

Pharmacological Studies on Bleomycin as Cancer Therapy and Protective Effect of Aged Garlic

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Abstract

The pharmacological impacts of bleomycin as anticarcinogenic agents and the protective effect of aged garlic were done in current study. Eighty mature male albino mice were divided into 8 groups (each ten mice), group one was kept as a negative control, group two which was injected intraperitoneally with bleomycin. Group 3, which was given orally aged garlic, group 4 was inoculated with Ehrlich Ascites Carcinoma cells (EAC), animals of group 5 were administered aged garlic and injected with EAC cells while group 6 was given simultaneously EAC cells and bleomycin I/P, group 7 was injected bleomycin I/P and administered garlic 8 days later and group 8 took both bleomycin and garlic before inoculation of EAC cells by 8 days. Our results revealed that there were side effects of bleomycin on GSH, ROS, CAT, GPX and GR in the inoculated groups with EAC cells and confirmed by biochemical analysis. Aged garlic suppresses the progression of EAC cells and prevents their developmental effects.

Keywords: Bleomycin, Cancer Therapy, Protective Effect, Aged Garlic

Introduction

Cancer is a major public health threatening disease in developed countries and it's still one of the most serious causes of death in the world with no major progress in reducing its morbidity and mortality [1]. Chemotherapeutic agents are medications that destroy dividing cancer cells by targeting different phases in the cancer cell's life cycle. Since various agents attack the cancer cell differently, they are commonly given in combination for maximum beneficial effect. Once you understand that chemotherapy is designed to kill rapidly dividing cancer cells, you realize that it can also attack other rapidly dividing cells within the body. As hair skin, nails, gastrointestinal tract and bone marrow will be under assault. This hit on healthy cells is which leads to the most common side effects of chemotherapy [2].

The use of chemical agents to destroy cancer cells is a main stay in the treatment of malignancies. The possible role in treating illness was discovered when bone marrow suppressive effect of nitrogen mustard was noted in the early 1900's. Since that time, the search for drugs with anticancer activity has continued, and the goal of treatment with

chemotherapy has evolved from relief of symptoms for cancer cure, A major advantage of chemotherapy is its ability to treat wide spread or metastatic cancer, whereas surgery and radiation therapy are limited to treat cancer, confined to specific areas [3].

Cancer chemotherapy can be divided into several categories; Alkylating agents (eg: cyclophosphamide, ifosfamide). Antibiotics which affect nucleic acids (eg: doxorubicin, bleomycin), platinum compounds (eg: cisplatin), Mitotic inhibitors (eg: vincristine), antimetabolites (eg: 5-Fluorouracil), Comptothecin derivatives (eg: Topotecan), biological response modifiers (eg: Interferon) and hormone therapies (eg: Tamoxifen). The alkylating agents and the antitumor antibiotics are the most creating cellular damage by initiating free radical oxidants [4]. Antitumor Antibiotic includes: Bleomycin, Dactinomycin, Daunorubicin, Doxorubicin, Idarubicin, Mitomycin, Mitoxantrone, Plicamycin [5].

Bleomycin (BLM) is an antitumor antibiotic which is a mixture of a purified glycopeptides produced by a fermentation process isolated originally from fungus

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streptomyces - Verticillium discovered by Umezawa *et al.* [6]. It is commonly used as part of the cytotoxic treatment of several tumors such as germ cell tumors, Lymphomas including Hodgkin's disease, non-Hodgkin's lymphoma, kaposi's sarcoma and squamous cell carcinomas of head and neck including: mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingivae, epiglottis, skin, larynx, penis, cervix, and vulva. The response to BLM is poorer in patients with previously irradiated head and neck cancer. This medication may also be used to control the buildup of the fluid around the lungs (pleural effusion) caused by tumors that have spread in the lungs. For this condition bleomycin is placed in the space around the lungs through a chest tube [7].

BLM is also one component of the ABCD system (doxorubicin, (adriamycin), bleomycin, vinblastine and dacarbazine) that is a standard schedule treatment for advanced Hodgkin's lymphoma. This system has little toxicity compared to others as MOPP and minimal potential for inducing second neoplasias or patient sterility also it is used to treat early disease stages [8]. BLM is widely used in experimental models to cause lung injury leading to oxidant-induced inflammatory and fibrotic lesions in the lung interstitium of various animal species [9]. This animal model of pulmonary fibrosis resembles that seen in humans and it is useful to assess the effects of potential therapeutic agents including antioxidants [10].

