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The Isoquinoline Alkaloid Berberine Augments Radiation Effect by Enhancing the DNA Damage at Molecular Level in HeLa Cells Irradiated with Various Doses of γ -Radiation: Correlation Between DNA Damage and Clonogenicity

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Abstract

The passage of ionizing radiations through aqueous medium of biological material leads into the generation of a burst of free radicals owing to radiolysis of water. These free radicals are extremely reactive and interact with important macromolecules of cells resulting in the cytotoxicity. It is well known that ionizing radiation induce damage to the DNA triggering a cascade of events that result in eventual cell kill. Earlier we found that berberine chloride an isoquinoline alkaloid present in certain plants inflicts damage to the molecular DNA. Therefore we wanted to know whether berberine chloride will increase the effects of radiation in HeLa cells exposed to various doses of γ- radiation. HeLa cells were treated or not with 0, 1, 2, 4, 6 or 8 µg/ml of berberine chloride prior to 0, 0.5, 1, 2, 3 or 4 Gy γ-irrradiation and the molecular DNA damage was assessed immediately after irradiation (within 15 minute of irradiation) by single cell gel electrophoresis. The migration of fragmented DNA into comet tails was considered as a measure of molecular damage to DNA and has been expressed as Olive tail moment. Irradiation of HeLa cells to 0, 0.5, 1, 2, 3 or 4 Gy γ-irradiation caused a radiation dose-dependent rise in the Olive tail moment indicating an elevation in the DNA damage in HeLa cells. Treatment of HeLa cells with different concentrations of berberine chloride for 2 or 4 h before irradiation further raised the DNA damage denoted by a rise in the amount of tail DNA of the comets and Olive tail moment immediately after irradiation. The clonogenic assay revealed that clonogenic potential of HeLa cells alleviated with an increase in irradiation dose and treatment of HeLa cells with 1, 2 or 4 µg/ml berberine chloride further reduced the clonogenicity of cells. Our study indicates that berberine is a potent DNA damaging agent and could enhance radiation damage during cancer treatment in clinical conditions and clonogenicity of cells is directly related to the ability of berberine to inflict damage to DNA.

Introduction

A longstanding paradigm in radiation biology has been that cell killing and carcinogenic effects of ionizing radiation are the result of DNA damage arising from the actions of ionizing radiation in the cell nuclei, especially by interactions of ionizing radiation and its products with nuclear DNA [1-3]. Consistent with this view, ionizing radiations undoubtedly damage DNA by directly ionizing DNA itself and by indirect processes in which DNA reacts with numerous radiolytic reactive products including H•, OH•, $O_2•^-$ and H_2O_2, that are generated in aqueous fluid surrounding DNA [2,4,5]. Many attempts have been made to increase the effect of ionizing radiations by combining hypoxic cell sensitizers with radiation. Although successful in the experimental setup, clinical success has always been elusive, which has led to the use of alternative approach, where radiotherapy has been combined with chemotherapeutic drugs with a remarkable success to treat various malignant tumors [6,7]. Several chemptherapeutic agents including cis-dichlorodiammine-platinum (II), 5-fluorouracil, mitomycin C, paclitaxel, docetaxel, topotecan, irinotecan, crytophycins, camptothecin and combretastatin A-4, and gemcitabine have been successfully combined with radiotherapy to treat difficult neoplasia [8-15]. The use of chemotherapy in conjunction with radiotherapy proved beneficial in the treatment of solid neoplastic disorders in randomized clinical trials, however, it is fraught with consequences of high toxicity and development of second malignancies [16,17]. Therefore, newer approaches are required to alleviate the toxic side effects of combination regimens, and give optimum therapeutic benefits to the patients with good quality of life.

With recent advances in molecular Radiation Biology, attempts have been made to use target specific drugs including prenyl transferase inhibitors, ErbB receptor tyrokinase inhibitors and several others but with limited success [18]. Out of several molecular targets studied in an attempt to sensitize the cells to radiation, it is fairly well established that DNA damage plays a crucial role in determining the mechanism/s of action of anticancer drugs and ionizing radiations. It is therefore an imperative task in cancer therapy to determine the DNA-damaging action of antineoplastic and radiosensitizing agents on the normal as well as cancer cells [19]. The DNA damage at molecular level can be examined easily and precisely by single cell gel electrophoresis also known as comet assay, which was first performed by Östling and Johanson [20], followed by independent modification of the assay technique by Singh et al. [21] and Olive et al. [22]. In comet assay the cells are usually first embedded into agarose, lysed in alkaline buffer and finally subjected to an electric current. The electric current pulls the charged DNA out of the confines of nucleus, where the relaxed and broken DNA fragments migrate farther from the nucleus in comparison with the intact DNA that looks like a celestial comet and this analogy

gives this technique the name comet assay [23]. The resulting comet images are acquired and analyzed under a fluorescence microscope to estimate the extent of lesion/s induced in the DNA [23-25].

