CHAPTER -5

EX VIVO AND *IN VITRO* RADIOPROTECTIVE PROPERTIES OF POLYSACCHARIDES FROM *GANODERMA LUCIDUM*

CHAPTER 5

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5.1. INTRODUCTION

The cellular membrane and DNA are the two main targets of radiation induced lethal effects and mutagenicity. The formation of lipid peroxides in the tissues exposed to γ-radiation is one of the markers of the membrane damage. DNA strand break assays have been used to test the genotoxicity of various chemical and physical agents in different cell types. *In vitro* radioprotective properties of the polysaccharides from *Ganoderma lucidum* was carried out by studying the extent of protection offered against radiation induced damage to plasmid pBR322 and mice liver microsomal membrane. Similarly the *ex vivo* radioprotective properties were assessed by alkaline comet assay, where human lymphocytes were exposed to gamma radiation. The alkaline comet assay is an elegant and effective technique to monitor the extent of the DNA damage and its protection. The Comet Assay can be used to detect DNA damage caused by double strand breaks, single strand breaks, alkali labile sites, oxidative base damage, and DNA cross-linking with DNA or protein (Figure .5.1).

5.2. MATERIALS AND METHODS

5.2.1. Chemicals

TRIS base, high melting agarose, low melting point agarose, Na_2-EDTA, TritonX-100, sodium sarcosinate, DMSO and propidium iodide were obtained from Sigma chemicals (St.Louis, Missouri), NaCl was from S.D. Fine Chemicals (Mumbai, India), and NaOH was from Thomas Baker Chemicals (Mumbai, India). All other chemicals were purchased from Bangalore Genei. Plasmid pBR322 DNA was also obtained from Bangalore Genei, Bangalore, India.

5.2.2. Animals

Male Swiss mice, 8-10 weeks old and weighing 20-25 g, were selected from an inbred group maintained under standard conditions of temperature (25±2°C) and humidity. All the experiments were conducted strictly according to the ethical guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division of the Government of India.

5.2.3. Collection of human blood

Human blood samples were collected from three healthy non smoking volunteers, with a mean age of 25 ± 2 years.

5.2.4. Isolation of microsomes

Rat liver microsomal fractions were isolated by standardized protocol in our laboratory (Gandhi et al., 2003 and shety et al., 2002).The protein concentration of the microsomal fraction was determined by Bradford's (1976) method.

5.2.5. Irradiation

Plasmid pBR322 DNA (20-25 μg/ml) in 0.1M sodium phosphate buffer pH 7.0 was exposed to ^{60}Co-gamma rays in a Gamma Cell 220 (AECL, Canada) at a dose rate of 5.9 Gy/min in the presence and absence of polysaccharides. Rat liver microsomal fractions were also exposed to ^{60}Co-gamma rays in a Gamma Cell 220 (AECL, Canada) at a dose rate of 5.9 Gy/min in the presence and absence of polysaccharides. *Ex vivo* irradiation of human peripheral leukocytes in the presence and absence of polysaccharides was done in Junior Theratron unit with a dose rate of approximately 0.4 Gy/min.

5.2.6. Analysis of DNA damage

The plasmid pBR322 DNA (250ng in 10 μl in 0.01M sodium phosphate buffer) was exposed to various doses of γ–rays in the presence and absence of polysaccharides. After irradiation, the DNA was electrophoresed in 1% agarose gel by using 0.8mM Tris borate/2mM EDTA buffer, pH 8.3. The Ethidium-bromide-stained DNA bands were photographed and analyzed by use of the Biorad GelDoc system.

