Enabling Mannan oligosaccharide pretreatment restores mitochondrial dynamics and ETC function in γ-irradiated cells

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ABSTRACT
Mitochondrial dynamics is important for mitochondrial inheritance and for the maintenance of mitochondrial functions and thereby ensuring cell survival. Mitofusin 1 (Mfn1), Mitofusin 2 (Mfn2) and Optic Atrophy 1 (OPA1) are important proteins are required in fusion process of mitochondria. Whereas proteins like, Dynamin related protein 1 (Drp1) and mitochondrial fission 1 (Fis1) play important role in mitochondrial fission in cells. Ionizing radiation (IR) exposure to cells has been known to disrupt fission and fusion homeostasis directly resulting in mitochondrial fragmentation and reduction in respiratory chain functions. Therefore, protection of mitochondria from IR, is crucial in order to minimize damage to the cells. This damage to the cells may be minimized by restoration of mitochondrial structure and functions; and therefore the effect of radiomodifying agents that restores mitochondrial functioning is worth investigating. In the present study we have identified that mannan oligosaccharide (MOS) pretreatment to cells followed by IR exposure reduces alterations in mitochondrial dynamics with respect to irradiated cell. We generated fluorescent reporter cells, stably expressing Su9 and Mfn2 proteins and treated them with MOS and/or exposed to 3Gy radiation dose, to study alterations in mean fluorescence 24 h post irradiation by flow cytometry. We expended TLR expressing normal cells (NKE cells) to study effect of MOS pretreatment in alterations in expression of proteins responsible for both mitochondrial dynamics and ETC functions with respect to irradiated cell 16 h and 24 h post irradiation. We have shown that mannan treatment to cells prior to IR exposure restored the levels of proteins responsible for both mitochondrial dynamics and ETC functions to the levels that of un-irradiated control cells.

KEYWORDS: Mannan oligosaccharide, mitochondria, radiation protection, ROS, Mitochondrial dynamics

INTRODUCTION

Ever since the discovery of radioactivity, it has been increasingly utilized in multiple ways for remuneration of world. The exploitation includes but are not limited to energy generation, medical imaging, diagnosis, clinical radiotherapy, sterilization of medical instruments, food, military, agriculture and industrial utilities. On the other hand, in case of accidental exposure or act of terrorism it can cause extensive damage that is practically irreversible and also reflects in impending generation. High dose radiation exposure in potential disaster scenarios causes acute radiation effects resulting near-term mortality as well as long-term adverse health effects.

Exposure of cells to gamma radiation adversely affects cells, nucleus and mitochondria (alterations in ETC, MMP, energy production etc.) and significantly increases oxidative stress resulting in oxidative modification of various biomolecules. The increased leakage of electrons from ETC further increases ROS generation, which may results in bio-energetic catastrophe and thereby cell death (Azzam et al., 2012; Detmer and Chan, 2007). Mitochondrial DNA is extensively damaged due to its close proximity to the electron transport chain and inefficient DNA repair systems (Aouacheria et al., 2017). Therefore, protection of mitochondria form IR induced injury is crucial for cell survival (Goel et al., 2005; Gupta et al., 2008a) and identification of agent(s), which can reduce IR induced oxidative stress in mitochondria and stabilize the cells is imperative.

Mitochondria are known to regulates various vital processes viz. OXPHOS, apoptosis, cell signaling, heme synthesis etc. Moreover, mitochondria possess high amount of unsaturated lipids, inorganic metals as a part of electron transport chain and also known as endogenous source of reactive oxygen species (ROS). These structural components and physiological processes (OXPHOS) makes them vulnerable to radiation (McBride et al., 2006). Among the different radiation-induced changes, mitochondrial elongation, branching, dilation of cristae, development of giant form, vacuolization and oxidation of mitochondrial biomolecules are the most common manifestations. Mitochondria constantly undergo process like fission, fusion and are most dynamic cell organelle known (Quintana-Cabrera et al., 2017; Westermann, 2010). On exposure to IR, the finely regulated homeostasis between fission and fusion (under physiological conditions) is disrupted resulting in mitochondrial fragmentation, which contributes to mitochondrial outer membrane permeabilization (MOMP) and cristae remodeling resulting in the release of apoptogenic factors and cell death (Detmer and Chan, 2007; Suen et al., 2008). Mitochondrial dynamics is an important constituent of cellular quality control (Cipolat et al., 2004; Meeusen et al., 2006; Westermann, 2010). A complex interaction between ROS and mitochondrial dynamics has been reported (Hung et al., 2017; Zhan et al., 2013). Dynamic change in mitochondrial morphology (mitochondrial fragmentation) has been shown as a significant contributing factor to ROS overproduction in high glucose conditions.