BLM exerts its antitumor effect by inducing tumor cell death by induction of free radicals causing DNA breaks [11]. It can be given by several routes; IV, IM, or SC. In case of malignant effusion it can be administered IP or intra pleural [12]. BLM is known to produce pulmonary fibrosis in humans as well as in experimental animals. Application of BLM is featured by the occurrence of sometimes fatal side effects. Due to the lack of the BLM-inactivating enzyme, BLM hydrolase, in the lungs and the skin, bleomycin-induced toxicity occurs predominantly in these organs directly after its administration, the occurrence of fever, chills, and sometimes hypotension is well known [13].

The most frequent event is pneumonitis occasionally progressing to pulmonary fibrosis. Approximately 10% of patients treated with BLM have died by pulmonary fibrosis. The most common changes in pulmonary function tests are decrease in total lung volume and decrease in vital capacity. The process of fibrosis is important in the lung since its progression may result in respiratory failure and poor prognosis in patients with a heterogeneous group of disorders of known and unknown etiology. The progression of fibrosis is closely controlled by a complicated network consisting of many cytokines, chemical mediators, growth factors and biophysical peptides derived from inflammatory immune cells and alveolar type II cells. These cells which accumulate in the lower airways release a harmful amount of (ROS) [14].

Recently it has been suggested that reactive nitrogen species (RNS) including nitric oxide (NO), peroxynitrite (ONOO⁻) and nitrogen dioxide which are released from neutrophils and macrophages play an important role in the pathogenesis of lung disease [15]. The cytotoxic activity of BLM depends on generation of single- and double-strand breaks in DNA [16]. Indispensable for this BLM activity is the presence of metal ions, specifically Fe²⁺, Fe³⁺. BLM with O₂ or Fe³⁺ BLM complexed with H₂O₂ leads to formation of an active BLM molecule, HOO⁻ Fe²⁺ BLM. BLM specifically induces DNA double strand breaks and apurinic / apyrimidinic sites in the complementary strands with a higher frequency than would result from randomly induced damage. It is expected that double strand break is generated by a BM molecule which is reactivated after nicking the first strand. The reactivation consists of reduction of BLM Fe³⁺ (formed after reaction with DKA) to BLM Fe²⁺ and a subsequent addition of an oxygen molecule leading to the generation of the active form of BLM [17].

BLM induces both chromatid and chromosome type aberrations (deletions, di- and multicentrics, rings, exchanges, breaks), but does not induce sister chromatid exchanges. Accordingly, interphase cells after BLM treatment show the presence of micronuclei [18]. The viability test for detection the viability of Ehrlich Ascites

carcinoma cells (EAC) in mature male albino mice was done in this study. Using bleomycin as anti-tumorigenic and detecting its side effects. Besides determination of sperm abnormalities in male mature mice and determination of antioxidants enzymes as glutathione reductase, reduced glutathione, glutathione -S-transferase, superoxide dismutase and catalase enzymes were also studied .

Material and Methods

Experimental animals

Eighty adult male Swiss albino mice weighted 30-50 gm were obtained from the Laboratory Animal Housing Unit, Animal Health, Research Institute, Dokki, Cairo, Egypt. The animals were clinically healthy, kept under hygienic conditions. Housed in metal cages with hard wood shavings as bedding. They were maintained on balanced ration, water and the food were giving ad libitum throughout the experimental period. The animals were accommodated to the laboratory conditions for one week before beginning of experiment.

Tumor Cell Line

Ehrlich Ascites Carcinoma cells (EAC)

The parent line of Ehrlich Ascites Carcinoma cells was initially supplied by the National Cancer Institute, Cairo University, Egypt, and maintained in mature male Swiss albino mice through serial intraperitoneal

inoculation of [EAC] 2.5×10^6 tumor cells/0.2 ml at 7-8 day intervals in the laboratory in an ascites form was detected.

Transplantation of Tumor in mice

EAC cells were collected from moderately growing 7 days old donor. After making appropriating dilution, the viability was checked by the trypan blue exclusion test 0.2 ml portions of ascetic fluid containing 2.5×10^6 viable cells were intraperitoneally injected in a group of healthy mice for maintenance of the line and for experimental work.

Bleomycin (BLM)

BLM was obtained in commercial form (Bleocin). The drug was obtained from Salides Pharma. The drug was produced by Nippon kayaku.Co. Ltd (Tokyo-Japan). Each vial contain Bleomycin Hydrochloride 15 mg potency.