5.2.7. Analysis of membrane damage *in vitro*

The damage to microsomal membrane by gamma irradiation was assessed in terms of lipid peroxidation (Shety et al., 2002). At 350 Gy gamma irradiation formation of n moles of TBARS per mg protein was studied. Microsomal membranes were suspended in 250μl of 10mM potassium phosphate buffer pH 7.4 to have a protein equivalent of 200 to 300μg and exposed to gamma irradiation in the absence and presence of polysaccharides. After radiation exposure, 750μl TBA reagent [0.375% thiobarbituric acid (TBA), 0.25 M HCI, 15% trichloroacetic acid (TCA), and 6mM EDTA was added. The reaction mixture

was incubated at 85°C for 20 min, cooled to ambient temperature, and centrifuged at 12000 x g for 10min at 25°C. Thiobarbituric acid reacting substances (TBARS) in the supernatant was estimated by measuring the absorbance at 535 nm by the use of a Varian DMS 200 UV-visible spectrophotometer. Lipid peroxidation values are expressed as n moles of TBARS per mg of protein.

5.2.8. Comet assay (Measurement of DNA damage by the use of Single Cell Gel Electrophoresis)

Alkaline single-cell gel electrophoresis was performed using the method of Singh with minor modifications (Rajagopalan et al, 2000, and Singh., 2000). In order to estimate DNA damage in blood leukocytes, 10µl heparinised whole blood was mixed with 200µl of low melting point agarose at 37°C and layered on frosted slides pre-coated with 200µl high melting point agarose. After solidification of agarose, the cover slips are removed and the slides were kept in pre-chilled lysing solution containing 2.5M NaCl, 100mM Na_2-EDTA: pH10.0, 10mM Tris HCI, 1% sodium sarcosinate with freshly added 1% Triton X-100 and 1% DMSO at 4°C for 1 hour. The slides were removed from the lysis solution and placed on a horizontal electrophoresis tank filled with the alkaline buffer (300mM NaOH, 1mM Na_2-EDTA, 0.2%DMSO, pH13.0). The slides were equilibrated in the same buffer for 20 minutes. Electrophoresis was carried out for 20 minutes at 25 V using a compact power supply. After electrophoresis, the slides were stained by layering on the top with 50µL of propidium iodide (20µg/ml) and was visualized using a Carl Ziess Axioskop microscope with bright field, phase contrast and epi-fluorescence facility (HBO 50 high pressure mercury lamp), 40X camera adaptor lens. The integral frame grabber used in his system (Cvfb01p) is a PC based card made in the Electronics Division of Bhabha Atomic Research Centre, and it accepts color composite video output of the camera.

The quantitation of the DNA strand breaks of the stored images was done using the imaging software Casp by which the percentage DNA in tail, tail length, tail moment, and olive tail moment can be obtained directly (Chaubey et al., 2000). The tail length of comet indicates the extent of damage because the smaller molecules move faster on the agarose gel. Thus, the longer tails of the comets indicate that the strand breaks are more frequent

and the DNA is fragmented into several small molecules. The tail moment is a commonly accepted unit of DNA damage that normalizes the difference in the size of the nucleus studied (e.g., blood leukocytes) (Rajagopalan et al., 2000 and Chaubey et al., 2000). It is product of the percent DNA in the tail of the comet and tail length. For olive tail moment distance of center of gravity of DNA is considered instead of usual tail length.

5.3. RESULTS

5.3.1. Plasmid DNA damage

The presence of polysaccharides from *G. lucidum* along with plasmid pBR322 DNA during irradiation protected DNA from radiation induced lesions (Figure 5.2) (Table 5.1). During exposure to ionizing radiation, the plasmid DNA suffered strand breaks, which converted the supercoiled (ccc) form of plasmid DNA to open circular form (oc). The radiation induced conversion of supercoiled form to open circular form was considerably reduced in the presence of the polysaccharides. Exposure of plasmid DNA to 25 Gy and 50Gy gamma irradiation resulted in complete damage of the (ccc) form. The polysaccharides of *G.lucidum* at a concentration of 50ug/ml along with the DNA during irradiation, rendered protection to an extend of 93.72% and 65.53% at 25Gy and 50 Gy respectively.

5.3.2. Lipid peroxidation

Exposure of mice liver microsome to gamma radiation resulted in peroxidative damage to membranous lipids. The peroxidation of membrane lipids in mice liver microsomal membrane estimated as n moles of malonaldehyde equivalent thiobarbituric reactive substance (TBARS) increased with increasing doses of γ–rays upto 350Gy. The effect of polysaccharides on radiation induced lipid peroxidation in the microsomes is represented in (Figure 5.3 and 5.4). The polysaccharides of *G.lucidum* prevented 98% of lipid peroxidation.