For the past several decades’ intensive efforts have been made towards reducing biological effects of Ionizing radiation (IR) and development of radiation countermeasure agents. Development of suitable radiation countermeasure agents for management of biological effects of radiation in human beings for protection, mitigation and therapeutics has become an inevitable requirement. Moreover, a safe radiation countermeasure agent will be useful for patients undergoing routine radiotherapy against cancer and are at increasing risk of acute radiation syndrome (ARS) associated side effects. Several approaches are currently being enforced to discover a countermeasure agent which is effective, and concomitantly safe. Under certain conditions, a possibility has been highlighted that, mitochondrial fragmentation and ROS may exacerbate each other to form a vicious cycle.
ultimately resulting in cell injury and death (Zhan et al., 2013). Therefore, it is of utmost importance to maintain mtDNA and mitochondrial functions, under all conditions, especially under oxidative stress. Once the radiation damage is inflicted, repair and restoration of normal metabolic activities is crucial and hold great significance. These processes are energy intensive and therefore the effect of radiomodifying agents that restores mitochondrial functioning is worth investigating. Recently, several agonists of toll like receptors have been shown to possess protective efficacy against lethal effects of IR and are currently under different stages of drug development (Burdelya et al., 2008; Singh and Pollard, 2015). Toll like receptors (TLRs) are components of innate immune signaling which helps in recognition of pathogens and danger signals (Singh et al., 2012). Activation of TLRs in turn activate intracellular signaling pathways resulting in activation of transcription factors including NFκB, MAPK etc. (Singh et al., 2015). Acute radiation syndrome involves substantial apoptosis in radiosensitive tissues such as the hematopoietic system and gastrointestinal tract. Pharmacological activation of NF-κB might be a promising strategy for both radioprotection and radio-mitigation. MOS is a TLR agonist and is used as nutritional supplement in farm animals, for its gastrointestinal and immunological responses (Hutsko et al., 2016; Momeni-Moghaddam et al., 2015; Razeghi Mansour et al., 2012; Zhang et al., 2012). Mannan improves overall health and growth status, enhances performance, resurgence of the systemic and local immune system in animals and stimulate epithelial barrier structure and functionality of intestinal mucosa (Bozkurt et al., 2012; Razeghi Mansour et al., 2012; Santos et al., 2013; Yamabhai et al., 2016; Zhang et al., 2012).

We hypothesised that, activation of NF-κB by TLR agonist might protect normal tissues against IR induced damage to hematopoietic system and gastrointestinal tract and also reduce the incidence of secondary cancers (immunostimulatory effect of NF-κB). We have identified Mannan oligosaccharide (MOS) as a potential radiation countermeasure agent and we have shown that, MOS pretreatment mediates alteration in mitochondrial physiology in immortalized normal cells exposed to γ-radiation. It reduces biological effects of γ-radiation and enhances the cell survival (data communicated elsewhere). In the present study, we have investigated MOS mediated alterations in mitochondrial dynamics and function in vitro after exposure of cells to γ-radiation and shown that MOS pretreatment restores mitochondrial dynamics and ETC function comparable to sham irradiated cells.

MATERIALS AND METHODS

Chemicals

All chemicals used in study were of analytical grade and were either procured from Indian manufacturer (SRL India, HiMedia chemicals) or obtained from Sigma Aldrich (St Louis, MO), Thermo Fisher Scientific Inc (USA) etc. Roswell Park Memorial Institute (RPMI-1640) medium, penicillin, streptomycin, G418, trypsin, bovine serum albumin (BSA), protease and phosphatase inhibitor cocktails, REDTaq ReadyMix PCR reaction mix, antibodies (anti-complex I, anti-complex V, anti-Mouse-HRP, anti-Rabbit-HRP, anti β-actin-HRP) etc. were obtained from Sigma Aldrich (St Louis, MO), antibodies (anti-Mfn1, anti-Mfn2, anti-Fis 1, anti-Drp1, anti-OPA1, anti- complex II), sulphorhodamine-B (SRB), fetal bovine serum (FBS) were procured from Thermo Fisher Scientific Inc (USA). Su9-EGFP (Addgene plasmid # 23214), Mfn2-YFP (Addgene plasmid # 28010) was gift from David Chan and Richard Youle.