Aged garlic extract (AGE)

Preparation n of AGE was carried out [19]. The Egyptian garlic gloves (*Alium sativum*) were sliced and soaked in 20% ethanol (80% water and 20% ethanol mixture) in dark coloured glass bottles (200 gm/liter). The garlic was aged naturally for 20 months at room temperature. The alcohol was found to have no effect except to inhibit bacterial and fungi growth. The extract was then filtered and tested bacteriologically and fungi on nutrient agar and S.S. media respectively.

Table 1 Experimental design and grouping

Groups	Treatment	Aged garlic 2 gm/kg b.wt. stomach lavage	bleomycin 15 gm/kg b.wt. I/P	Ehrlich Ascites carcinoma (EAC) I/P
Negative control		-ve	-ve	-ve
Positive control		-ve	+ve	-ve
Positive Control (inoculated) control		+ve	-ve	-ve
Aged garlic and inoculated with EAC cells		-ve	-ve	+ve
at the moment F in collection		+ve	-ve	+ve
after inoculation by 8 days.		-ve	+ve	+ve
Before inoculation by 8 days		+ve	+ve	+ve before inoculation 8 days

Sampling

Blood samples were collected at the end of the experiment and harvesting the blood serum and other half were collected in heparinated test tube and used immediately for counting the blood cells using haemocytometer. At the end of the experimental period, animals were fasted overnight sacrificed by cervical dislocation. The mice epididymis was obtained for collection the semen for application the semen analysis.

Semen analysis

The Canada epididymis of one testis was excised and received in a sterilized Petridis containing warm normal saline of 37 °C, then it was macerated by sterilized scissors to obtain the epididymal content in a suspension that was handled as a semen [20].

- 1) Sperm abnormalities: It was carried out after the method described [21].
- 2) Sperm cell concentration per Mel of semen: It was performed according to the method [22].
- 3) Sperm abnormalities: It was determined using the method [23].

Biochemical analysis

- 1) Determination of glutathione reductase (GR) activity according to the method described [24].
- 2) Determination of reduced glutathione (GSH) according to the method described modified [25].

- 3) Determination of superoxide dismutase (SOD) activity. According to the method described [26].
- 4) Determination of glutathione peroxidase activity (GPX) using the biodiagnostic assay [27].
- 5) Determination of Catalase (CAT) activity [28].

Statistical analysis

The obtained results were analyzed and graphically represented using the statistical package for social science (SPSS 8.0 software) [29] for obtaining mean and standard error. The data analyzed one –way ANOVA to determine the statistical of difference among groups.

Results and Discussion

1.Evaluation of anticarcinogenicity of Bleomycin and side effects on EAC

Examination of peritoneal fluid for EAC cells using trypan blue stain 0.5%

Viability test Was done by using trypan blue 0.5% for staining of ascetic fluid samples and counting of the total, life and dead EAC cells by using hemocytometer and the results were statistically analysed (Table 2). Ascitic fluid samples were stained trypan blue 0.5%, we made ascetic fluid film using glass slides and examined under microscope and it have been noticed that EAC in the fourth group showing numerous life cells not stained blue.

Table 2: Examination viability test revealed total tumor, life and dead cells.

Groups	EAC cells	Mean ± SEM (no of cells per ml x 10 ⁴)		
		Total tumor cell count	Life cell count	Dead cell count
1		-ve	-ve	-ve
2		74.5±3.10a	73.5±2.85a	1.00±0.55a
3		-ve	-ve	-ve
4		65.35±2.75a	59.65±2.60a	5.75±0.60a
5		62.50±2.55a	58.40±2.45a	4.40±0.55a
6		61.5±2.35a	9.75±0.85b	51.25±2.60a
7		63.10±2.65 b	4.50±0.25c	58.60±0.60a
8		10.50±0.65c	3.25±0.25c	7.25±0.75c

* There is significant difference (P≤0.05)

** There is highly significant difference (≤0.01)

Mean carrying different superscripts are significantly. Oxidative stress was considered to be one of the mechanisms implicated in bleomycin induced treatment [30]. Bleomycin oxygen dependent toxicity occurs via cyclic one electron reduction of the bleomycin molecule, followed by oxidation with molecule and the superoxide radical, an extremely reactive species that produces a variety of toxic radicals and results in several types of damage to biological molecules [31]. In fact, reactive oxygen species (ROS) can attack biomolecules, such as lipid and thiols, in proteins and glutathione, leading to indication of enzymes, cell dysfunction and cell death [32].