5.3.3. Comet assay

An exposure to γ-radiation *ex vivo* induces damage to the DNA of human peripheral blood leukocytes, as can be inferred from Figure. 5. An exposure of human peripheral blood leukocytes to 0Gy, 1Gy, 2Gy and 4Gy γ-radiation *ex vivo* resulted in

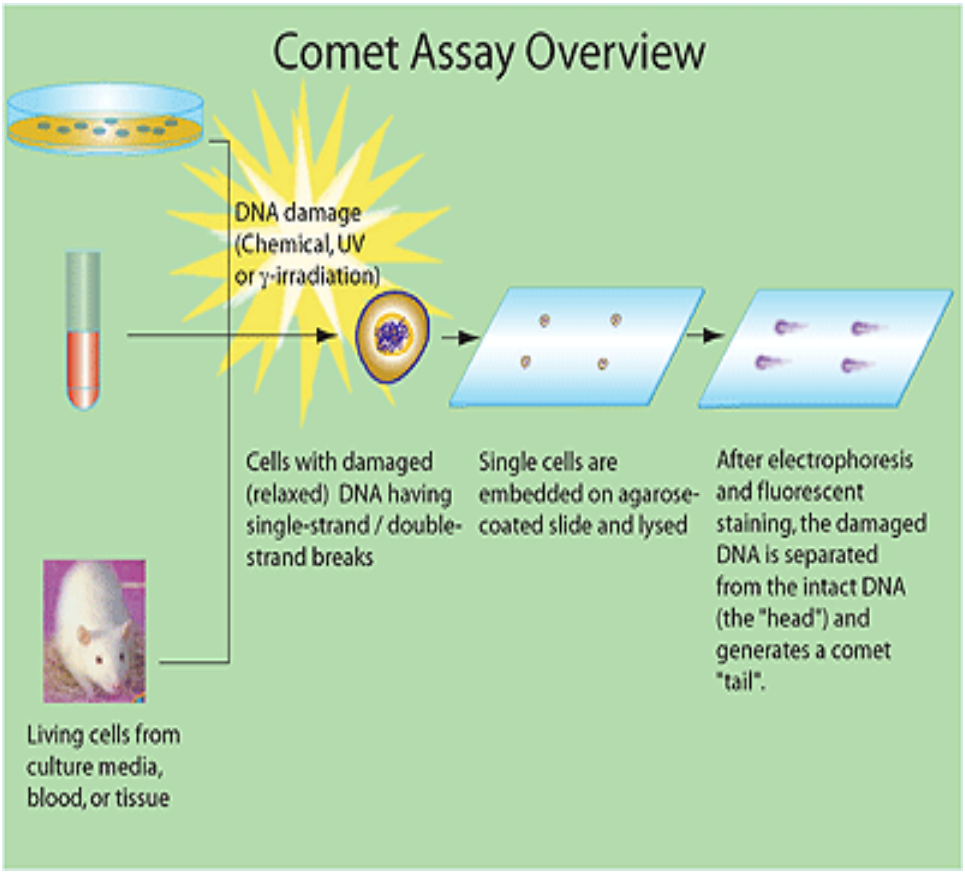
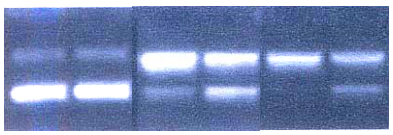


Figure 5.1. Comet assay.



1 2 3 4 5 6

Figure 5.2.

Protection of plasmid pBR 322 against gamma radiation induced strand breaks by polysaccharides isolated from *Ganoderma lucidum*.

