Effect of Bleomycin on Oral Leukoplakia Cell line- an in Vitro Study

Al Moutassem B. Khair
Ajman University
Alexander Luke (a.luke@ajman.ac.ae)
Ajman University
Shishir R. Shetty
Ajman University
Simy Mathew
Ajman University
Rajashree Patnaik
Ajman University

Research article

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Abstract

Background Leukoplakia generally refers to a firmly attached white patch on a mucous membrane which is associated with an increased risk of cancer. So it is important to diagnose leukoplakia in early stage. In this study we are trying to treat leukoplakia as a marker for prediagnosis of cancer. Since, bleomycin as a chemotherapeutic agent, is mainly used in the treatment of multiple tumors, and several cancers. Hence in this study effect of bleomycin was studied in D38, a leukoplakia cell line.

Methods Percent lethality values of leukoplakia cells after treated with several concentations of bleomycin was studied by acridine orange/ethidium bromide staining (AO/EB), propidium iodide (PI) staining, 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay and comet assay. Minimum inhibitory concentration (MIC), lethal concentration$_{25}$ (LC_{25}), lethal concentration$_{50}$ (LC_{50}), lethal concentration$_{75}$ (LC_{75}) and highest permissive concentration (HPC) was calculated from probit computational method.

Results From AO/EB staining, PI staining, MTT assay and comet assay the MIC value was 15 mg/L concentration of bleomycin, where as the HPC was found at 100 mg/L of bleomycin. The comet tail length gradually increased from 15 mg/L to 100 mg/L of bleomycin. Percent lethality values were also increased from 15 to 100 mg/L concentration of bleomycin in all the staining protocols as well as in comet assay technique.

Conclusion It is important to diagnose leukoplakia in early stage. Since, bleomycin as a chemotherapeutic agent, it is mainly used in the treatment of multiple tumors, testicular carcinomas, lymphomas, and head, neck cancers. From the results, it was found that bleomycin can be used for treatment of oral leukoplakia to prevent its development to SCC.

Background

Over the years potentially malignant disorders like oral leukoplakia (OL) and oral erythroplakia (OE) are associated with dysplastic cellular changes and hence carry a risk of undergoing malignant transformation leading to oral cancer (OC) [1]. Numerous surgical and nonsurgical modalities have been used for treatment of OL [2]. Some of the non-surgical modalities used for the treatment of OL include photodynamic therapy, beta-carotene, lycopene, or vitamin A [2, 3]. Bleomycin a chemotherapeutic agent has also been used for treatment of leukoplakia and other malignant lesions [4, 5, 6, 7, 8]. Apart from topical application report intralesional injection of bleomycin into OL lesion has been performed with good results [9]. Another study suggested the delivery of bleomycin into head and neck tumors using iontophoresis [10]. Bleomycin is isolated from the bacteria *Streptomyces verticillis* [11, 12, 13], which is a glycopeptide antibiotic. As a chemotherapeutic agent, it is mainly used in the treatment of multiple tumors, testicular carcinomas, lymphomas, and head, neck cancers [14, 15]. The biological action of bleomycin is through a sequence-selective, metal-dependent oxidative cleavage of DNA and RNA in the presence of oxygen. It can mediate the oxidative degradation of all major classes of cellular RNAs and

inhibition of DNA synthesis [13, 16]. Still its mechanism of action has not been elucidated. It has been reported that Bleomycin is responsible for lipid peroxidation and mitochondrial DNA damage [17]. Bleomycin induces G2/M cell cycle arrest, which is important for genomic stability, in cancer cell lines. It causes senescence, apoptosis and mitotic cell death [18, 19, 20].

Studies have suggested intralesional injection of bleomycin to be highly effective in treatment of warts [21]. Off-label use of intralesional bleomycin is another primary and/or adjunctive therapy for different cutaneous lesions dermatology as several types of cutaneous malignancies, telangiectasias, vascular malformations, hemangiomas, and lesions of leishmaniasis cutisand condyloma acuminate [22]. Studies have also suggested intralesional bleomycin to be more effective in treatment of warts when compared to surgical modalities like cryotherapy [23]. Recent research has revealed that bleomycin is a reliable and safe treatment modality for warts resistant to other therapeutics [24].