Cell culture

Human normal kidney epithelial (NKE) cells were received as kind support for research from Dr Andrei V. Gudkov, Roswell Park Cancer Institute, Buffalo, USA. 293/hTLR4 cells (HEK 293...
cells stably transfected with the human TLR4 gene were obtained from InvivoGen (San Diego, USA). NKE cells and 293/hTLR4 cells were maintained in RPMI-1640 and High glucose DMEM medium respectively, supplemented with 10% (v/v) heat-inactivated FBS, 1% non essential amino acids, 100 units/ml of penicillin and 100 µg/ml of streptomycin, pH 7.4 to maintain optimal growth of cells at 37°C in humidified atmosphere of 5% CO₂. NKE cells were specifically maintained as indicated with additional supplementation of 1% β-mercaptoethanol. All experiments were performed on exponentially growing cells and were subcultured twice a week.

**Preparation of Mannan solution**

Mannan was dissolved (20mg/ml stock) in sterile PBS (1X) under aseptic conditions. Treatments of cells with mannan were performed as per indicated concentration(s), and time.

**Gamma Irradiation of cells**

Irradiation was done using Bhabhatron- II Telecobalt unit (Bhabha Atomic Research Center, Mumbai, India) at dose rate 2.25-2.55 Gy/min. Radiation dosimetry of unit was carried out by certified radiation safety officer in the institute and Baldwin Farmer secondary dosimeter and Frickie’s chemical dosimeter methodologies were used (Gupta et al., 2003). Briefly, logarithmically growing cells were treated with mannan and/or exposed to γ-radiation. Cells were washed with ice-cold PBS, and thereafter lysed in RIPA buffer containing protease and phosphatase inhibitors [PMSF, sodium orthovanadate, protease and phosphatase inhibitor cocktail (Sigma Aldrich, USA), sodium fluoride]. Samples were vortexed at 4°C for 20 min followed by centrifugation (12000g, 4°C for 20min) and collection of supernatant for western blotting.

**Western blotting**

Expression of various mitochondrial dynamics related proteins (Mfn1, Mfn2, Fis 1, Drp1, OPA1) and ETC complex I, complex II, complex V were measured by immune–blotting technique 24 h post treatment as described by Gupta et al with minor modifications (Gupta et al., 2008b). Briefly, logarithmically growing NKE cells were treated with mannan and/ or exposed to γ-radiation. Cells were washed with ice-cold PBS, and thereafter lysed in RIPA buffer containing protease and phosphatase inhibitors [PMSF, sodium orthovanadate, protease and phosphatase inhibitor cocktail (Sigma Aldrich, USA), sodium fluoride]. Samples were vortexed at 4°C for 20 min followed by centrifugation (12000g, 4°C for 20min) and collection of supernatant for western blotting. Protein estimation was performed using BCA reagent (Sigma Aldrich, USA) and samples were loaded in equal quantity (40µg protein/ well) for separation on 8-20% gradient SDS-polyacrylamide gel. After gel electrophoresis proteins were transferred into PVDF membrane (Amersham, GE healthcare, Germany), using Tris-glycine transfer buffer containing 10% Methanol. After transfer, membranes were blocked using 5% skimmed milk (in TBST) for 1 h and thereafter the membranes were incubated overnight (at 4°C) with anti-Mfn1

**Treatment protocol**

NKE cells were divided into seven groups: Control (sham treatment), mannan alone (16 h), radiation alone (16 h), and combination (mannan + radiation; 16 h), mannan alone (24 h), radiation alone (24 h), and combination (mannan + radiation; 24 h). For all experiments 20 µg/ml concentration of mannan was used, 30 min prior to IR exposure, unless otherwise stated.