Excess generation of ROS and a deficient cellular antioxidant defense system may lead to a state of oxidative stress that can cause oxidative damage to cells by affecting the cell membrane, genetic material, and various enzymatic reactions. ROS levels are normally controlled by intracellular antioxidant defense mechanism, which include the enzymes catalase (CAT) GR, GSH and (Gpx) as well as non-enzymatic antioxidant system [33]. Glutathione peroxidase (Gpx) is a seleno enzyme, two third of which is present in-

cystol and one-thirds in the mitochondria in the liver. It plays a major role in the reduction of hydrogen peroxide and hydroperoxide converted to non-toxic products [34].

The results of the present study revealed depletion in GSH level of Bleomycin in the mice liver and administration of aged garlic after inoculation of EAC cells and replenishes intracellular GSH levels as presented in Table (3). Aged garlic attenuates oxidative stress by increasing the content of hepatic glutathione, leading to the reduction in the level of lipid hydro peroxide. These finding indicate that the increase in free radicals scavenging enzyme followed by a decrease in GSH by bleomycin could be initiated by oxidative stress. The reduction of GSH after bleomycin treatment may have resulted from activity of GPX in reducing hydro peroxides to stable non-radical lipid alcohols utilizing GSH as a source of reducing equivalents. Alternatively, GSH concentration may have been reduced by the direct utilization of GSH as an antioxidant in termination free radical reaction initiated by bleomycin. AGE supplementation, however, provokes a sustained higher level of GSH while nullifying the increasing in the level of antioxidant enzymes.

Table 3: The effects Bleomycin & AGE on sperm quality in mature mice against EAC cells. (Means± SE.)

Parameters	Sperm cell concentration 10 ⁶ /mm ³	Sperm motility %	Speram abnormalities %
Groups			
1	22.65±2.35 ^c	65.15±1.75 ^c	19.80±0.80 ^c
2	16.5±1.75 ^b	48.55±1.35 ^b	26.45±1.35 ^b
3	25.60±2.40 ^a	80.50±3.10 ^a	14.10±0.55 ^a
4	14.30±1.15 ^b	40.70±1.80 ^b	36.40±1.85 ^c
5	18.65±1.80 ^b	52.60±1.45 ^c	29.15±0.90 ^b
6	20.90±2.15 ^c	56.60±1.60 ^c	21.80±0.70 ^c
7	23.55±2.60 ^c	63.40±1.70 ^c	20.00±0.95 ^c
8	23.90±2.70 ^c	67.80±2.10 ^c	15.70±0.65 ^c

Means within the same column have different superscripts are significantly different at (P≤0.05).

Previous study confirmed that the depletion of antioxidants including glutathione and protein bound sulfhydryl groups and the increase in the activity of various anti-oxidant enzymes indicative damage of cell molecules [35]. Under normal physiological conditions, ROS are easily neutralized by antioxidant defense such as glutathione (GSH) and several

antioxidant enzymes. However, upon bleomycin treatment, an excess of ROS production can occur and the antioxidant defended available may be overwhelmed, which may lead to damage in cellular components [36].

Hepatic damage induced by bleomycin treatment in mice, has possibly caused sever

irritation of oxidant system in these cells [37]. Garlic has been as a medicinal remedy as well as presents disease and enhances vigor. Modern science and medicine have validated many of the traditional uses of garlic and identified its health-promoting compounds. Most of the benefits of garlic are attributed to its high content of organosulfur compounds and

antioxidant activity, which protect against oxidative damage by reactive oxygen species (ROS) and other beneficial effects (groups 3,4,5,7, and 8, Table 4). Thus, intake of AGE and the potential increase in glutathione provide additional antioxidant protection in cancer.

Table 4: Mean \pm S.E. of biochemical analysis of antioxidant enzymes activities of the EAC cells inoculated in mature male albino mice and treatment bleomycin and AGE.