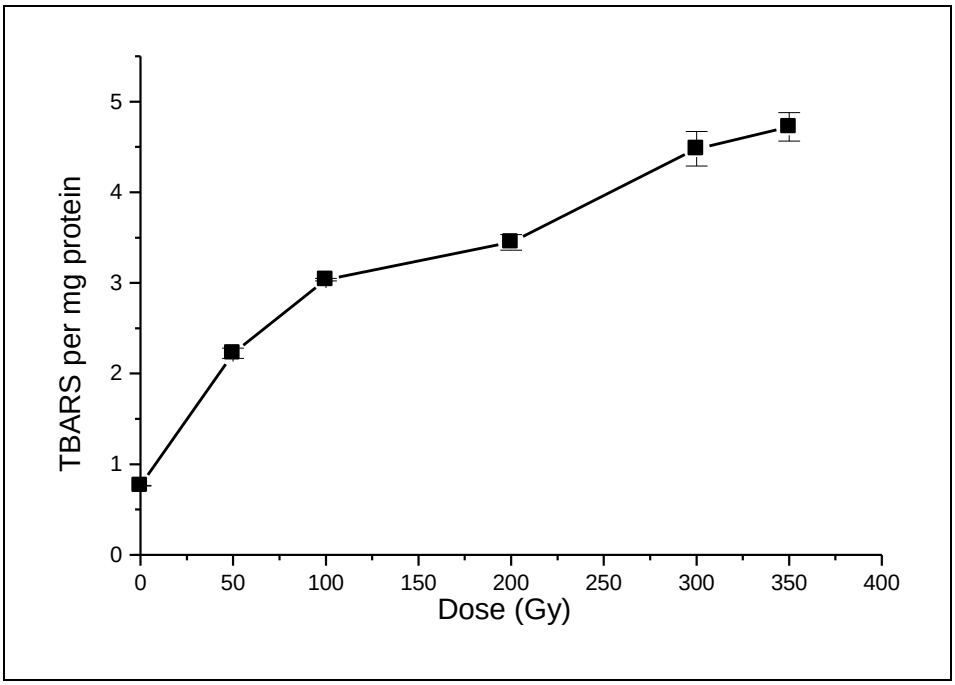


Figure 5.3. Lipid peroxidation status in microsomal membrane with increasing dose of gamma radiation.

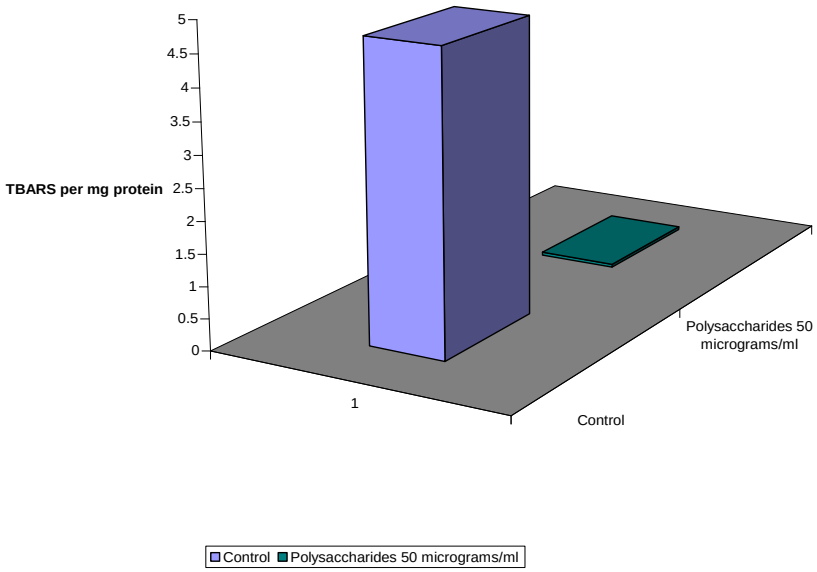


Figure.5.4. Protection of microsomal membrane against gamma-radition induced lipid peroxidation at 350 Gy by Polysaccharides of *Ganoderma lucidum*

increase of comet parameters such as % DNA in tail, tail length, tail moment and olive tail moment and the presence of polysaccharides reduced the comet parameters (Table 5.2).