Berberine is an isoquinoline alkaloid synthesized by various plants [3]. Berberine has been reported to exert anti-bacterial, anti-cancer, anti-depressant, anti-diabetic, anti-diarrheal, anti-inflammatory, anti-angiogenic and anti-arrhythmic activities [21,26-33]. Berberine administration has been shown to improve the cardiac performance in patients with heart failure in clinical trials and it also acts as a hypolipidemic [34,35]. It has been found to protect diabetic rats against cardiac dysfunction [36]. Berberine has been reported to protect against memory impairment in rats and also inhibit inflammatory colitis in mice [37,38]. It has been reported to exert anti-cholesterolemic activity [39,40]. An earlier study has indicated a significant reduction in tumor yield and tumor incidence in 2-stage skin carcinogenesis model in mouse administered with berberine [41]. Berberine was found to exert the anti-cancer activity *in vivo*, where it increased tumor free survival in mice transplanted with Ehrlich ascites carcinoma [29]. Berberine has been also found to inhibit growth of prostate cancer cells *in vitro* by inducing apoptosis and activation of Bax and caspase 3 [42]. The DNA damage induction is one of the important mechanisms of cell death. Therefore, the present study was undertaken to obtain an insight into the induction of molecular DNA damage in HeLa cells treated with berberine chloride before exposure to different doses of γ- radiation using comet assay.

Materials and Methods

Drugs and chemicals

Berberine chloride (BCL), fetal calf serum, Minimum Essential Medium (MEM), L-glutamine, gentamicin sulfate, normal and low melting agarose (Cat No. A-9418), Ethylene Diamine Tertra-Acetic Acid (EDTA), trizama base, ethidium bromide and triton X-100 were supplied by Sigma Chemical Co. St. Louis, USA. The other routine chemicals were obtained from Ranbaxy fine Chemicals, Mumbai, India.

Dissolution of drug

Berberine chloride was dissolved in sterile double distilled water (DDW) before use (at a concentration of 5 mg/ml), filter sterilized and diluted in sterile MEM in such a way so as to get the desired concentration.

Cell line and cell culture

The entire study was carried out in HeLa S3 cells with a doubling time of 20 ± 2 h. The cells were supplied by the National Centre for Cell Science, Pune, India. The cells were usually cultured in 25 cm^2 culture flasks (Techno Plastic Products, Trasadingën, Switzerland) containing 5 ml Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum, 1% L-glutamine and 50 μg/ml gentamicin sulfate with their caps loosened. The flasks were incubated at 37°C in a CO_2 incubator (NuAir, Plymouth, USA). in an atmosphere of 5% CO_2 and 95% humidified air.

Experimental protocol

Generally 5 × 10^5 exponentially growing HeLa cells were seeded into several culture flasks (Techno Plastic Products, Trasadingën, Switzerland) and allowed to grow until plateau phase. The cells were harvested by trypsin EDTA treatment, embedded into agarose (details are given in comet assay section) and were divided into the following groups according to the treatment:

MEM + sham-irradiation: The cells of this group were sham-irradiated that is exposed to 0 Gy of γ-radiation.

BCL + sham-irradiation: This group of cells was treated with 1, 2, 4, 6 or 8 μg/ml of BCL for 2 or 4 h before sham-irradiation (0 Gy).

MEM + irradiation: The cells set in agarose were exposed to 0.5, 1, 2, 3 or 4 Gy γ-radiation.

BCL + irradiation: This group of cells was embedded in agarose and treated with 1, 2, 4, 6 or 8 μg/ml of BCL for 2 or 4 h before exposure to different doses of γ- radiation.

Irradiation

The slides embedded with cells in agarose were irradiated on ice using a Telecobalt therapy source (Theratron Atomic Energy Agency, Ontario, Canada). The γ-radiation was delivered at a dose rate of 1 Gy/min. at a distance (SSD) of 91 cm. Ten cell embedded slides for each irradiation dose from both groups were placed on a flat glass plate in close thermal contact with ice and exposed to 0, 0.5, 1, 2, 3 or 4 Gy γ-radiation.

Assessment of DNA damage

The DNA damage was assessed by alkaline comet assay within 15 minutes of exposure to 0, 0.5, 1, 2, 3 or 4 Gy γ-radiation.

Alkaline comet assay

The DNA damage at molecular level was determined using alkaline comet assay [21,43-45]. The frosted slides at one end were layered with 100 μl of 0.6% low melting agarose dissolved in Ca^{+2} and Mg^{+2} free PBS at 37°C and a coverslip was placed over the molten agarose. The coverslips were removed after the congealing of agarose. Usually, 1 × 10^5 HeLa cells harvested in one ml of MEM were pelleted by centrifugation at 1,500 rpm for 5 min. The cell pellets were resuspended in 80 μl of 0.6% low melting agarose spread on to the first layer, covered with a coverslip and allowed to solidify on ice. The whole procedure was carried out under a diffused light so as to avoid additional DNA damage.