Bleomycin along with Adriamycin, vinblastine and dacarbazine is the standard chemotherapy regimen for Hodgkin's, and non-Hodgkin's lymphoma disease squamous cell cancers, sarcoma, melanoma, and testicular cancer. Also it is used to treat malignant pleural effusion and Leukemias [25]. Bleomycin is found to concentrate more in lymphoid tissue and does not cause excessive myelo-suppression, thus is the preferred agent in chemotherapeutic regimens for non hodgkins lymphoma also [26]. Bleomycin is one of the important drugs in induction chemotherapy for testicular cancer [27]. Bleomycin also is a key component in the chemotherapy regimens for cervical and ovarian cancer [28, 29].

In this study, effect of bleomycin was studied by using D38 cell line which is a leukoplakia cell line. Percent lethal values after the leukoplakia cells treated with several concentration of bleomycin, were calculated from probit table. LC_{25}, LC_{50}, and LC_{75} values were calculated by taking the $\log_{10}$ concentrations of obtained percent lethality. MIC and HPC values were calculated directly from the experiment.

Methods

Cell Culture

Human leukoplakia cell line D38, were used for this study. The cell line was purchased from NCCS, Pune, India. After getting the cell line, cells were washed thoroughly with PBS and the cell number was counted. The cells were cultured in Dulbecco's modified eagle media (DMEM) containing 10% FBS (Fetal Bovine Serum) and 1% penicillin-streptomycin solution in six well culture plate. The cells were kept in an incubator at 5% CO_2 and at 37°C temperature. The cells were maintained by changing the media in two days interval. After getting confluent cells were transferred to 96-well culture plates at concentration of $1X10^4$ cells/well for study of effect of bleomycin. The cells were treated with several concentrations of bleomycin (0, 15, 25, 50, 75, 90 and 100 mg/L) and were incubated for 24 hours. After the incubation period, viability of cells were studied by acridine orange/ethidium bromide staining (AO/EB), propidium

iodide (PI) staining, 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay and comet assay.

AO/EB staining

The AO/EB solution was prepared in PBS at the concentration of 100 µg/mL and is applied to *in vitro* cultured cells. When observed under the fluorescent microscope at 400X, green colour indicated live cells, whereas cells with orange and red colour were recorded as apoptotic and necrotic cells, respectively. AO is taken up by both live and dead cells and emits green fluorescence whereas EB is only taken up by dead cells, as the integrity cytoplasmic membrane is lost and it stains nucleus orange. Hence live cells, apoptotic cells and necrotic cells were green, orange and red in appearance, respectively.

PI staining

The working solution for PI staining was prepared after mixing PI stock solution (1 mg/mL) with PBS in the ratio 1:9. For monitoring the viability, an aliquot of 20 µL of cell suspension grown in the presence of bleomycin was mixed properly with an aliquot of 20 µL of the working PI solution. The viable cells would appear green and the nucleus of non-viable cells would appear red in colour, under the fluorescent microscope. Toxicity values were obtained after a 24 h of incubation. Probits of observed lethality percentage values were used for analysis of toxicity. The dye, PI binds to DNA by entering dead cells only, which appear red in colour whereas the live cells appear green in fluorescent light.

MTT assay

The MTT solution was prepared at the concentration of 5 mg/mL in PBS. After 24 h of bleomycin treatment in a 6-well culture plate, 80 µL of MTT solution was added to each well to study the toxicity effect. The plate was kept in an incubator (37º C, 5% CO_2) for 4 h. Then, it was found that the media containing the cells and chemicals converted to blue colour after incubated with MTT. Then gently the mass was centrifuged at 1000 rpm for 10 min at 22º C. The supernatant was removed and the pellet was dissolved in an aliquot of 1 mL 100% dimethyl sulfoxide (DMSO) and kept in the incubator (37º C, 5% CO_2) for 1 h. The mixture turned purple and its optical density (OD) was measured with a spectrophotometer at the 570 nm wavelength.

Percentage of cell density = 100 X (OD_{sample} - OD_{blank})/ $OD_{control}$; MTT in DMSO solution was taken as the blank. Probits of observed lethality percentage values were used for analysis of toxicity.