Groups	Parameters	Reduced Glutathione (GSH) (nmol/mg hepatic protein)	Glutathione reductase (μ mole/gm. hepatic tissue GR)	Glutathione GRX peroxidase (u/mg hepatic protein)	Catalase (CAT) (u/mg hepatic protein)	Superoxide (SOD) dismutase (u/mg hepatic protein)
1		40.50 \pm 0.40 ^b	23.70 \pm 0.30 ^c	76.10 \pm 0.50 ^c	24.30 \pm 0.35 ^c	19.15 \pm 0.16 ^c
2		22.60 \pm 0.50 ^c	11.15 \pm 0.70 ^a	30.90 \pm 1.20 ^a	19.25 \pm 0.55 ^b	15.20 \pm 0.25 ^b
3		35.70 \pm 0.75 ^b	26.35 \pm 12.30 ^b	79.25 \pm 0.90 ^b	26.20 \pm 0.70 ^a	21.40 \pm 0.30 ^a
4		47.10 \pm 1.25 ^a	21.50 \pm 0.50 ^a	73.40 \pm 0.35 ^a	23.60 \pm 0.50 ^c	20.25 \pm 0.60 ^c
5		28.45 \pm 0.60 ^c	14.30 \pm 0.45 ^c	49.10 \pm 0.60 ^c	18.40 \pm 0.60 ^b	16.30 \pm 0.40 ^b
6		33.65 \pm 0.70 ^c	17.60 \pm 0.35 ^c	56.40 \pm 0.40 ^c	15.80 \pm 0.75 ^b	14.25 \pm 0.35 ^b
7		37.15 \pm 0.15 ^b	20.10 \pm 0.55 ^c	69.80 \pm 1.15 ^c	21.20 \pm 0.70 ^c	17.30 \pm 0.40 ^c
8		42.20 \pm 0.30 ^a	25.20 \pm 0.50 ^a	79.30 \pm 1.40 ^a	25.90 \pm 1.30 ^c	20.30 \pm 0.50 ^c

Means within the same column have different superscripts are significantly different at ($P \leq 0.05$).

The results showed that in the all treated groups the number of adenomas increased continuous over the year, but in the aged garlic group, AGE suppresses the progression of pre. cancerous colon lesions and thus would prevent them from developing into full blown colon cancer [38]. AGE and bleomycin, with

their high antioxidant activity, which would help prevention of pathological complications in diabetes. The potential therapeutic role of AGE in diabetes is now being tested in clinical trial involving patient with type 2 diabetes [39].

Figure 1: Negative control, mature male mice spermatozoon. (Bar = 100 μ m).



Aged Garlic Extract could protect cells which line the veins and arteries from damage caused by free radicals by enhancing the activity of antioxidant enzymes (the glutathione (GSH) redox cycle). SOD is

another potent intra cellular antioxidant. AGE was found to time – and dose – dependently enhance intra cellular GSH level, glutathione disulfide reductase and SOD and thus may help to protect the cardio vascular system [40]. Bleomycin exerts its antitumor effect by

inducing tumor cell death, while inhibition of tumor angiogenesis may also be important. The cytotoxicity occurs by induction of free

radicals. Bleomycin forms a complex with Fe^{+++} , resulting in reduction of oxygen to free radicals.



Figure 2: Mature male mice spermatozoon of groups inoculated with EAC cell and treated by Bleomycin and AGE showing the following. A. Abnormal tail shape, B. protoplasmic droplet. C. Curved Tail, D. Detached head and Tail. (Bar = 100 μ m).

Conflict of interest

The authors declare that they have no conflict of interest.

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الملخص العربي

دراسات فارماكولوجية على عقار البليوميسين كعلاج للسرطان والعلاج الوقائي بالثوم المعتق

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اجريت هذه الدراسة للكشف عن الأثار الجانبية لاستخدام عقار البليوميسين المستخدم فى علاج السرطان ومحاولة درء هذه الأثار باستخدام الثوم المعتق ، تمت هذه الدراسة على ٨٠ جرذ أبيض ذكر بالغ (٣٠ - ٤٠ جرام) وقسمت الى ثمانية مجاميع بالتساوى (كل مجموعة ١٠ جرذان). المجموعة الأولى ضابطة تم حقنها بمحلول ملح فسيولوجى فى الغشاء البريتونى . المجموعة الثانية : حقنت بعقار البليوميسين فى الغشاء البريتونى . المجموعة الثالثة : جرعت بالثوم المعتق . المجموعة الرابعة : تم نقل خلايا السرطانية لها. المجموعة الخامسة : تم تجريع الجرذان المصابة بالخلايا السرطانية بالثوم المعتق فى نفس الوقت . المجموعة السادسة: تم علاج الجرذان المصابة بالسرطان بعقار البليوميسين . المجموعة السابعة : تم تجريع جميع الجرذان المصابة بالسرطان بالبليوميسين والثوم المعتق بعد اصابتها بثمانية أيام المجموعة الثامنة: تم تجريعها قبل اصابتها بثمانية أيام وكانت النتائج كالتالى: لعقار البليوميسين تأثير سلبى على جميع الأختبارات البيوكيميائية (GSH, GR, CAT, ROS , GPX) وتأثير سلبى مدمر على تحليل السائل المنوى كما وجد أن الثوم المعتق له تأثير وقائى فعال للأثار السلبية التى لعقار البليوميسين.