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'LYCOPENE LESSENS BLEOMYCIN INDUCED MICRONUCLEI FREQUENCY IN CULTURED HUMAN LYMPHOCYTES *IN VITRO*'

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ABSTRACT

The cytokinesis-block micronucleus (CBMN) assay is a comprehensive technique for measuring DNA damage, cytostasis, and cytotoxicity in different tissue types, including human lymphocytes. In present study protective effects of lycopene in cultured human lymphocytes against Bleomycin-induced DNA damage in the form of micronuclei (MN) has been reported. Four different cultures were set up. First culture vial was kept untreated, second was treated with lycopene (8µg/ml), third was treated with Bleomycin (15µg/ml) and fourth received combined treatment of lycopene (8µg/ml) and Bleomycin (15µg/ml). Positive implications were recorded about protective effects by lycopene on Bleomycin induced DNA damage in the form of micronuclei (MNi), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs).

KEYWORDS: Lycopene, Bleomycin, Micronuclei Assay.

INTRODUCTION

Lycopene from the neo-Latin *lycopersicum*, the tomato species, is a bright red carotene and carotenoid pigment and phytochemical found in tomatoes and other red fruits and vegetables such as red carrots, watermelons, gac, and papayas, but not in strawberries, red bell peppers, or cherries. Although lycopene is chemically a carotene, it has no vitamin-A activity.[1] Foods that are not red may also contain lycopene, such as brown beans or parsley. In plants, algae, and other photosynthetic organisms, lycopene is an important intermediate in the biosynthesis

of many carotenoids, including beta carotene, which is responsible for yellow, orange, or red pigmentation, photosynthesis, and photo-protection. Like all carotenoids, lycopene is a polyunsaturated hydrocarbon, i.e. an unsubstituted alkene. Structurally, lycopene is a tetraterpene and assembled from eight isoprene units that are composed entirely of carbon and hydrogen. It is insoluble in water. Lycopene's eleven conjugated double bonds give its deep red color and its antioxidant activity. Owing to the strong color and non-toxicity, lycopene is a useful food coloring and approved for usage.

Bleomycin sulfate is a mixture of glycopeptides antibiotic containing approximately primarily BleomycinA2 (70%) and B2 (30%). It is isolated from *Streptomyces verticillus*. The drug binds to DNA, inhibits DNA synthesis, and causes single strand scission of DNA *in vivo* and *in vitro* at specific base sequences.[2] Bleomycin has been shown to cause cell cycle arrest in G2 and in mitosis. *In vitro* treatment of Bleomycin produces chromosomal aberrations similar to those caused by radiation and hence is often referred as radiomimetic drug.

Free radicals are very short lived, with half-lives in milli-, micro- or nanoseconds. Free radicals have been implicated in the etiology of several human diseases as well as ageing.[3, 4] It has to be emphasized that reactive oxygen species (ROS) and reactive nitrogen species (RNS) are both produced in a well regulated manner to help maintain homeostasis at the cellular level in the normal healthy tissues and play an important role as signaling molecules. Most cells can produce superoxide (O_2^-), hydrogen peroxide (H_2O_2). Sources of free radical generation include redox cycling of xenobiotics, exposure to physicochemical agents like ionizing radiations, visible light or UV in the presence of oxygen and an endogenous compound or a drug that act as photo sensitizer. Most of the damage induced by ionizing radiations in biological systems is indirect and is mediated by products of radiolysis of water including hydrogen radical, hydrated electron (eaq-), H_2O_2, peroxyl radical, etc.[5, 6]

Oxidative damage to DNA is a result of interaction of DNA with ROS or RNS. Free radicals such as hydrogen radical, eaq- and peroxyl radical react with DNA by addition to bases or abstractions of hydrogen atoms from the sugar moiety.[7] The C4-C5 double bond of pyrimidine is particularly sensitive to attack by peroxyl radical, generating a spectrum of oxidative pyrimidine damage products, including thymine glycol, uracil glycol, urea residue, 5-hydroxydeoxyuridine, 5-hydroxydeoxycytidine, hydantoin and others. Similarly, interaction of OH with purines will generate 8-hydroxydeoxyguanosine (8-OHdG), 8-

hydroxydeoxyadenosine, formamidopyrimidines and other less characterized purine oxidative products.