The cells set in agarose were treated or not with different concentrations of BCL as described above and irradiated on ice using a Telecobalt therapy source as described in irradiation section. Immediately after irradiation was over (within 15 min), these slides were transferred into cold lysis buffer containing 2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM Trizma base, pH 10 and 1% Triton X-100 (added fresh) and left undisturbed for 2 h in cold. This resulted in the complete removal of cellular proteins and left DNA as nucleoids. Once the cell lysis was over, the lysis buffer was decanted from the slides. These slides were transferred into a horizontal gel electrophoresis tank filled with a fresh electrophoresis buffer consisting of 300 mM NaOH, 1 mM Na_2 EDTA, at pH 13.0, up to a level of ~0.25 cm above the slides and left undisturbed for next 20 min to unwind the DNA. The horizontal electrophoresis tank was connected to the power supply and slides were electrophoresed for 20 min at 1.25 V cm^{-1} and 300 mA in cold. The slides were pulled out from the electrophoresis tank and the buffer was allowed to drain off. The slides were then flooded gently with three changes of neutralization buffer (0.4 M Trizma base, pH 7.5) for 5 min each and stained with 50 μl of ethidium bromide (2 mg/ml) and covered with a coverslip for immediate analysis.

The data of molecular DNA damage were collected from each ethidium bomide stained slide at 40 X magnification under a fluorescence microscope as "comets" with a fluorescent head and a

Berberine chloride	Olive Tail Moment (Mean ± SEM)						Linear correlation
(µg/ml)	Exposure dose (Gy)						(r²)
	0	0.5	1	2	3	4	
0	0.76 ± 0.06	1.13 ± 0.08	2.84 ± 0.02	4.58 ± 0.04	5.84 ± 0.06	8.22 ± 0.07	0.97
1	2.14 ± 0.05	4.13 ± 0.05	6.51 ± 0.03	8.12 ± 0.06	11.13 ± 0.03	15.13 ± 0.03	0.98
2	5.14 ± 0.04	7.34 ± 0.01	9.54 ± 0.06	11.87 ± 0.04	12.59 ± 0.02	16.72 ± 0.05	0.96
4	8.11 ± 0.08	10.15 ± 0.02	12.08 ± 0.05	14.07 ± 0.03	18.15 ± 0.02	22.05 ± 0.06	0.99
6	11.11 ± 0.04	17.18 ± 0.02	19.26 ± 0.05	23.20 ± 0.04	25.75 ± 0.07	30.32 ± 0.06	0.89
8	23.23 ± 0.07	26.34 ± 0.04	29.57 ± 0.03	34.17 ± 0.05	42.82 ± 0.08	54.21 ± 0.03	0.98
p<0.001 BCL + irradiation group compared to MEM + irradiation group							

Table 1: Alteration in the radiation-induced DNA damage in HeLa cells treated with berberine chloride for 2 hours before exposure to various doses of γ-

Berberine chloride	Olive Tail Moment (Mean ± SEM)						Linear correlation
(µg/ml)	Exposure dose (Gy)						(r²)
	0	0.5	1	2	3	4	
0	0.84 ± 0.04	1.32 ± 0.05	3.62 ± 0.02	6.55 ± 0.04	9.88 ± 0.09	12.27 ± 0.06	0.99
1	4.11 ± 0.02	5.05 ± 0.05	6.34 ± 0.04	8.14 ± 0.07	12.87 ± 0.06	15.23 ± 0.04	0.99
2	8.21 ± 0.06	10.86 ± 0.06	12.34 ± 0.04	13.42 ± 0.03	15.93 ± 0.07	19.09 ± 0.01	0.98
4	10.55 ± 0.04	13.43 ± 0.05	16.26 ± 0.06	18.16 ± 0.04	21.17 ± 0.08	25.35 ± 0.04	0.98
6	15.34 ± 0.03	19.14 ± 0.05	22.64 ± 0.04	26.18 ± 0.02	30.23 ± 0.06	35.10 ± 0.03	0.98
8	35.12 ± 0.06	38.23 ± 0.04	42.11 ± 0.03	45.24 ± 0.04	50.52 ± 0.06	53.12 ± 0.04	0.97
p<0.001 BCL + irradiation group compared to MEM + irradiation group							

Table 2: Alteration in the radiation-induced DNA damage in HeLa cells treated with berberine chloride for 4 hours before exposure to various doses of **γ**-radiation.

tail and the images [44,45] were acquired using an epifluorescence microscope (Olympus BX51, Olympus Microscopes, Tokyo, Japan) equipped with a 515-535 nm excitation filter, a 590 nm barrier filter and a CCD camera (CoolSNAP-Pro$_{cf}$ Digital Color Camera Kit Ver 4.1, Media Cybergenetics, Silver Spring, Maryland, USA). Generally one hundred cells from each slide were scored so as to give a representative result for the population of cells [46]. The comet images thus acquired by Azzam et al. [47] were analysed using Komet software (Version 5.5, Kinetic Imaging Ltd, Bromborough, UK). The mean olive tail moment (OTM) was selected as the parameter that best reflects DNA damage (defined as the distance between the profile centres of gravity for DNA in the head and tail). OTM was measured from three independent experiments, each containing quintuplicate measures and presented as Mean ± SEM.

