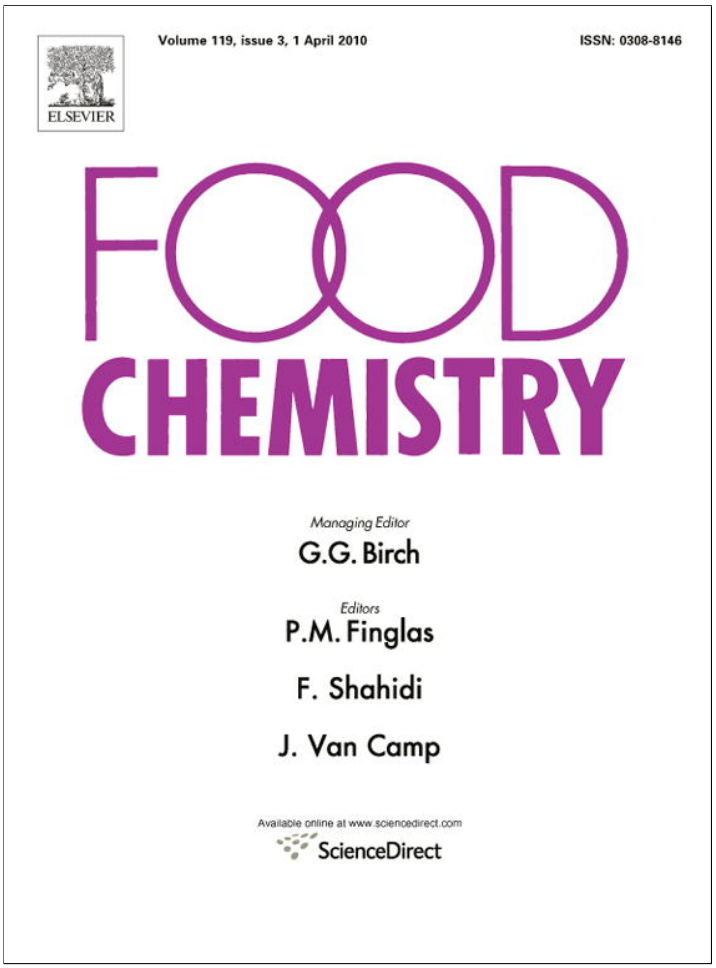

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Enhancement of repair of radiation induced DNA strand breaks in human cells by *Ganoderma* mushroom polysaccharides

Thulasi G. Pillai *, C.K.K. Nair, K.K. Janardhanan

Amala Cancer Research Centre, Thrissur, Kerala, India

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ABSTRACT

The DNA repair ability of a cell is vital to the integrity of its genome and thus to its normal functioning and that of the organism. The repair-enhancing property of polysaccharides isolated from *Ganoderma lucidum* which belongs to the polyporaceae family was determined by comet assay in human peripheral blood leukocytes. Comet parameters were studied at 2 Gy gamma irradiation with 15 min intervals. The comet parameters after 2 Gy exposures to γ-radiation were reduced to nearly normal levels after 120 min of exposure. The polysaccharides from *G. lucidum* enhance the repair process, which is a promising approach for protection from radiation exposure, but a detailed study of the molecular mechanism is needed for further application.

1. Introduction

Ganoderma lucidum (Fr) P. Karst, commonly known as Reishi in Japan and Ling Zhi in China, belongs to the polyporaceae family and is well known for its medicinal properties. Because of its perceived health benefits, Ling Zhi has gained wide popularity as a health food in China, Japan, Korea and Taiwan. *G. lucidum* contains a number of components among which the polysaccharides and triterpenoids have been isolated and identified as the major active components. Crude or partially purified polysaccharides of *G. lucidum* were found to inhibit tumour metastasis in mice (Gao, Zhou, Jiang, Huang, & Dai, 2003). The polysaccharides are among the major source of its pharmacologically active constituents (Gao, Wenbo, Gao, Lan, & Zhou, 2004). The immunomodulating property of this mushroom provides a promising approach for cancer prevention and its administration is found useful alone or in combination with chemotherapy and radiotherapy (Gao et al., 2003). Our earlier reports suggest that the aqueous extract of this mushroom have significant radioprotective activity *ex vivo* (Pillai, Salvi, Maurya, Nair, & Janardhanan, 2006). The major constituent of the aqueous extract of this mushroom is polysaccharides. The present study was thus undertaken to examine the enhancement of DNA repairing capacity of the polysaccharides isolated from this mushroom.