5.4. DISCUSSION

DNA constitutes the primary vital target of living systems by ionizing radiation. Ionizing radiation damages to cellular DNA are mainly strand breaks, elimination of bases and sugar damage. When plasmid is exposed to γ–radiation, the ccc form of the molecule is converted to the oc form, with a difference in the mobility in the agarose gel because of the induction of strand breaks in the DNA. The damages by ionizing radiation to DNA can cause the loss of viability of the cells exposed to radiation. The alkaline comet assay is an elegant and effective technique to monitor the extent of the DNA damage and its protection. When human leukocytes are exposed to γ–radiation e*x vivo*, the cellular DNA undergoes damage, as reflected in the increase of the comet parameters (% DNA, Tail Length, Tail Moment and Olive Tail Moment). The presence of aqueous extract of G. *lucidum* during irradiation of the cells decreases the comet parameters, indicating its significant role in protection. One of the deleterious consequences of DNA damage from exposure to ionizing radiation is the induction of cancer. Protecting cellular DNA from radiation damage might result in the prevention of the cancers induced by the radiation.

Studies also reveal that treatment of the *G.lucidum* polysaccharides enhanced lymphocyte mitogenic reactivity to concanavalin A and PHA and natural killer cell activity in late – stage cancer patients. The use of ionizing radiation has become an integral part of modern medicine as it is used both for diagnostic and therapeutic purposes. In some cases radiation may be the single best treatment for cancer. The development of radiation protectors is important not only to enhance the effectiveness of cancer treatment, but also for the study of the underlying mechanisms of radiation cytotoxicity. As polysaccharides are free from cytotoxicity, their potential can be fully utilized.

Table .5.1: Protection of plasmid pBR 322 against gamma radiation induced strand breaks by aqueous extract isolated from *Ganoderma lucidum*.

Lane	Treatment	% of SC from remaining
1	0 Gy, without polysaccharides.	100
2	0 Gy, with polysaccharides	100
3	25 Gy, without polysaccharides	74.758
4	25Gy, with polysaccharides	93.723
5	50 Gy, without polysaccharides	61.835
6	50Gy, with polysaccharides	65.534