The potentiating factor (PF) was calculated by the following formula:

$$PF = \frac{BCL + IR - BCL}{IR - SIR}$$

Where IR = Irradiation; BCL = Berberine Chloride; SIR = Sham-Irradiation.

Clonogenic assay

Another experiment was undertaken to estimate the relationship of molecular DNA damage with the clonogenicity of cells, where the grouping and other conditions were exactly similar to that described above except that stationary phase HeLa cells were inoculated into several culture flasks and the cells were treated with 1, 2 or 4 µg/ml of BCL for four h before exposure to 0, 0.5, 1, 2, 3 and 4 Gy of γ-radiation. Immediately after irradiation, the medium from each flask of both groups was decanted and all the flasks were washed twice with sterile PBS before trypsin-EDTA treatment. The clonogenic assay was performed as described earlier [48]. Briefly, 300 HeLa cells were inoculated in quintuplicate in 25 cm² petriplates (Techno Plastic Products, Trasadingën, Switzerland) for each exposure dose or each dose of BCL. The petriplates containing cells were transferred into a

CO_2 incubator and left undisturbed for 11 days for colony formation. The colonies thus formed were stained with 1% crystal violet in methanol and the cluster containing 50 or more cells were scored as a colony. The data thus obtained were fitted on to a linear quadratic model = $exp^{-\alpha D + \beta D^2}$

Statistical analyses

The statistical analyses were performed using Origin Pro 2015 statistical software (Origin Lab. Corporation Northampton, MA, USA). The significance between the treatments was determined by one-way ANOVA and Bonferroni's post-hoc test was applied for multiple comparisons. The results were confirmed by running repeat experiments at least twice. The results are the average of three individual experiments. The test of homogeneity was applied to find out variation among each experiment if any. Since the data of each experiment did not differ significantly from one another, all the data have been combined and means calculated. A p value of <0.05 was considered statistically significant.

Results

The results of DNA damage as mean olive tail moment (OTM) and cell survival are expressed in (Tables 1-3) and (Figures 1-5).
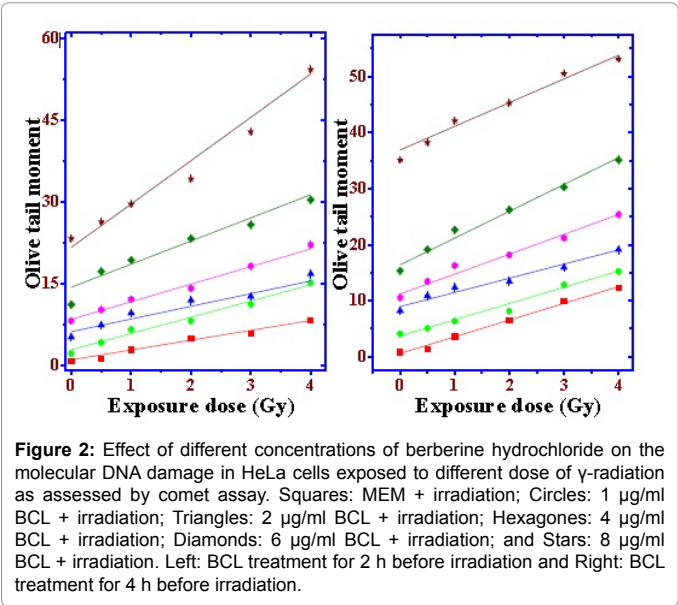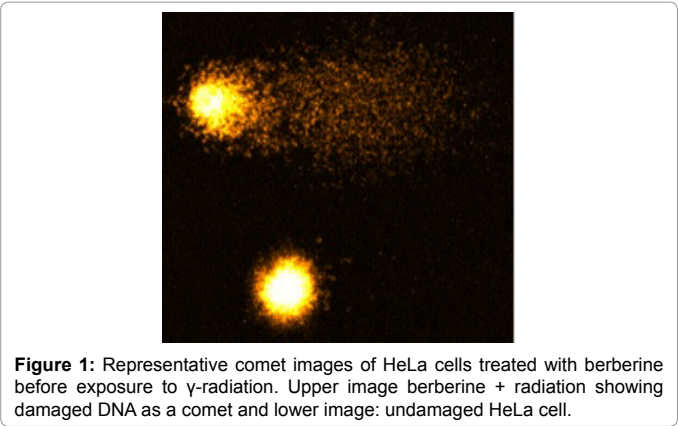
Assessment of DNA damage by Comet assay