DNA repair refers to a collection of processes by which a cell identifies and corrects damage to the DNA molecules that encode its genome. The genetic information required for the identity and function of eukaryotic cells resides within the DNA and during the lifetime of the cell DNA can be continuously damaged from a variety of factors. Perhaps the most remarkable features of the molecular structures that control the reproduction and development of living organisms are their stability and the fidelity of their replication process.

The stability though remarkable is not perfect. The structures can be altered and exposure to radiation is one way of producing such alterations (Goyal, 1999). The lesions in DNA produced by ionising radiation can be intra or inter strand cross-linking and single and double strand breaks. The cellular responses include arrest in cell cycle, progression at cell cycle checkpoints and the induction of DNA repair. Unrepaired or misrepaired DNA damage can result in genetic or genomic instability, changes in cellular identity and function, cell death, and in multi-cellular organisms, neoplastic transformation. Eukaryotic cells have evolved efficient mechanisms to detect and repair DNA lesions induced within each phase of the cell cycle (Craig & Alt, 2004). Damage to chromosomes is manifested as breaks and fragments which appears as micronuclei in the rapidly proliferating cells (Hofer, Mazur, Pospisil, Weiterova, & Znojil, 2000). In human cells, both normal metabolic activities and environmental factors such as UV light can cause DNA damage, resulting in as many as one million individual molecular lesions per cell per day (Lodish et al., 2004).

Many of these lesions cause structural damage to the DNA molecule and can alter or eliminate the cell's ability to transcribe the gene that the affected DNA encodes. Other lesions induce potentially harmful mutations in the cellular genome, which affect the survival of its daughter cells after it undergoes mitosis. Conse-

* Corresponding author.
E-mail address: amala_research@rediffmail.com (T.G. Pillai).

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quently, the DNA repair process must be constantly active so it can respond rapidly to any damage in the DNA structure (Lodish et al., 2004).

Failure to correct molecular lesions in cells that form gametes can introduce mutations into the genomes of the offspring and thus influence the rate of evolution. The rate of DNA repair is dependent on many factors, including the cell type, the age of the cell, and the extracellular environment. DNA repair rate is an important determinant of cell pathology.

In the present study, the radiation induced repair in cellular DNA of human peripheral blood leukocytes in the presence and absence of polysaccharides was examined by the use of an alkaline comet assay.

2. Materials and methods

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.

2.1. Isolation of polysaccharides

The fruiting bodies of *G. lucidum* were collected from the outskirts of Thrissur district, Kerala. The type specimen was deposited in the herbarium of Centre for Advanced Studies in Botany, University of Madras, Chennai, India with the identification number (HERB. MUBL. 3175). Sporocarps were cut into small pieces, dried at 40–50 °C for 48 h and powdered. Polysaccharides were isolated by the method of Mizuno (2000) with slight modifications (Pillai, Nair, & Janardhanan, 2008). The powdered sporocarps were defatted with petroleum ether and extracted with double distilled water at 80 °C for 8–10 h in several batches.

The extracts were combined, filtered, and concentrated to about one third of the original volume and chilled ethanol about five times the original volume was added and kept at 4 °C for 48 h. The precipitate was collected after centrifugation, redissolved in distilled water and treated with Sevags reagent (Staub, 1969) several times to remove protein and then dialysed against deionised water for 48 h at 4 °C. The precipitate was dissolved in water and reprecipitated with an equal volume of cetyl trimethyl ammonium hydroxide and kept overnight. The supernatant obtained was precipitated with chilled ethanol. After centrifugation, the precipitate obtained was run through a diethyl amino ethyl cellulose column and eluted with deionised water. The precipitate thus obtained was lyophilised to get a light brown powder (polysaccharide). Confirmation of the polysaccharides was done by Anthrone (Yemn & Wills, 1954) and phenol sulphuric acid tests (Dubois, Gilles, & Hamilton, 1956). Further characterisation of the polysaccharides was done by IR and NMR spectrum and MALDI TOF analysis which were recorded at the Sophisticated Analytical Instrument Facility, Indian Institute of Technology, Bombay, Mumbai. Gel filtration chromatography of the polysaccharides was carried out using Sephadex G – 200 in order to determine the molecular weight. The polysaccharides were hydrolysed by trifluoroacetic acid and analysed by paper chromatography in order to identify the sugar components.