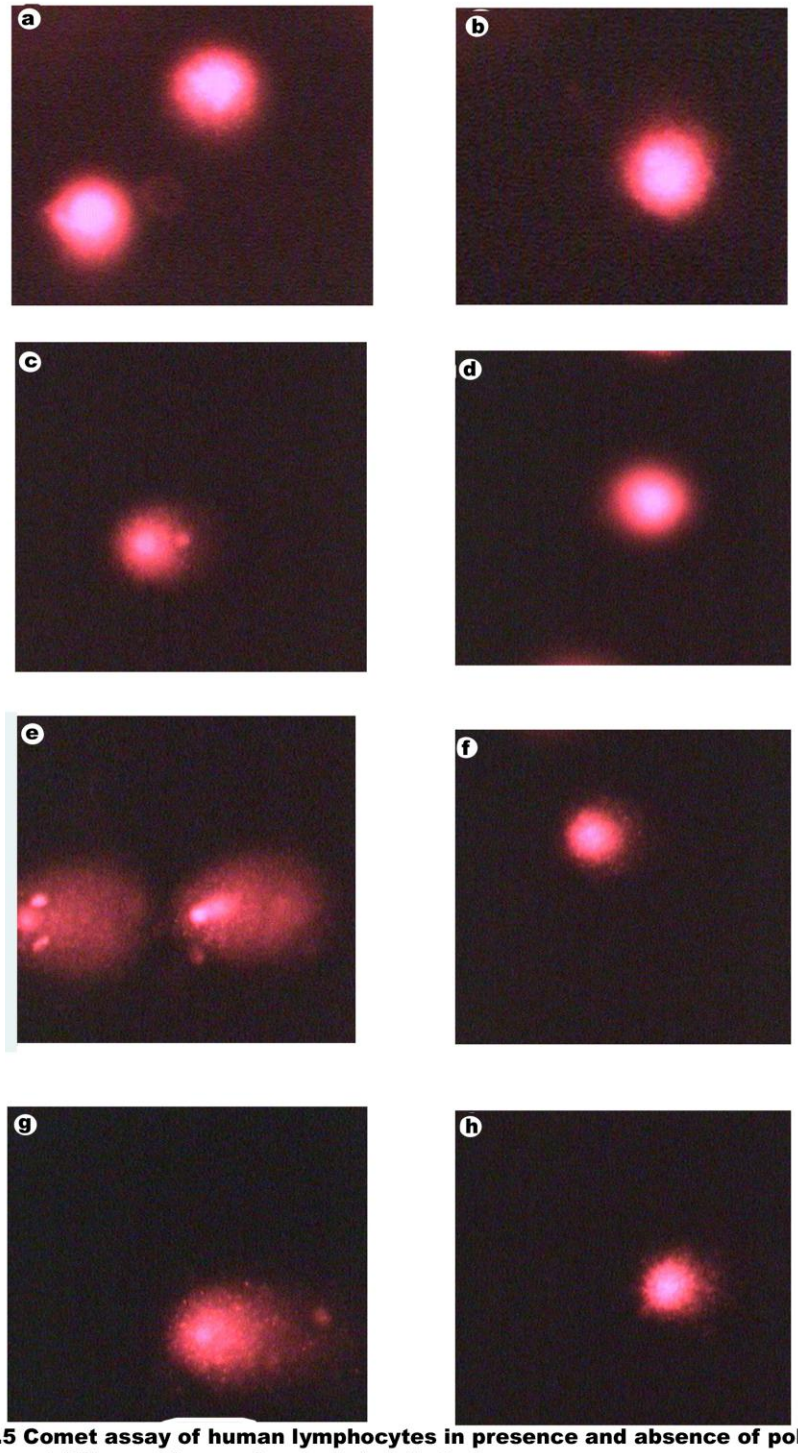


Figure 5.5 Comet assay of human lymphocytes in presence and absence of polysaccharides at different doses of gamma irradiation.

a . Control
b . Control + PS
c . 1 Gy without PS
d . 1 Gy + PS
e . 2 Gy without PS
f . 2 Gy +PS
g . 4 Gy without PS
h . 4 Gy +PS

PS - Polysaccharides [50 ug/ml]

Table. 5.2.

Comet parameters in the presence and absence of polysaccharides at different doses of gamma irradiation.

TREATMENT	0Gy	1Gy	2 Gy	4 Gy
% DNA				
RT alone	1.9718±0.21096	2.7315±0.4736	3.426±0.3952	6.6679±0.3743
RT+PS 50μg/ml	1.0258±0.2008	2.1259±0.1806	2.7436±0.3610	5.2055±0.3728
RT+100 μg/ml	0.8718±0.1707	0.91974±0.2842	2.1560±0.4541	4.3003±0.5664
Tail length				
RT alone	9.7137±0.3456	13.1368±1.182	11.8648±.61625	21.7296±0.6288
RT+PS 50μg/ml	8.2476±0.6154	11.1730±0.7463	9.3694±.7929	19.8316±0.7294
RT + 100 μg/ml	6.7807±0.3258	9.5267±0.4194	7.0196±1.0938	16.5423±1.1118
Tail moment				
RT alone	0.3051±.0.0463	0.9531±0.5304	0.5925±0.0908	2.0148±0.1665
RT+PS 50μg/ml	0.2324±0.0501	0.6531±0.5327	0.4260±0.978	2.011±0.2160
RT + 100 μg/ml	0.2161±0.0311	0.4110±0.1105	0.2575±0.1055	0.6887±0.1243
Olive tail moment				
RT alone	0.7326±0.0637	1.5144±.4783	1.0849±.1197	2.7278±0.14507
RT+PS 50μg/ml	0.3617±0.0691	1.288±0.2876	0.9041±0.1345	2.2012±0.2299
RT+100 μg/ml	0.1327±0.0691	0.7790±0.1202	0.4017±0.1025	1.1450±0.1434

RT –Radiation alone, PS-Polysaccharides.