$ on intracellur ROS generation

3. Discussion

Morphological changes and death of apoptotic cells are caused by a series of proteases termed caspases, such as caspase-3, -6, -7, -8 and -9, which degrade proteins [16]. Caspases in mammals can be broadly classified by their roles in apoptosis (caspases-3, -6, -7, -8 and -9). With regards to apoptosis, caspases-8 and -9 are initiator caspases in the extrinsic and intrinsic pathways, respectively, while caspase-3, -6 and -7 are executioner caspases in both pathways [17,18]. In the present study $Ni(BTSC)_2$ induced apoptosis was confirmed by the observation of the changes in nuclear morphology and cell shape as compared to that of the control EAC cells. Moreover the activity of the caspase-3 and caspase-8 were blocked significantly by the z-DEDV-fmk and z-IETD-fmk inhibitors respectively. Form our result it is clear that $Ni(BTSC)_2$ induced apoptosis was blocked by caspases-3 inhibitors, which suggesting that apoptosis occurred mainly through caspase-3 dependent intrinsic pathway.

Similarly, this study showed induction of apoptosis in $Ni(BTSC)_2$-treated EAC cells is regulated by numerous growth related genes. Among them, Bcl-2 (B cell lymphoma gene 2) family gene was believed to be the first one attributed in the apoptotic process [19]. Among the apoptosis-related genes, Bax, Bid, and Bak act as proapoptotic, and other members Bcl-2, Bcl-xL, and Bcl-W act as antiapoptotic class [20]. In the present study, we found that Bcl-2 and Bcl-xL expressions were observed in control mice, and they were absent in mice treated with $Ni(BTSC)_2$, whereas proapoptotic genes such as Bax, P53, and PARP-1 expressions showed opposite results. We found over expression of Bax, P53, and PARP-1 genes in $Ni(BTSC)_2$-treated mice in comparison with that of control mice. Increased expression of these genes could lead to cytochrome c release from mitochondria and binds with apoptotic protease activating factor-1 (Apaf-1) and ATP. Which then bind to procaspase-9 to create a protein complex known as an apoptosome. The apoptosome then activates caspase-3 and -9, leading to the cleavage of PARP-1 [21]. Altered expression of these proapoptotic and antiapoptotic genes in EAC cells upon $Ni(BTSC)_2$ treatment further confirmed the induction of programmed cell death of cancer cell.

It has been believed that in cancer progression and prevention ROS acts as a double-edged sword. Moderate levels of ROS maintain essential mechanisms of cancer cell survival, such as proliferation and angiogenesis [22], while high levels of ROS lead to destructive effects on cancer cells through pathways such as apoptosis and autophagy [23,24]. In this study, it is revealed that treatment with $Ni(BTSC)_2$ for 24 h increased ROS production in EAC cells in a dose-dependent manner. Since the stability of the mitochondria is closely related to ROS balance, it can be said that apoptosis occurred in this study mainly through the mitochondrial pathway.

4. Conclusion

In the light of above observations $Ni(BTSC)_2$ may therefore be considered primarily as potent anticancer agents and the possible mechanism for the anticancer effect is ROS dependent mitochondria-mediated intrinsic pathway rather than an extrinsic pathway and was regulated by the Bcl-2 protein family. In order to ascertain these compounds as novel potential anticancer drugs, it is necessary to carry out further experiments against other cancer cell lines with higher test animals and with advanced techniques. These findings definitely give positive support to carry out further researches in a way to formulate novel anticancer drugs.

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Conflict of Interest

The authors have no conflict of interest.

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