The DNA damage was evaluated by comet assay as a measure of DNA tail fragmentattion (OTM) immediately after irradiation in HeLa cells treated with 1, 2, 3, 6 or 8 µg/ml BCL for 2 h or 4 h before exposure to different doses of γ-radiation (Figure 1). HeLa cells treated with different concentrations of BCL either for 2 h or 4 h caused an increase in the tail DNA (OTM) in a BCL concentration dependent manner and the greatest DNA damage was observed in the cells that received 8 µg/ml BCL treatment (Figure 2). The DNA damage was greater in the cell treated with BCL for 4 h than that of to 2 h (Table 2). The γ-irradiation of HeLa cells to 0, 0.5, 1, 2, 3 or 4 Gy resulted in a significant rise in the migration of framented DNA into comet tails leading to a subsequent increase in OTM (p < 0.001) in the MEM + irradiation group when compared with non-drug treated sham-

Exposure dose (Gy)	Berberine chloride treatment (h)									
	2					4				
	Berberine chloride (µg/ml)					Berberine chloride (µg/ml)				
	1	2	4	6	8	1	2	4	6	8
0.5	5.38	5.95	5.51	16.4	8.4	1.96	5.52	6	7.92	6.48
1	2.1	2.11	1.91	3.92	3.05	0.8	1.49	2.05	2.63	2.51
2	1.56	1.76	1.56	3.16	2.86	0.7	0.91	1.33	1.9	1.77
3	1.77	1.47	1.98	2.88	3.86	1.09	0.85	1.17	1.65	1.7
4	1.74	1.55	1.87	2.57	4.15	0.97	0.95	1.29	1.73	1.57

Table 3: Potentiating factor for various concentrations of berberine chloride in HeLa cells exposed to different doses of γ- radiation.

Figure 1: Representative comet images of HeLa cells treated with berberine before exposure to γ-radiation. Upper image berberine + radiation showing damaged DNA as a comet and lower image: undamaged HeLa cell.