Comet assay

Single cell gel electrophoresis was carried out to study DNA damage of the treated cell lines. Cultured cells were harvested and used in the alkaline comet assay technique. After coating slides with 1% agarose, the slides were allowed for air dried. Treated cells with different concentrations of bleomycin were centrifuged and pellets were washed with PBS; and the washed cells were mixed with three times the cell volume with the low melting point agarose (LMPA) 1% in sol state. The mixture of cells and LMPA sol was placed over the agarose coated slide that was dried at 4º C for 10 min. The slides were further treated with 1% Triton X 100, 10% DMSO, individually, and were placed in the lysing solution of the mixture of 100 mM Na_2EDTA, 10 mM Tris, 2.5 mM NaCl (pH, 10), at 4º C for 1 h. The slides were subsequently removed and placed in the electrophoretic buffer consisting of 1 mM Na_2EDTA and 300 mM NaOH (pH, 13) for 30 min. The slides were subjected to electrophoresis was carried out at 1.0 V/cm for 30 min, and the slides were placed in the neutralizing solution (0.4 M Tris HCl, pH, 7.5) for 5 min (Tice et al. 2000). The slides were stained with an aliquot of 40 µL of 10 µg/mL EB solution. Comets were scored with a fluorescence microscope at 400X and the DNA fragmentation index (DFI) as percent values were presented. Hydrogen peroxide 100 µM solution was used as the positive control. Values of comet tail length were measured with the help of an ocular micrometer.

Results

AO/EB staining

The number of dead cells gradually increased from the level of 15 to 100 mg/L level, assessed from colour variation of cells. Experimentally, the lethality was found seen at 15 mg /L, which was recorded as the MIC. From the plot, it was ascertained that for values of $LC_{25,}$ LC_{50}, and LC_{75} with probit values, 4.3255, 5.0000 and 5.6745, respectively, corresponding log_{10} concentration values were 1.53, 1.68 and 1.82, respectively. Antilog values of these log_{10} concentration values are 33.88 (LC_{25}), 47.86 (LC_{50}) and 66.07 (LC_{75}), which in mg/L are the computed LC values of bleomycin; the individual MIC and HPC values were noted directly from experiments.

PI staining

From PI staining results, the minimum live cell density was determined as 65.8% at 15 mg/L bleomycin, i.e., it was the MIC value. The HPC was 100 mg/L bleomycin. The probit plot yielded log_{10} concentrations as, 1.59, 1.72 and 1.85, which on extrapolation, yielded computed lethal values, 38.99 (LC_{25}), 52.06 (LC_{50}) and 70.96 mg/L (LC_{75}), respectively, by the probit computation.

MTT assay

From the cell density as OD_{570} and cell viability the level of 100 mg/L was recorded as the HPC. Experimentally, the MIC value was 15 mg/L bleomycin. Probits of percentage lethality (PL) and

corresponding log_{10} values of bleomycin concentrations were used for the plot that yielded log_{10} levels for $LC_{25,}$ LC_{50}, and LC_{75} log_{10}, as 1.42, 1.70 and 1.91, respectively; these log_{10} concentration values generated LC values: 26.36 (LC_{25}), 50.23 (LC_{50}) and 81.47 mg/L (LC_{75}).

Comet assay

The length of comet tail increased from 15 mg/L to 100 mg/L bleomycin. From probit analysis, it was found that the LC_{25}, LC_{50} and LC_{75} values were 26.36, 42.76 and 61.80 mg/L, respectively. Microphotographs of observed comets at 0 and 42.76 mg/L (LC_{50}) bleomycin, clearly demonstrated the nuclear damage by bleomycin. The mean DFI values from number of cells yielding comet tails and their length values are presented; the mean comet tail length was 16.41±0.45 with 100 µM H_2O_2.

Table 1
Lethality values during Bleomycin toxicity to D38 cells growing in DMEM, assessed by three methods, AO/EB staining, propidium iodide staining and MTT assay.