Reporter cells, stably expressing Su9-EGFP and Mfn2-YFP were treated with mannan and/or exposed to 3Gy radiation dose, and alteration in mean fluorescence was measured 24 h post irradiation by flow cytometry.
(1:1000), anti-Mfn2 (1:1000), anti-Fis 1 (1:500), anti-Drp1 (1:2000), anti-OPA1 (1:1000), anti-complex I (1:2000), anti-complex II (1:2500), anti-complex V (1:2000) and anti β-actin (1:5000) antibody in 2% skimmed milk (TBST). The membranes were washed thrice using TBST to remove unbound primary antibody and incubated with appropriate secondary antibodies conjugated with horseradish peroxidase (HRP) for 3hr. The expression of proteins was measured using super signal West Pico chemiluminescent substrate (Thermo Scientific, USA). Band intensity was determined using GS-900™ Calibrated Densitometer software. Results are expressed as fold changes from mean of densitometer data with respect to control ± SD of three independent experiments.

Data analyses and statistical evaluations

All experiments were performed at least three times independently. For the graphical representation of the data, y-axis error bars represents mean ±SD. Statistical analysis of data was done using one-way analysis of variance (ANOVA) followed post hoc analysis using Tukey’s multiple comparisons test (Prism 6.0, GraphPad Software, San Diego, CA, USA) unless otherwise stated. Differences were designated significant at values *p < 0.05 and were labeled with asterisks.

RESULTS

Pre-irradiation treatment of 293/hTLR4 cells (expressing Mfn2-YFP and Su9-EGFP) with mannan restores mean fluorescence

Fluorescent reporter cells, stably expressing Su9-EGFP and Mfn2-YFP genes are depicted in figure 1. Mfn2 is an essential protein required for continuous mitochondrial fusion process inside cells. Decrease in mean fluorescence (0.64±0.063) associated with Mfn2 protein after IR exposure in comparison to sham irradiated control (1±0.013) cells implies decrease in mitochondrial fusion and shift of equilibrium towards mitochondrial fission (Fig. 2a). Mannan treatment followed by IR exposure exhibits restitution of mean fluorescence (0.84±0.05) suggesting restoration of mitochondrial fusion homeostasis in these cells. Likewise, Su9 associated fluorescence shows expression of Su9 protein of complex V (ETC). IR exposure to cells shows increase in mean fluorescence (1.35±0.065) 24 h post irradiation with respect to control cells (1±0.001) suggesting increase in expression of Su9 protein (Fig. 2b). Mannan pretreatment in these cells restores Su9 associated fluorescence (1.06±0.074) comparable to control cells.

Fig 1: Fluorescent Reporter cells Representative images of cells expressing (a) Mfn2-YFP and (b) Su9-EGFP as described under materials and methods. Image acquisition was done using ZOE™ Fluorescent Cell Imager (BioRad, USA). Scale bar equals 100 µm
Fig 2: Fluorescent protein Reporter assay Effects of pre-irradiation treatment of cells with mannan (a) 293/hTLR4 cells expressing Mfn2-YFP (b) 293/hTLR4 cells expressing Su9-EGFP were treated with mannan and/or IR exposure. Cells were analyzed for change in mean fluorescence intensity by flow cytometry 24 h post treatment using Ex λ 488 and Em λ 530. Data was analyzed using FlowJo V10.1 software. Results are expressed as mean fluorescence (fold changes) with respect to control ± SD of three independent experiments. Differences were designated significant at values ** p < 0.001 and *** p < 0.0001 and were labeled as $ compared with the sham irradiated control group, * compared with the radiation only group.

Fig 3: Expression of various mitochondrial proteins associated with ETC function and mitochondrial fusion/fission homeostasis in NKE cells: Cells were treated, harvested and samples were resolved on 8-20% gradient SDS PAGE followed by transfer on to PVDF membrane and blotted using specific antibodies as described in materials and method section. β-actin was used as a loading control. Results are representative of three independent experiments.