Figure 2: Effect of different concentrations of berberine hydrochloride on the molecular DNA damage in HeLa cells exposed to different dose of γ-radiation as assessed by comet assay. Squares: MEM + irradiation; Circles: 1 µg/ml BCL + irradiation; Triangles: 2 µg/ml BCL + irradiation; Hexagones: 4 µg/ml BCL + irradiation; Diamonds: 6 µg/ml BCL + irradiation; and Stars: 8 µg/ml BCL + irradiation. Left: BCL treatment for 2 h before irradiation and Right: BCL treatment for 4 h before irradiation.
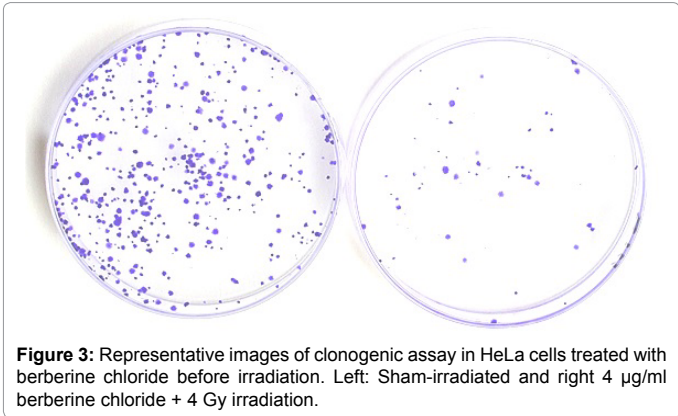
irradiation control (Figure 2). The expsoure of HeLa cells to various doses of γ-radiation caused an irradiation dose dependent accrual in the DNA damage as indicated by increasing migration of DNA into comet tails (Tables 1 and 2) and the highest DNA damage was scored in cells irradiated to 4 Gy of γ-radiation in MEM + irradiation group (Figure 2). The treatment of HeLa cell with 1, 2, 4, 6, or 8 µg/ml BCL for 2 h led to a significant increment in the DNA damage (OTM) and the quantitty of DNA damage elevated with rising concentration of BCL as well as increasing dose of irradiation (Table 1) in BCL + irradiation group (Figure 2). The DNA damage was greater in the cells treated with different concentrations of BCL for 4 h (Table 2) than 2 h
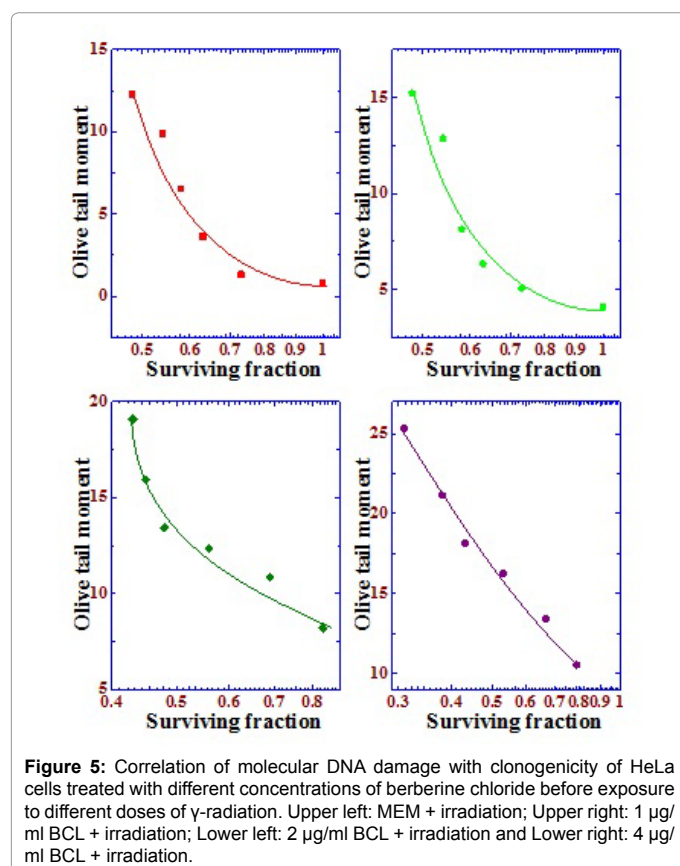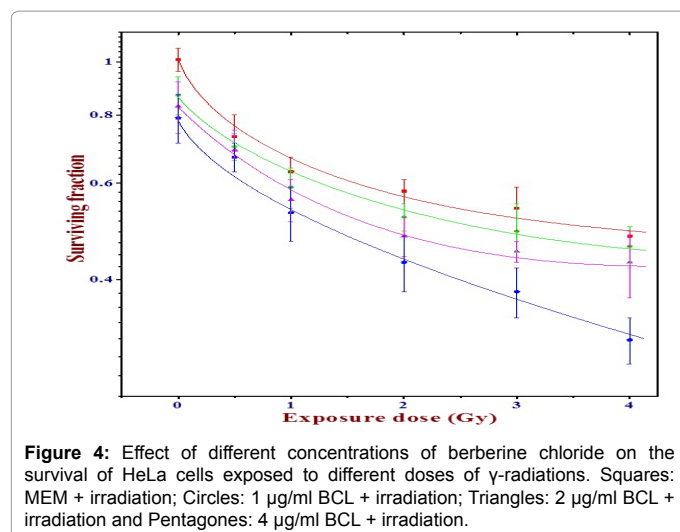
in BCL + irradiation group (Table 1). The greatest DNA damage was discerned in the cells treated with 8 µg/ml BCL and then exposed to 4 Gy γ-radiations in comparison to other concentration of BCL and 0.5-3 Gy of radiation (Figure 2). The dose response relationship was linear for both cells treated for 2 or 4 h BCL and then exposed to different doses of γ-radiation (Figure 2).

The determination of potentiating factor of BCL revealed that potentiating factor was consistently higher for 6 and 8 µg/ BCL at all exposure doses in HeLa cell that were treated to BCL for 2 h before exposure to different doses of γ-radiation (Table 3). Similarly, a maximum potentiating effect was obtained for all BCL concentrations in the cells exposed to 0.5 and 1 Gy when compared to higher irradiation doses (Table 3), despite the fact that absolute values of OTM were higher in the cells treated with different doses of BCL for 4 h before exposure to different doses of γ-radiation (Tables 1 and 2).

Clonogenic assay

Figure 3 shows representative images of clonogenic assay in HeLa cells. Treatment of HeLa cells with 1, 2 or 4 µg/ml BCL for 4 h reduced the cell survival and a greatest reduction in the cell survival was detected for 4 µg/ml BCL (Figure 4). Irradiation of HeLa cells to various doses of γ-radiation led to an increasing reduction in the cell survival (Figure 4). This attrition in cell survival was greater for 0.5 and 1 Gy when compared to the 2, 3 and 4 Gy γ-radiation (Figure 4). A further decline in the survival of HeLa cells was detected after treatment with 1, 2 or 4 µg/ml BCL for 4 h before exposure to different doses of γ-radiation in BCL + irradiation group when compared to MEM + irradiation group (Figure 4). The maximum decline in the cell survival was registered in the cells treated with 4 µg/ml BCL in BCL + irradiation group (Figure 4). The data fitted on the linear quadratic model.