Concentration of Bleomycin (mg/L)	Lethality of cells by AO/EB staining (%)	Lethality of cells by PI staining (%)	Lethality of cells by MTT assay (%)
0	0	0	0
15	32.8±1.20	34.2±0.79	5.9±0.43
25	32.8±1.31	37.6±2.09	12.8±0.45
50	37.5±2.28	40.2±1.91	52.7±0.81
75	85.9±0.87	87.9±2.32	75.4±1.32
100	90.6±1.57	93.7±0.72	85.9±0.70

Note: AO/EB, Acridine orange/ethidium bromide; DMEM, Dulbecco's modified Eagle's medium; MTT, 3-[4, 5- dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide; PI, Propidium iodide; —, Not applicable.

Table 2
Toxicity values of Bleomycin to D38 cells obtained by experimentation and the probit computation.

Asssay methods	Toxicity values (mg/L)				
	MIC*	LC_{25}	LC_{50}	LC_{75}	HPC*
AO/EB staining	15	33.88	47.86	66.07	100
PI staining	15	38.99	52.06	70.96	100
MTT assay	15	26.36	50.23	81.47	100
Comets seen	15	26.36	42.76	61.80	100
*From experiments. LC, lethal concentration.					

Table 3
DNA fragmentation index (DFI) and tail length of comets of D38 cells after growth in the presence of graded concentrations of Bleomycin.

Concentration of Bleomycin (mg/L)	DFI (%)	Tail length (μm)
0	0	0
15	4±2.0	4.26±0.50
25	19±1.73	8.16±0.75
50	62±2.0	13.3±0.56
75	84±5.29	18.5±1.50
100	93±3.60	21.2±0.86

Discussion

Since oral leukoplakia has a poor diagnosis, but it may be fatal due to its development to oral squamous-cell carcinoma (OSCC). Risk factors for oral carcinoma have been identified, but there are no reliable predictors of the outcome in individual patients with oral leukoplakia. Prevention of leukoplakia to OSCC is important because tramsformation of dysplastic leukoplakiato OSCC is higher in comparison with hyperkeratotic leukoplakia [30]. Chemotherapy may have toxic effects which may be reduced with topical therapy. In a study, it was found that vitamin A has some effect in snuff induced leukoplakia [31, 32]. Daily topical application of bleomycin in DMSO for 15–18 days reduces keratinization and dysplasia [8]. In another study, bleomycin at 0.5% and 1.0% has effect in reducing the size and severity of dysplasia after applying once daily for 2 weeks [1, 7].

In this study it was found that bleomycin is effective against leukoplakia cell line D38. After treating bleomycin at several concentrations to D38 cell line, it was observed that the MIC values were 15 mg/L of bleomycin, whereas the HPC values were 100 mg/L of bleomycin after 24 hours of incubation, by AO/EB staining, PI staining, MTT assay and comet assay techniques.

Eliminating the use of tobacco and alcohol is the most important aspect of treatment of oral leukoplakia. Now-a-days surgical removal is the common method of management and a choice of treatment by using lasers. But there are some difficult excision sites. In some cases posttreatment complications are also found. So it is difficult to treat the leukoplakia by excision only, in all cases. Hence, bleomycin can be used to prevent the leukoplakia developing to OSCC. Advantages of this approach are easy for application, that doesn't require treatment at a health care centre and relatively low cost as compared to surgical intervention.

Conclusion

Advances in the management of oral leukoplakia will help in prevention of potential progression of oral leukoplakia to SCC through dysplasia. From this study, it is clear that bleomycin can be used as an active drug for the treatment and management of oral leukoplakia.

Abbreviations

AO/EB: Acridine orange/ethidium bromide staining; PI: Propidium iodide; MTT: 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; MIC: Minimum inhibitory concentration; LC: Lethal concentration; HPC: Highest permissive concentration; DMEM: Dulbecco's modified eagle media; FBS: Fetal Bovine Serum; LMPA: Low melting point agarose

Declarations

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Authors' Contributions

Al MB, Khair, AM, Luke conceived and designed the experiment. AM, Luke, SR Shetty, and R Patnaik performed the experiments. S Mathew, SR Shetty interpreted and analysed the data. Al MB, Khair and AM, Luke contributed reagents, materials, analysis tools or data. Al MB, Khair, and R Patnaik wrote the manuscript.