Mannan treatment prior to IR exposure restores mitochondrial fusion fission homeostasis and expression of ETC complexes

Expression of various mitochondrial proteins associated with ETC function and mitochondrial fusion-fission homeostasis in NKE cells following treatment with MOS and/or IR showed alterations (Fig. 3). Results show increase in fold expression of Drp1, mitochondrial fission related protein (1.767±0.031; Fig. 5) 16 h post irradiation with respect to control cells (1±0.001) and corresponding decrease in Mfn1, mitochondrial fusion related protein (0.613±0.032 and 0.447±0.040; Fig. 4) both at 16 h and 24 h post irradiation in comparison to their respective unirradiated cells.
Fig 4: Expression of mitochondrial fusion related in NKE cells: Cells were treated, harvested and samples were resolved on 8-20% gradient SDS PAGE followed by transfer on to PVDF membrane and blotted using specific antibodies (a) Mfn1, (b) Mfn2, and (c) OPA1 as described in materials and method section. β-actin was used as a loading control. Band intensity was determined using GS-900™ Calibrated Densitometer software. Results are expressed as fold changes from mean of densitometer data with respect to control ± SD of three independent experiments. Differences were designated significant at values ** p < 0.001 and *** p < 0.0001 and were labeled as $ compared with the sham irradiated control group, * compared with the radiation only group.

Fig 5: Expression of mitochondrial fission related in NKE cells: Cells were treated, harvested and samples were resolved on 8-20% gradient SDS PAGE followed by transfer on to PVDF membrane and blotted using specific antibodies (a) Fis1 and (b) Drp 1, as described in materials and method section. β-actin was used as a loading control. Results are expressed as fold changes from mean of densitometer data with respect to control ± SD of three independent experiments. Differences were designated significant at values ** p < 0.001 and *** p < 0.0001 and were labeled as $ compared with the sham irradiated control group, * compared with the radiation only group.

IR has been shown to trigger mitochondrial fission by the Drp1 and its inhibition has been shown to attenuate radiation-induced mitotic catastrophe (Kobashigawa et al., 2011; Yamamori et al., 2015). Mannan treatment decreases fold expression of Fis1 protein (0.427±0.039) and increases fold expression of Mfn2 protein (1.260±0.066 and 1.320±0.075) both at 16 h and 24 h post treatment in comparison to un-irradiated control cells. Mannan treatment prior to irradiation restored normal Drp1 levels (countering radiation
induced increase in Drp expression) 16 h post irradiation (Fig. 5). Drp1 and Fis1 proteins play important role in mitochondrial fission in cells (Meeusen et al., 2006). The dynamics is coordinated and finely regulated by a fusion and fission machinery proteins on both the outer and inner membranes of mitochondria (Zhang et al., 2013). Results also show increase in fold expression of complex I, II and V in irradiated cells 16 h post irradiation in comparison to control cells and mannan pretreatment restored the expression of proteins comparable to their respective controls (Fig. 6).

**DISCUSSION**

Ionizing radiation is known to cause considerable damage to cellular biomolecules, cells, tissues and organs (Azzam et al., 2012; Singh et al., 2015). Mitochondria, being an endogenous source of ROS itself, further increase the leakage of electrons after oxidative damage to mitochondrial ETC components and lipids, which leads to depolarization of mitochondrial membrane potential (MMP), opening of mitochondrial permeability transition (MPT) pores, bioenergetics catastrophe and thereby release of cytochrome C into the cytosol which finally signals for cell death or apoptosis (Gupta et al., 2008a; Leach et al., 2001).