2.2. Collection of human blood

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

2.3. Irradiation

Ex vivo irradiation of human peripheral leukocytes was done in a Junior Theratron unit with a dose rate of approximately 0.4 Gy/min.

2.4. Treatment

Lymphocytes were treated with polysaccharides just after irradiation.

2.5. Measurement of DNA damage by the use of single-cell gel electrophoresis (comet assay)

Alkaline single-cell gel electrophoresis was performed using the method of Singh (2000) with minor modifications (Maurya, Salvi, & Nair, 2004; Gandhi et al., 2004). In order to estimate DNA damage in blood leukocytes, 10 μl heparinized whole blood after irradiation with or without polysaccharides treatment was mixed with 200 μl of low melting point agarose at 37 °C and layered on frosted slides pre-coated with 200 μl high melting point agarose. After solidification of agarose, the cover slips are removed and the slides were kept in pre-chilled lysing solution containing 2.5 M NaCl, 100 mM Na_2–EDTA: pH 10.0, 10 mM Tris HCl, 1% sodium sarcosinate with freshly added 1% TritonX-100 and 1% DMSO at 4 °C for 1 h. The slides were removed from the lysis solution and placed on a horizontal electrophoresis tank filled with the alkaline buffer (300 mM NaOH, 1 mM Na_2–EDTA, 0.2% DMSO, pH 13.0). The slides were equilibrated in the same buffer for 20 min.

Electrophoresis was carried out for 20 min at 25 V (180 mA) using a compact power supply. After electrophoresis, the slides were stained by layering on the top with 50 μl of propidium iodide (20 μg/ml) was visualised using a Carl Ziess Axioskop microscope with bright field, phase contrast and epi-fluorescence facility (HBO 50 high pressure mercury lamp), 40× camera adaptor lens. The integral frame grabber used in the system (Cvfb01p) is a PC based card made in the Electronics Division of Bhabha Atomic Research Centre, and it accepts a colour 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 (Rajagopalan, Ranjan, & Nair, 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 normalises the difference in the size of the nucleus studied (e.g., blood leukocytes) (Rajagopalan et al., 2000; Chaubey, Bhilwade, Rajagopalan, & Bannur, 2001). It is a product of the percent DNA in the tail of the comet and tail length. For olive tail moment distance of centre of gravity of DNA is considered instead of usual tail length.

3. Results

The identity of the polysaccharide nature of the isolated compound was confirmed by anthrone and phenol sulphuric acid tests. The IR spectrum showed three absorption bands at 1153.4 cm^{-1}, 1091.6 cm^{-1} and 1029.9 cm^{-1}. In the ^1H NMR spectrum, H^{-1} signals were observed at less than 4.8 ppm (4.762, 4.683, 4.667, 4.658, and 4.402 ppm), which suggest that the component sugars have a beta configuration (Pillai et al., 2008). In the ^{13}C NMR spectrum C-4 and C-5 signals were observed at less than 80 ppm. This

result suggests that the component sugars are in the pyranoid form (Pillai et al., 2008). MALDI TOF analysis suggested that the molecular weight of polysaccharides was between 10 and 20 lakhs Dalton (Pillai et al., 2008). From the gel filtration chromatography, the molecular weight of the polysaccharides were found to be 1.5×10^6 Daltons.

Sugar analysis revealed that component sugars were glucose, mannose and rhamnose (Pillai et al., 2008). The repair process in peripheral blood leukocytes was found to be enhanced by the polysaccharides at 50 μg/ml concentration (Fig. 1).

The % DNA, tail length, tail moment and olive tail moment at 0 Gy was 7.4429 ± 0.4966, 33.5283 ± 1.2234, 2.7445 ± 0.2592, and 3.9444 ± 0.2582, respectively. At 2 Gy 0 min, the comet parameters increased to 36.5343 ± 0.5951, 82.2989 ± 1.1414, 30.6186 ± 0.8378, and 26.1602 ± 0.5566. After 15 min of irradiation the comet parameters were 25.1835 ± 0.6434, 69.0571 ± 1.4956, 17.7173 ± 0.7408, 15.6947 ± 0.5193. The presence of the polysaccharide reduced the comet parameters after 15 min of irradiation to 22.4603 ± 1.0740, 69.2549 ± 2.3163, 16.2134 ± 1.1699 and 15.0996 ± 0.7832.