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Availability of data and materials

All data generated and analysed during this study are included in this article. The raw datas during the current study are available from the corresponding author on reasonable requests.

Ethics approval and consent

This study was approved by institutional ethical committee, Ajman University, Ajman, UAE.

Competing interests

Authors declare no conflict of interest.

Author details

[1]Department of Surgical sciences, College of Dentistry, Ajman University, Ajman, UAE. [2]Department of Surgical sciences, College of Dentistry, Ajman University, Ajman, UAE.[3]Department of oral Radiology, College of Dental Medicine, Ubiversity of Sharjah.[4] Department of Surgical sciences, College of Dentistry, Ajman University, Ajman, UAE. [5]Department of Surgical sciences, College of Dentistry, Ajman University, Ajman, UAE.

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Figures

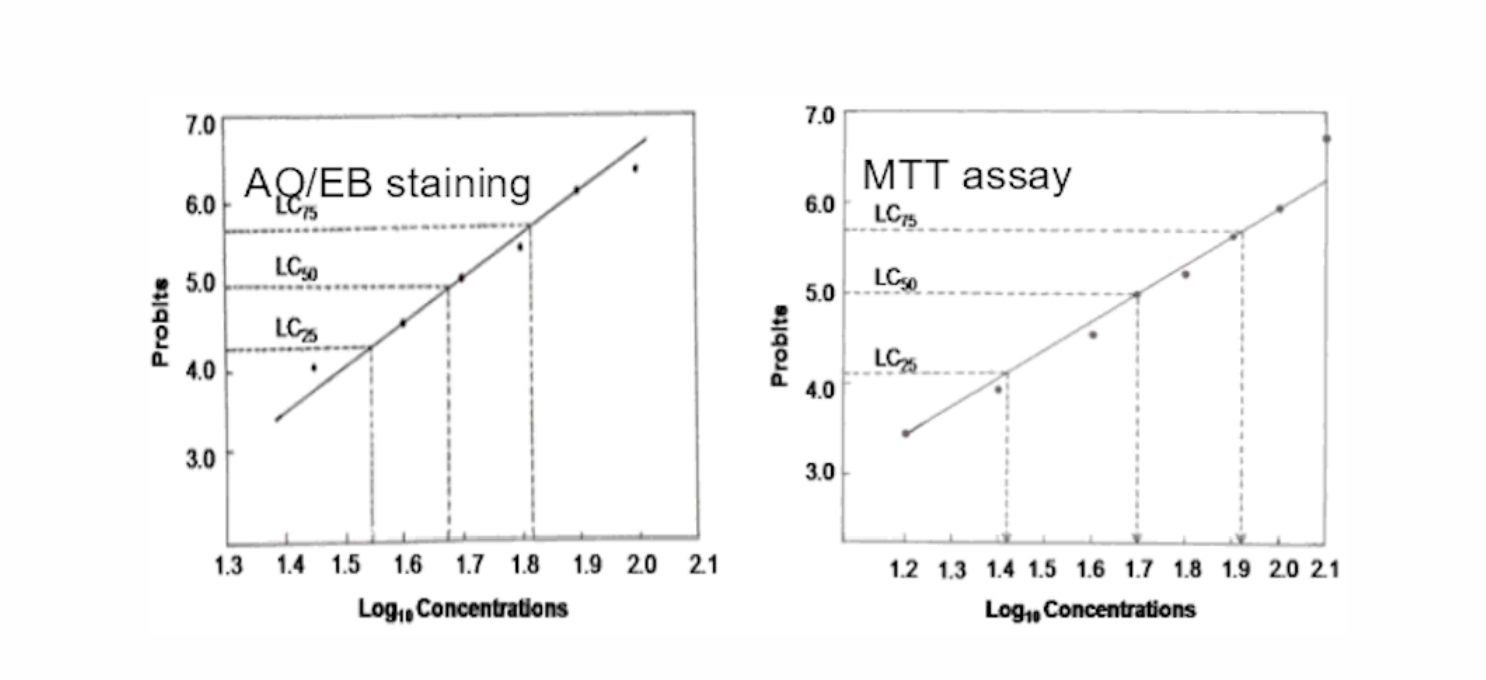

Figure 1

Probits of percentage lethality values plotted against log10 concentrations of Bleomycin in the toxicity study of leukoplakia cell line, D38; three log10 concentration values were determined taking probit points, from which, log10 concentrations for LC25, LC50 and LC75 by AO/EB staining and MTT assay were determined.