During research investigations, we developed reporter cells for high throughput screening of variety of molecules that may possess radio-modulatory potential Fig. 1a-b). We utilized TLR expressing cells and transfected them with suitable vectors including Su9-EGFP (Addgene) and Mfn2-EYFP (Addgene). Several potential molecules (preferably TLR agonists) were tested for their ability to modify radiation response of mitochondria, in terms of Su9 expression (an indirect measure of ETC function) and mitochondrial dynamics (Mfn2 expression). MOS, an important TLR agonist was identified as activator of NF-κB, and modulator of mitochondrial function (data communicated...
MOS pretreatment (identified concentration) was also found to restore normal mitochondrial Su9 expression and dynamics post IR exposure (Fig. 2a-b). Mitochondrial ETC generates potential gradient across the inner mitochondrial membrane along with ROS as a by-product of the vital oxidative phosphorylation process (Hamanaka and Chandel, 2010). We have shown that irradiation of MOS pretreated cells in state of reduced state of ΔΨm and ROS enhances cell survival (data communicated elsewhere) (Sanguri et al., 2016). IR induced ROS can cause substantial damage to ETC components causing alterations in the structure and organization leading to amplification of ROS (Leach et al., 2001). Compromised mitochondrial ETC function may lead to bioenergetic catastrophe or cell death (Orrenius et al., 2007; Paradies et al., 2009). Mitochondrial dynamics is vital for the structural integrity and function of normal mitochondrial function (Sanguri and Gupta, 2017). Mitochondria compensate for the defect in genetic material or cellular machinery by fusion process and maintain energy output even in stress condition (Youle and van der Bliek, 2012). Mfn1, Mfn2 and OPA1 are important proteins required in fusion process of mitochondria (Cipolat et al., 2004; Meeusen et al., 2006). Mitochondrial fission regulates healthy mitochondrial morphology and facilitates mitochondrial transfer to daughter cells in events of cells division. It separates the damaged mitochondria and marks it for removal by mitophagy (Babbar and Sheikh, 2013). Drp1 and Fis1 proteins play important role in mitochondrial fission in cells (Meeusen et al., 2006). The dynamics is coordinated and finely regulated by a fusion and fission machinery proteins on both the outer and inner membranes of mitochondria. In stress condition, when the damage to mitochondria cannot be compensated by fusion alone, mitochondrial fission process takes over to eliminate the damaged mitochondria. NKE cells demonstrated increased expression of Drp1 and Fis1 16 h post irradiation. In addition there was decrease in expression of Mfn1 and Mfn2 proteins (Fig. 3). Ionizing radiation exposure has been shown to directly alter mitochondrial morphology, and cause mitochondrial dysfunction (Kobashigawa et al., 2011). It has been demonstrated that ionizing radiation causes mitochondrial dysfunction through interruption of mitochondrial dynamics (Kobashigawa et al., 2011; Wu et al., 2011). In the present study, MOS treatment to cells prior to IR exposure restored altered fusion in cells as well as fusion related proteins to the levels of un-irradiated control cells (Fig. 2a-b). MOS treatment alone to NKE cells was found to significantly decrease expression of proteins responsible for mitochondrial fission and increase expression of proteins responsible for mitochondrial fusion (Fig. 3). Alteration in expression of proteins involved in maintenance of mitochondrial dynamics and ETC function should impact physiological and metabolic state of cell. Furthermore, in our earlier studies we have shown that, MOS pretreatment in NKE cells results in abrupt decrease in mitochondrial membrane potential and endogenous ROS levels. However, these alterations are transient and the parameters returns to normal levels after 24 h (data communicated elsewhere). Disruption in fission or fusion homeostasis directly impacts mitochondrial function resulting in excessive production of free radicals, altered mitochondrial enzymatic activities, impaired calcium homeostasis, low ATP production, and overall reduced energy metabolism in mammalian cells (Reddy, 2014). Down-regulation of either Fis1 or Drp1 has been shown to inhibit mitochondrial fission and reduces apoptosis and demonstrated that multiple components of the mitochondrial fission and fusion machinery can positively and negatively regulate apoptosis (Lee et al., 2004). During stress, the homeostasis between fusion and fission is disrupted contributing to mitochondrial damage and consequent cell injury (Detmer and Chandel, 2007; Suen et al., 2008). A complex interaction between ROS and mitochondrial dynamics has been reported and a possibility has been
emphasized that under certain conditions, mitochondrial fragmentation and ROS may exacerbate each other to form a vicious cycle ultimately resulting in cell injury and death (Zhan et al., 2013). MOS treatment in NKE cells increases expression of fusion related proteins and decreases expression of fission related proteins in cells. MOS treatment prior to IR exposure counteracts IR induced increase in expression of fission related proteins and restores the homeostasis between mitochondrial fission and fusion process to ensure cell survival.

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Conflict of interest statement
The author has declared that no competing or conflict of interests exist. The funders had no role in study design, writing of the manuscript and decision to publish.

Authors’ contributions
SS and DG designed and performed the experiments. SS analyzed and interpreted the data. Both SS and DG contributed to manuscript preparation. DG conceived and supervised the work done.

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