Figure 3: Representative images of clonogenic assay in HeLa cells treated with berberine chloride before irradiation. Left: Sham-irradiated and right 4 µg/ml berberine chloride + 4 Gy irradiation.

Figure 4: Effect of different concentrations of berberine chloride on the survival of HeLa cells exposed to different doses of γ-radiations. Squares: MEM + irradiation; Circles: 1 μg/ml BCL + irradiation; Triangles: 2 μg/ml BCL + irradiation and Pentagones: 4 μg/ml BCL + irradiation.

Figure 5: Correlation of molecular DNA damage with clonogenicity of HeLa cells treated with different concentrations of berberine chloride before exposure to different doses of γ-radiation. Upper left: MEM + irradiation; Upper right: 1 μg/ml BCL + irradiation; Lower left: 2 μg/ml BCL + irradiation and Lower right: 4 μg/ml BCL + irradiation.

Biological response

The correlation between the molecular DNA damage and cell survival was determined by plotting molecular DNA damage on Y axis and cell survival on X-axis (Figure 5). The cell survival declined with increasing molecular DNA damage (Figure 5) indicating that the cell survival is directly related to the molecular damage to DNA. The greater was the damage to DNA higher was the cell kill (Figure 5). The relationship between DNA damage and cell survival was linear quadratic (Figure 5).

Discussion

Natural products have drawn the attention of human beings for healthcare since time immemorial and they have been traditionally used as remedies due to the popular belief that they are non-toxic or produce fewer adverse side effects [49]. Therefore, it is essential to determine the beneficial or adverse influence of natural products, which are extensively used by humans, especially to implement public health safety measures. Development of resistance after administration of different chemotherapeutic drugs is generally a major stumbling block in successful chemotherapy of cancer and usually it is extremely difficult to prognosticate the degree and timing of the appearance of tumor resistance in most chemotherapy regimens [50,51]. Modern developments in the single-cell gel electrophoresis or 'comet' assay to estimate DNA damage at the single-cell level indicate that this technique might provide a useful method in identifying and potentially monitoring the response of tumor cells to numerous chemotherapeutic agents including radiotherapy *in situ* [52]. Technically comet assay can be applied to any tumor undergoing chemotherapy that cause overt DNA damage and is accessible for sample collection. The comet assay or single-cell gel electrophoresis assay is a simple technique that may use for brisk estimation and quantitation of DNA damage from single cells [23,25,43,45,53]. The comet assay is based on lysis of labile DNA at sites of damage in the alkaline condition. Cells are immobilized in a thin agarose matrix on slides and gently lysed and subjected to electrophoresis, where the unwound and relaxed DNA comes out of the cells. After staining with a nucleic acid stain, the cells that have accumulated DNA damage appear as fluorescent comets, with tails of DNA fragmentation or unwinding [23,25,45].

In radiobiology, there is always a need for the development of new rapid and more sensitive techniques for DNA damage evaluation [25,54]. The comet assay, also called single-cell gel electrophoresis (SCGE) assay is a method of choice because it is a rapid and sensitive method for the detection of various DNA damages (strand breaks and alkali-labile sites) in individual cells, induced by a variety of genotoxic agents including ionizing radiations [23,25]. Radiation may cause SSB, DSB, DNA-DNA as well as DNA-protein crosslinks and damage to bases, which can be detected by comet assay and hence this method could provide information on any type of DNA damage caused by ionizing radiations. This is not the case for standard cytogenetic methods that provide only average DNA damage information [24].

Radiation therapists would like to have a tool that allows the quantitative assessment of radiosensitivity of normal tissues. Comet assay is one such method that allows estimation of DNA damage in short duration and may allow precise treatment planning of cancer patients undergoing radiotherapy or chemotherapy or both. DNA damage and consequent cell killing are the main effects on cells after exposure to ionizing radiation. The first effect can be measured by comet assay and the second by evaluating the reproductive integrity using clonogenic assay [25,44,48]. Meanwhile, research has identified quite a number of factors that affect individual's radiation sensitivity including the cells' ability to repair DNA damage. The results from the present study show that with increasing BCL concentration and irradiation dose the possibility of DNA repair constantly dwindled as evidenced by the reduced clonogenicity of HeLa cells. A similar observation has been made earlier [43,45,55]. Berberine treatment of HeLa cells has been reported to up regulate Fas, FasL, TNF-α and TRAF-1 which are invoved in the activation of death receptor and apoptosis causing reduced survival [56].