After 30 min of irradiation the comet parameters were 17.2774 ± 0.7690, 56.0714 ± 1.7367, 10.1087 ± 0.6405, 10.0415 ± 0.5287 and the presence of polysaccharide reduced it to 14.7731 ± 0.8322, 43.8035 ± 1.6428, 7.0179 ± 0.5363 and 7.9954 ± 0.57714, respectively. After 45 min of irradiation the comet parameters were noted as 15.6961 ± 0.8115, 47.9315 ± 1.9092, 8.4409 ± 0.6919, 7.2821 ± 0.5541 and the presence of the polysaccharide reduced it to 11.3869 ± 0.9237, 41.1428 ± 2.3391, 5.7261 ± 0.7103 and 6.1824 ± 0.5673.

After 1 h of irradiation the comet parameters were 15.6263 ± 1.0402, 49.7254 ± 2.4741, 8.7273 ± 0.8750 and 7.5109 ± 0.5966. The presence of polysaccharides further reduced these parameters to 9.7326 ± 0.5818, 38.7058 ± 1.9318, 4.1722 ± 0.3784 and 4.4504 ± 0.3189, respectively. After 2 h of irradiation the comet parameters were reduced to 14.6320 ± 0.8875, 44.3207 ± 1.5216, 6.8811 ± 0.5640 and 6.2424 ± 0.3847, which was further reduced to 6.5463 ± 0.5300, 32.1029 ± 1.3700, 2.4508 ± 0.2925, and 3.6330 ± 0.3214 in the presence of polysaccharides and the comet parameters were comparable to the control level.

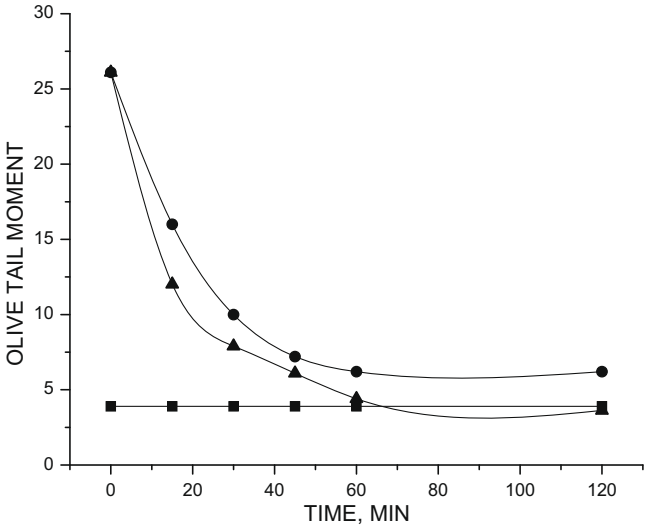

Fig. 1. Effect of *Ganoderma* polysaccharides on repair enhancement of human peripheral blood leukocytes at 2 Gy γ- irradiation. -■- unirradiated, - ▲ - 2 Gy, incubated with *Ganoderma* polysaccharide, -●- 2 Gy, incubated in medium without *Ganoderma* polysaccharides.

4. Discussion

The susceptibility of cells to radiation leading to a loss of cell viability can be attributed to damage to cellular DNA, but this can be greatly influenced by the ability of cells to repair damaged DNA. Radiation induced DNA damage may temporarily shut down DNA replication and thus allow repair to occur. This would involve a well coordinated event involving DNA repair enzymes such as DNA repair polymerase and DNA ligase (Hanawalt, Cooper, Ganesan, & Smith, 1979). The factors that influence the response of living cells to radiation are the DNA repair status, the physiological state of cells, the presence of oxygen and chemicals as well as pre and post irradiation treatments (Pasupathy, Nair, & Kagiya, 2001).