Radiation is a well-known inducer of free radicals that causes DNA and chromosomal damage. Exposure of cells to ionizing radiation during G_0 & G_1 phase of cell cycle causes chromosomal aberration (CAs) as breaks, dicentrics, acentrics, fragments, rings, translocation, and micronuclei formation. These CAs are used as biomarkers of radio sensitivity or radiation damage after medical, accidental and occupational exposure. With respect to radiation damage to human, it is important to protect humans from adverse effects induced by ionizing radiation.[8] The use of certain materials may help to decrease the genotoxicity created by radiation and may inhibit mutagenesis and carcinogenesis.[9] Recently carotenoids have been used to decrease the effects of radiation.[10 - 13]

In 2006, the radio-protective effect of Carnosic acid (CA), Carnosol (COL) and Rosmaric acid (RO) against chromosomal damage induced by γ-rays in Human lymphocytes, compared with those of L-ascorbic acid (AA) and the S-containing compound dimethyl sulfoxide (DMSO) has been determined by micronucleus test.[14] Their study showed the reduction in micronuclei frequency (MN) in cytokinesis – blocked human lymphocytes before and after gamma ray irradiation. Another study.[15] revealed that the Bangla variety of piper betel (pan) leaf ethanol extract possesses best antioxidant activity and radioprotective effects as well.

The present study was initiated to know whether lycopene has any radioprotective activity. Human lymphocytes can easily be cultured in laboratory and forms an excellent *in vitro* culture system for early and rapid detection of activities of various compounds.[8] Any change induced in micronuclei formation after addition of compounds reflects genotoxicity of those compounds. We therefore analyzed the effects of lycopene on Bleomycin induced genotoxic damage from *in vitro* cultured human lymphocytes by CBMN assay.

MATERIALS AND METHODS

Human lymphocyte culture

Whole blood culture was set up by following the standard methods.[16] with some modifications.[8] To 5ml of RPMI 1640 (Hikaryo, ready mix) medium taken in sterile culture vial, 50µl Heparin and 0.6ml of whole blood was added. Cultures were incubated at 37 °C for duration of 72 hrs. At 44th hour of incubation, 6 µg/ml of Cytochalasin – B was added. After 72 hrs incubation, cultures were terminated by centrifugation at 1500 rpm for 10 min.

Supernatant was discarded and pellet was treated with 5ml of chilled hypotonic potassium chloride (0.075 M) solution for 5-9 min, followed by chilled fixative (3:1methanol: acetic acid) washes so that clear white pellet was obtained.. This was suspended in about half ml of fixative for final preparation of slides. On chilled sterile slides, about 4-5 drops of cell suspension was dropped from convenient height with the help of pasture pipette. Slides were air dried and immediately blind coded. Air – dried slides were stained in 2% Giemsa/ May Grundwald staining solution prepared in Sorenson's buffer. Optimum staining time varied between slides and the stain batches, however, was generally of the order of about 5–7 minutes.

Treatment protocols

The present study was carried out on blood samples collected from 12 healthy, non smoking, and randomly selected individuals of either sex. The age of the donors ranged from 20-30 years. Four separate culture vials were set up from each of the twelve blood samples collected. First culture vial was kept untreated so as to act as control. After 24 hours of initiation, second culture vial was treated with lycopene (8µg/ml). The third culture vial was treated with Bleomycin (15µg/ml) and the fourth vial received both lycopene (8µg/ml) and Bleomycin (15µg/ml). At 44th hr, 6 µg/ml of Cytochalasin-B was added to all four vials. The presence of micronuclei was recorded keeping in mind the standard guidelines.[17] For counting micronuclei frequency, one thousand cells were considered.

RESULTS

During scoring of slides, both cytotoxicity score as well as DNA damage indices were considered. Cytotoxicity measurement parameters included number of binucleated, multinucleated, apoptotic and necrotic cells. While presence and frequency of micronuclei in either mononucleated, binucleated or multinucleated cells were considered as DNA damage indices. Further number of cells with nuclear bridges and nuclear buds were also recorded**.** The results of the present study **(Table I)** revealed that there was statistically significant reduction ($P< 0.01$), in total number of micronuclei after addition of lycopene (8µg/ml) along with Bleomycin (15µg/ml) as compared to alone treatment of Bleomycin at the same concentration. Further, significant ($P< 0.05$) reduction was observed in nuclear buds (NBud), nucleoplasmic bridges (NPB), number of apoptotic as well as necrotic cells from those cultures treated with both lycopene as well as Bleomycin when compared to the cultures treated alone with Bleomycin. **Fig1** represents binucleated cell, **Fig 2** indicates the

binucleated cell with single micronuclei formation, **Fig 3** represents the binucleated cell with two micronuclei formation, **Fig 4** indicates mononucleated cell with micronuclei, **Fig 5** indicates mononucleated cell with multiple micronuclei, **Fig 6** represents nuclear bud, **Fig 7** indicates nucleoplasmic bridge, **Fig 8** represents apoptosis while **Fig 9** indicates cell necrosis.