Treatment of HeLa cells with 1-8 μg/ml BCL before exposure to

different doses of γ-radiation increased the OTM in BCL concentration and radiation dose dependent manner and this rise was significantly greater in BCL pretreated group when compared with the concurrent non-BCL treated irradiated control. The accelerated DNA migration in HeLa cells after BCL treatment prior to γ-irradiation may be a likely a sum of genotoxic or cytotoxic effect of BCL. In our earlier study berberine treatment increased molecular damage to DNA in HeLa cells in a concentration dependent manner [45]. The radiosensitizing effect of BCL on HeLa cells may be due its ability to inflict the molecular damage to cellular genomic indicated by a steady increase in the OTM after BCL treatment before irradiation. Berberine was found to induce DNA fragmentation in human leukemic HL-60 cells earlier [26]. The DNA damage elevated with increase in BCL treatment time in the irradiated group. However, the potentiating factor was higher in the cell treated with BCL for two hours before exposure to different doses of γ-radiation despite the fact that absolute DNA damage was greater in the cells treated with BCL for 4 h (Table 3). This may be due to the fact that increasing incubation of HeLa cells with BCL, the BCL per se induced higher DNA damage reducing the potentiating factor, which is clear from the data presented in the (Tables 1 and 2). It is conspicuously clear that low doses of radiation had greater sensitization and cell killing ability than the higher doses of radiation. This observation is clinically significant as the fraction size of radiation for cancer treatment may be reduced, indicating that its clinical application may reduce the adverse effect of radiotherapy and at the same time bringing out effective tumor control if berberine is administered before irradiation. This was evident also in the clonogenic assay where low doses provided more cell killing effect in BCL + irradiation group.

Earlier reports suggest that increased DNA damage is the hallmark of cell death and the increasing DNA damage caused a corresponding reduction in cell survival [43-45,55,57-59]. This is also clear from the biological response data that clearly indicate that increasing OTM actually reduced the clonognenicity of HeLa cells (Figure 3). A similar effect has been reported earlier [43-45,55,57,58]. Our results indicate that BCL pretreatment did not allow repair and the amount of DNA damage actually increased with increasing concentration of BCL and radiation dose, which is translated into cell killing. Likewise, 1-methylxanthine has also been reported to disallow repair of DNA strand breaks in RKO human colorectal cancer cells [60].

The exact mechanism by which BCL enhanced the radiation-induced DNA damage is not well understood. The increased DNA damage by BCL may not be due to a single mechanism but several putative mechanisms may have acted independently or in concert with each other to bring this effect. Ionizing radiation interacts with cellular genome by induction of OH free radicals [4,5,61] and presence of BCL would have further enhanced the induction of radiation-induced reactive oxygen species (ROS) resulting in the augmented radiation-induced DNA damage. BCL actually induces reactive oxygen species [62,63] that supports this contention. Ionizing radiations have been reported to cause single and double strand breaks in DNA sugar and base damage as well as protein crosslinks [3,64]. The double strand breaks are considered cell lethal and the main causative factor of cell death [3,65,66]. The presence of BCL before irradiation might have minimized the chances of DNA repair thus bringing effective killing of cells. The berberine has been reported to inhibit DNA repair mechanisms earlier [31]. Lipid peroxidation and lipid peroxides produce excessive damage to the cell DNA [66] and BCL would have increased the radiation-induced lipid peroxidation in HeLa cells thereby increasing DNA damage further. We have observed that BCL actually increases radiation-induced lipid peroxidation in cultured HeLa cells

(data not shown). The topoisomerases are involved the transcription, recombination, DNA repair and chromatin remodeling [67]. The presence of BCL before irradiation may have suppressed the action of topoisomerase II, which may have caused an elevation in the radiation-induced DNA damage in BCL pretreated group in comparison with non-drug treated irradiation group. The increased OTM can originate from stabilization of topoisomerase II, which can introduce DNA strand breaks when it interacts for a prolonged time with the action of free radicals [67,68] which may have been generated by irradiation. An earlier study has reported that berberine treatment caused internucleosomal DNA fragmentation resulting in the formation of a complex with DNA that inhibited topoisomerase II enzyme *in vitro* [69]. A relationship between topoisomerase II and radiosensitivity has also been reported [70]. Apart from these mechanisms, operation of other molecular mechanisms including inhibition of nuclear factor κB, cyclooxygenase II, activator protein 1, cyclins, p53 and PARP may have played a crucial role in increasing the radiation-induced DNA damage [69,71-75], which may have contributed in their own way to enhance the radiation-induced DNA damage in the present study. The increased DNA damage by berberine may have also resulted in the apoptosis of cells leading to decreased cell survival. Berberine has been reported to activate caspase 8 and 9 which are essential in induction of apoptosis [75].

Conclusion

Berberine might have enhanced radiation-induced DNA damage either by increasing the radiation-induced ROS, lipid peroxidation, lactate dehydrogenase release or by suppressing topoisomerase II. It may also have blocked the transactivation of nuclear factor κB, suppressed the expression of cyclooxygenase II, activator protein 1, cyclins, p53 and PARP and other DNA repair enzymes leading to increased DNA damage and reduction in the survival of HeLa cells.

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