The effect of polysaccharides on DNA repair was ascertained by examining the comet parameters of human peripheral blood leucocytes. It can be seen that during the initial 30 min repair of most of the DNA damages were completed. The repair of DNA takes place at a faster rate in the presence of polysaccharide. Both in the irradiated control and polysaccharide treated group, the comet parameters exhibited a peak at 30 min post irradiation, which could be due to the commencement of an excision repair process (Mendiola-Cruz & Morales-Ramirez, 1999). After 45 min there was not much difference in the comet parameters, in the control group. The comet parameters keep on reducing in the presence of polysaccharides and at 120 min the comet parameters were almost similar to the unirradiated control.

The comet assay for repair analysis was done at a dose of 2 Gy and the compound was added just after the irradiation and incubated at 37 °C and study was done at different time intervals. We know little about the variation in the efficacy of DNA repair process among individuals, even though this is likely to be an important determinant of individual susceptibility to cancer. A suitably robust and sensitive assay to monitor this has not yet been available but the comet assay has the potential to fill this gap. DNA repair can be monitored by incubating cells after treatment with a damaging agent and measuring the damage remaining at intervals. Rejoining of DNA strand breaks by most cell types is known to be a rapid process, with a half time of a few minutes (Frankenberg-Schwage, 1989) and these kinetics are seen in the comet assay too. However freshly isolated lymphocytes appear to repair H_2O_2 induced breaks very slowly. This may be because they suffer an additional DNA breakage as a result of sudden exposure to atmospheric oxygen during the repair incubation (Torbergsen & Collins, 2000). Repair of endonuclease III- or FPG – sensitive sites (i.e., oxidised purine and pyrimidines) by base excision repair, is a slower process, requiring a few hours (Collins & Horvathova, 2001).

DNA repair rate is an important determinant of cell pathology. Experimental animals with genetic deficiencies in DNA repair often show decreased lifespan and increased cancer incidence. For example, mice deficient in the dominant NHEJ pathway and in telomere maintenance mechanisms get lymphoma and infections more often and consequently have shorter lifespans than wild-type mice (Dolle et al., 2006). Similarly, mice deficient in a key repair and transcription protein that unwinds DNA helices have premature onset of aging-related diseases and consequent shortening of lifespan (Espejel et al., 2004). However, not every DNA repair deficiency creates exactly the predicted effects; mice deficient in the NER pathway exhibited shortened lifespan without correspondingly higher rates of mutation (Kobayashi et al., 2004). *Ganoderma* polysaccharides have been reported to restore the TNF-α production inhibited by cyclophosphamide to normal levels and also was effective in repairing the damage of subset T cells in spleens of γ–irradiated mice (Gao, Zhou, Chen, Dai, & Ye, 2002). If the rate

of DNA damage exceeds the capacity of the cell to repair it, the accumulation of errors can overwhelm the cell and result in early senescence, apoptosis or cancer. Inherited diseases associated with faulty DNA repair functioning result in premature aging, increased sensitivity to carcinogens, and correspondingly increased cancer risk. On the other hand, organisms with enhanced DNA repair systems, such as *Deinococcus radiodurans*, the most radiation-resistant known organism, exhibit remarkable resistance to the double strand break-inducing effects of radioactivity, likely due to enhanced efficiency of DNA repair and especially NHEJ (Kobayashi et al., 2004). It is noteworthy that agents which can enhance the repair process can help to minimise the deficiency in DNA repair. The comet assay promises to provide answers to important questions concerning for example, background levels of DNA damage in normal cells, the variation in DNA repair capacity within human populations, and the regulation of DNA repair at the molecular level within the nucleus (Gantt, 1987). The successful functioning of a cell and the faithful transmission of the genetic information contained into its progeny depend on the maintenance of the structural integrity of each molecule of DNA.

Changes in the sequence of nucleotides or alterations in the structure of bases or sugars, which make up the double helix of DNA, can interfere with the replication or transcription of the cellular genome. Damage to molecules of DNA is the primary cause of radiobiological effects (cell death, loss of reproductive capacity, mutation). Mechanisms of repair of damaged DNA molecules therefore play an essential role. The current investigations indicate that the polysaccharides from *G. lucidum* enhance the repair process, which is a promising approach to protect deleterious effect of radiation exposure, but a detailed study in the molecular mechanism is needed for further application. Further studies will shed light in understanding the mechanism of the repair process by the polysaccharides. As a traditional medicine this mushroom is used for several therapeutic uses and is freely available all over world. Our experimental findings demonstrate that the polysaccharide from *G. lucidum* possess a profound DNA repair-enhancing property.

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