Plate I

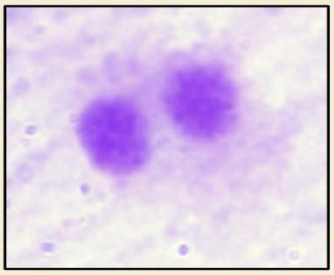

Fig 1

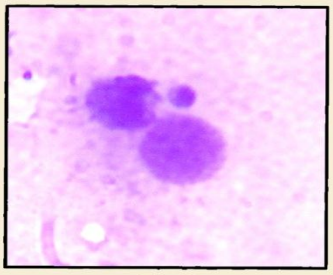

Fig 2

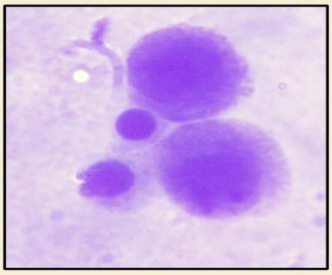

Fig 3

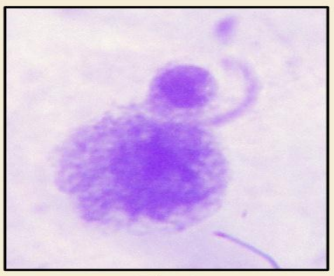

Fig 4

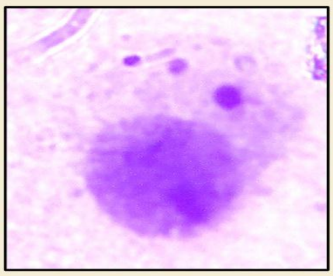

Fig 5

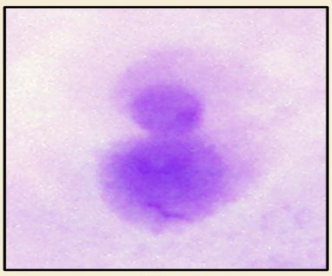

Fig 6

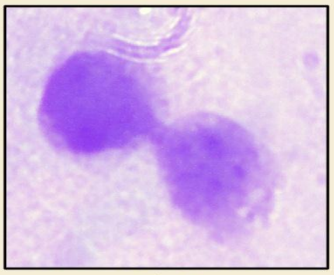

Fig 7

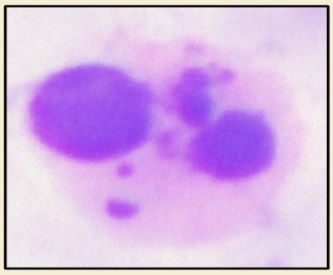

Fig 8

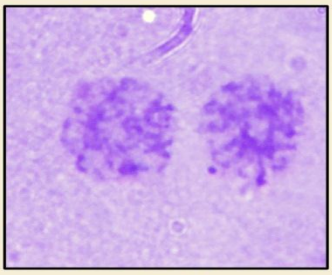

Fig 9

Fig 1: Binucleated cell
Fig 2: Binucleated cell with single micronuclei
Fig 3: Binucleated cell with two micronuclei
Fig 4: Mononucleated cell
Fig 5: Mononucleated cell with single micronuclei
Fig 6: Nuclear bud
FIg 7: Nucleoplasmic bridge
Fig 8: Apoptosis
Fig 9: Cell Necrosis

Micronuclei induced in *in vitro* cultured human lymphocytes after addition of lycopene alone as well as with bleomycin.

