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Nuclear magnetic resonance spectroscopy-based metabonomic investigation of biochemical effects in serum of γ-irradiated mice

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Abstract

Purpose: Radiation exposure induces change in many biological compounds. It is important to assess the physiological and biochemical response to an absorbed dose of ionising radiation due to intentional or accidental event and to predict medical consequences for medical management. In the present study, nuclear magnetic resonance (NMR) spectroscopy-based metabolic profiling was used in mice serum for identification of radiation-induced changes at metabolite level.

Materials and methods: Mice were irradiated with 3, 5 and 8 Gray of γ-radiation dose and serum samples collected at day 1, 3 and 5 post irradiation were analysed by proton nuclear magnetic resonance (1H NMR) spectroscopy. 1H NMR spectra of serum were analysed by pattern recognition using principal component analysis.

Results: Irradiated mice serum showed distinct metabonomic phenotypes and revealed dose- and time-dependent clustering of irradiated groups. 1H NMR spectral analysis exhibited increased lactate, amino acids, choline and lipid signals as well as decreased glucose signals. These findings indicate radiation-induced disturbed energy, lipid and protein metabolism.

Conclusions: The information obtained from this study reflects multiple physiological dysfunctions. The study promises the application of NMR-based metabonomics in the field of radiobiology, for development of metabolic-based markers for screening of risk populations and medical management in these cases.

Keywords: radiation, NMR spectroscopy, serum, metabonomics

Abbreviations: NMR, nuclear magnetic resonance; FID, free induction decay; PCA, principal components analysis; PR, pattern recognition; BCA, branched chain amino acid; PC, principal component; TSP, 3-(Trimethylsilyl) propionic-2, 2, 3, 3-d4 acid sodium salt; Gy, gray; LDL, low density lipoprotein; VLDL, very low density lipoprotein; ROS, reactive oxygen species.

Introduction

The study of normal tissue response and injury after exposure to moderate dose of ionising radiation is of great importance to patients with cancer, and people potentially subjected to military, nuclear power industry and radiological terrorism (Coleman et al. 2003). The resulting radiation injuries would be due to a series of molecular, cellular, tissue and whole organal processes. During the last decade, research in the field of radiation sciences has been more focused on the development of a multiparametric approach for studying gene, protein and other biochemical expressions after radiation exposure (Bertho et al. 2008). To develop a multiparametric approach, inputs from all relevant fields using high throughput technology are required at cellular, biochemical and molecular levels since damage from ionising radiation occurs at cellular and systemic levels.

Since the last decade, the metabonomics approach is becoming increasingly popular as the source of biomedical data. Mass spectrometry and proton nuclear magnetic resonance (1H NMR) spectroscopy are the two key experimental methods applied in this area of global biochemistry. In particular, 1H NMR spectroscopy has the advantage of efficiently obtaining information on large number of metabolites in...
biofluids such as serum, in vitro, ex vivo and in vivo in various tissues that could serve as snapshots of physiological states (Ala-Korpela 2008). Proton nuclear magnetic resonance (NMR) spectrum enables the simultaneous identification and monitoring of a wide range of low molecular weight endogenous metabolites, thus providing a biochemical fingerprint of an organism (Nicholson and Wilson 1989). It is now well established that this profile gets altered in disease or toxic process and a metabonomic approach can be successfully applied to various toxicological, disease evaluation and risk assessment studies (Holmes et al. 2000, Lindon et al. 2004, Serkova and Niemann 2006, Ala-Korpela 2008).

Several biological compounds are altered in body tissues after radiation exposure (Walden and Farzaneh 1990). However, little is known on how the body responds to ionising radiation at a metabolite level, which may represent the most incisive indicator of cellular physiology. Recently, a few metabolomic studies based on ultra performance liquid chromatography (UPLC), and gas chromatography-mass spectrometry (GC-MS) have reported several metabolites of energy, purine and pyrimidine metabolism as urinary biomarkers in rodents with radiation exposure (Patterson et al. 2008, Tyburski et al. 2008, Lanz et al. 2009). It is necessary to explore metabolic markers of ionising radiation exposure based on a physiological and biochemical response that can be used for mass screening in the events of radiological incidents. Analysis of biofluids such as serum and urine is the simplest and minimally invasive approach to work at a mass screening level. The present study was undertaken to investigate the biochemical and physiological perturbations after ionising radiation exposure in mouse serum using metabonomic approach based on high resolution NMR spectroscopy.

Materials and methods

Animal handling, radiation exposure and sample collection

Strain ‘A’, male mice of 10 weeks of age obtained from experimental animal facility of the institute were placed in polypropylene cages. The mice received tap water, were fed chow ad libitum and were housed under a standard 12 h light/12 h dark cycle and at controlled temperature of 25 ± 2°C and relative humidity of 45–65%. All experimental protocols and animal handling practices adopted in this study were approved by institutional animal ethical committee. Three groups with an equal number of animals in each group (n = 18) were exposed to gamma radiation doses of 3 (group I), 5 (group II) and 8 Gray (Gy) (group III), respectively, and controls (n = 9, group IV) were sham irradiated. The mice were irradiated through gamma irradiation facility 60Co (Gammacell-220, Atomic Energy of Canada Ltd (AECL), Ottawa, Ontario, Canada). During the course of experimentation, the dose rate of the source was 0.22 Gy/min. Irradiation was performed on mice kept in pi-cage which was placed in irradiation chamber. Air supply was established through air pump in irradiation chamber to avoid hypoxic condition. Approximately 400 μl blood sample was collected between 09:00 and 10:00 h through retro-orbital plexus route from controls and irradiated animals. In the irradiated group, blood was collected at three time points (day 1, 3 and 5) post irradiation from one third of animals present in group at each time point (n = 6, animals for each dose level at each time point). Blood sample was allowed to clot for 30 min and serum was separated by centrifugation at 2665 g for 10 min. All serum samples were stored at −80°C until NMR measurement.