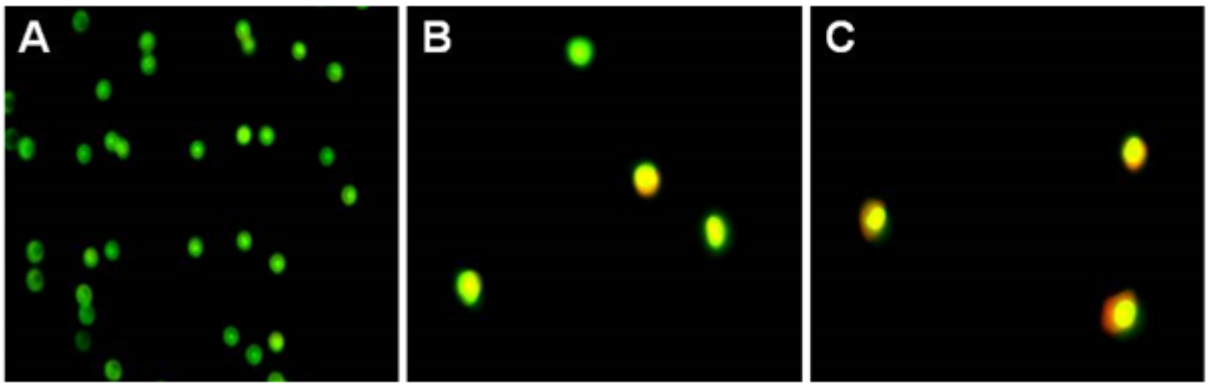

Figure 2

Acridine orange/ethidium bromide staining. (A) Control cells (B) Cells after treated with 25 mg/L Bleomycin, (C) 50 mg/L Bleomycin.

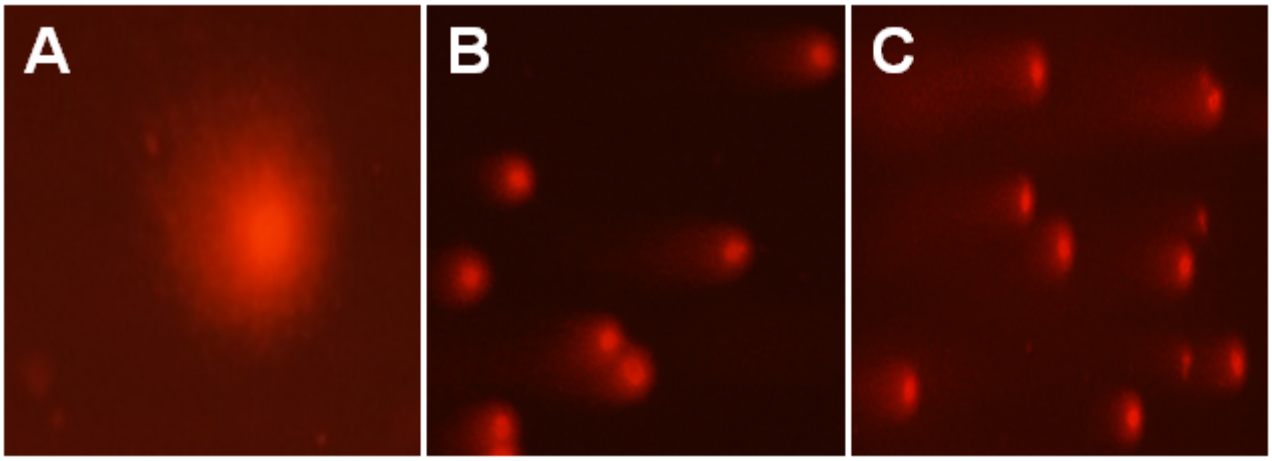

Figure 3

Comet assay. (A) Control cells, (B) Cells with comets after treatment with 100 µM H2O2 as positive control, (C) Cells with comets after treatment with 42.76 mg/L Bleomycin .