Treatment		Cytostatic /cytotoxicity score					DNA damage indices in BN cells				
		Total no of cells scored	**No of BN cells**	**No of MLN cells**	**No of apoptotic cells**	**No of necrotic cells**	**No of mono-nucleated cells with MN**	**No of BN cells with MN**	**Total no of MN**	**No of BN cells with NPB**	**No of BN cells with NBud**
Control	Pooled values	11707	11141	432	36	60	38	98	136	322	297
	Percentile values	1000	952	37	3	5	3	8	12	28	25
Lycopene 8µ/ml	Pooled values	12356	11867	346	41	59	43	97	140	417	348
	Percentile values	1000	960	28	3	5	3	8	11	34	28
Bleomycin 15µg/ml	Pooled values	11988	11246	441	87	94	120	228	348	820	540
	Percentile values	1000	938	37	7	8	10	19#	29#	68#	45#
Bleomycin + Lycopene 15µg/ml+8µ/ml	Pooled values	11560	10923	467	43	67	60	93	153	472	372
	Percentile values	1000	945	40	4*	4*	5	8*	13*	41*	32*

(BN= Binucleated; MLN= Multinucleated; MN= Micronuclei, NPB= Nucleoplasmic bridge, Nbud= Nuclear bud)

 # Significantly greater at (P< 0.001) than the control as well as alone lycopene treatment

** Significantly less at (P< 0.01) than only bleomycin treatment*

DISCUSSION

Biological effects of ionizing radiation begin with ROS generation.[18] Irradiation of biological material leads to a rapid burst of ROS, generated primarily due to the ionization of water molecules and direct ionization of target molecules.[19] ROS generation is important in the pathogenesis of radiation-induced tissue injury.[20] Cellular DNA is the primary target for the biological and lethal effects of ionizing radiation, and ROS interact with biological molecules, producing toxic free-radicals that cause DNA or protein damage and lipid peroxidation.[21, 22]

There is great interest in reducing radiation toxicity in normal tissues by administering naturally radioprotective agents.[23, 24] Such agents have several mechanisms of action they scavenge free radicals generated during radiolysis and induce cellular radioprotectors including prostaglandins, SOD and GSH.

Lycopene is a natural pigment, synthesized by plants and micro-organisms, but not by animals. It is a carotenoid, an acyclic isomer of β-carotene and has no vitamin A activity.[1] Epidemiological and small experimental animal studies[1] indicate that lycopene rich diets may have a protective effect against chronic diseases, including cancer and heart disease. These properties of lycopene are attributed to its ability to scavenge free radicals and physically quench singlet molecular oxygen.

Antioxidants are protective agents that inactivate reactive oxygen species and therefore significantly delay or prevent oxidative damage. Antioxidants such as superoxide dismutase, catalase and glutathione peroxidase are naturally present within human cells. In addition, antioxidants such as vitamin E, vitamin C, polyphenols and carotenoids are available from food. These antioxidants protect proteins, lipids, enzymes, chromosomes and DNA against free radical oxidations.[12, 13] The antioxidant activity of lycopene based on its ability to capture free radicals has been extensively evaluated in cell cultures and animal models. Experimental evidence suggests that Lycopene can quench singlet oxygen and capture nitrogen dioxide, thiyl and sulphonyl free radicals.[25] In their repeat-dose studies, synthetic Lycopene had a low-grade acute toxicity profile: no significant toxic or histo -pathological effects were observed with beadlet formulations of synthetic lycopene in rats at doses up to 500 mg/kg body weight per day for 14 weeks or 1000 mg/kg body weight per day for 4 weeks. A wide range of lycopene doses have been used (1 – 1000 mg/kg) in animal studies investigating the antioxidant potential of lycopene. The antioxidant status of lycopene could

be considered as a cofactor providing decrease in chromosomal aberrations and micronuclei frequency.

Lycopene has been considered as a potential agent for prevention of prostate cancer. Lycopene may be most powerful carotenoid quencher of singlet oxygen [26] being 100 times more efficient than vitamin E which in turn has 125 times the quenching action of glutathione singlet oxygen produced during exposure to UV light which is primary cause of skin ageing..[27]

Preliminary study[28] in which lymphocyte cultures were exposed to 15µg/ml Bleomycin and 8µg/ml Lycopene revealed significant reduction in the chromosomal aberration frequency, particularly chromosome gaps, chromosome breaks and dicentric chromosomes, in those cultures treated with both lycopene as well as bleomycin when compared to the culture treated alone with Bleomycin.

The present study was carried out on *in vitro* cultured human lymphocytes. The cultures were treated either alone with lycopene as well as in combination with radiomimetic drug Bleomycin. Positive implications about radioprotective effects by Lycopene on Bleomycin induced cytogenetic alteration in the form of Micronuclei in *in vitro* human lymphocyte culture were recorded. In conclusion, the evidence provided in the current study give reason to believe that the Lycopene possess a radioprotective potential. However to further justify, additional *in vitro* and *in vivo* studies need to be performed so as to confirm these data and to discover the mechanisms of the possible protective effects.

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