Sample preparation and NMR spectroscopy

NMR solvents, trimethylsilyl-2,2,3,3-tetradeteruero-propionic acid (TSP) and deuterium oxide (D2O) were obtained from Sigma-Aldrich (St Louis, MO, USA). Serum sample of 200 μl was mixed with 400 μl of D2O and transferred to 5 mm NMR tubes containing 1 mM TSP closed capillary (Wilmad, SP Industries, Buena, NJ, USA) as reference. All NMR spectra were acquired at 400.15 MHz, Bruker-AV spectrometer (Bruker, Rheinstetten, Karlsruhe Germany) at 298 K. Water suppressed Carr-Purcell-Meiboom-Gill (CPMG) spin echo pulse sequence (RD-90°-τ-180°-τ)  8 acquire with a total spin echo (2 πτ) of 200 milliseconds was used to attenuate broad signals from proteins and lipoproteins. Here RD represents relaxation delay of 2 sec during which water resonance is selectively irradiated, 90° represents a 90° radio frequency (RF) pulse, τ (τ) is spin echo delay, 180° is 180° RF pulse and n represents number of loops. Typically 64 free induction decays (FID) were collected into 32 K data points over a spectral width of 8223.68 Hz with an acquisition time of 3.98 sec. Each FID was weighted by an exponential function with a 0.3-Hz line broadening factor prior to Fourier transformation. 1H NMR chemical shifts (δ) in the spectra were referenced to TSP at δ 0.0 ppm and peaks identified on the basis of chemical shifts were assigned according to previously reported literature (Lindon et al. 1999).

Data reduction and pattern recognition (PR) analysis of 1H NMR spectra

All the spectra were phase and baseline-corrected using TOPSPIN (Bruker, Rheinstetten, Karlsruhe,
The spectra over the range (δ 0.2–10.0 ppm) were reduced to 245 regions, each 0.04 ppm wide and signal intensity in each region was integrated. The region between δ 4.5 and δ 5.0 ppm was removed prior to any statistical analysis in order to remove any spurious effects of variability in the suppression of the water resonance. Following removal of this region, each integral region was divided by the sum of all integral regions for data normalisation. PR analysis was done by carrying out principal components analysis (PCA) on normalised data. PCA is a well known statistical procedure for data dimensionality reduction. It allows the expression of most of the variance within a data set in a small number of newly transformed variables known as principal component (PC). Each PC is a linear combination of original variables whereby each successive PC explains the maximum amount of variance possible that is not accounted for by the previous PC. Hence, each PC is orthogonal and therefore, independent of the other PC. The output of the method results in two matrices known as ‘score’ and ‘loadings’. Data were visualised by plotting the Principal Component (PC) scores, where each point on the score plot represents an individual sample. The loading plots were used to detect spectral areas responsible for separation in the data, where each point represents one spectral region. The resulted score values from PCA were subjected to one way analysis of variance (ANOVA) to test the levels of statistically significant differences between treatment and controls. Pattern recognition of NMR data was performed using in house developed algorithm on Matlab 7.0 (MathWorks, Natick, MA, USA). PCA plot generation and statistical analysis were performed using Sigma plot 11.0 (Systat, San Jose, CA, USA).

Results

1H NMR analysis of serum samples from irradiated mice

A number of perturbations in endogenous metabolites were observed in 1H NMR spectra of serum samples of irradiated groups collected at different time points. Figure 1 illustrates 400 MHz 1H NMR spectra of serum samples from control and irradiated mice at day 5 post irradiation. The metabolites of serum affected by radiation exposure are tabulated in Table I. By visual inspection, at day 1 post irradiation, few metabolites were altered in group III compared to controls but no changes had been observed in Group I and II in respect to controls. Prominent changes in endogenous serum metabolites, branched chain amino acid (BCA), alanine, lactate, betaine, choline and phosphoethanolamine, were observed at day 5 post irradiation in all irradiated groups compared to controls. Although the lipid peaks of very low density lipoproteins (VLDL) and low density lipoprotein (LDL) were suppressed considerably by CPMG sequence, however, the peak intensities of VLDL and LDL for irradiated groups were stronger than those for control group.

Pattern recognition analysis of serum samples

PCA of NMR spectra was used initially to determine whether irradiated groups and controls could be distinguished post irradiation. The PC score plot from analysis of 1H NMR spectra of serum sampled at various time points (1, 3 and 5 days post irradiation) for irradiated mice groups are shown in Figure 2a–c.

At day 1, 1H NMR spectra and pattern recognition analysis indicated no obvious change in the serum samples from mice irradiated with 3 and 5 Gy of radiation dose. Only mice exposed to 8 Gy could be separated from controls at day 1 post irradiation. Significant difference (ANOVA, p < 0.05) on PC1 and PC2 scores were observed between group I, II and control on day 3 post irradiation. Therefore, at day 3 time point, mice group irradiated with 3 and 8 Gy dose of radiation could be separated from control in PC score plot. Remarkable differences (ANOVA, p < 0.05) between the control and all three irradiated groups were achieved along PC1 and PC2 axis at day 5 post irradiation that resulted in clear separation of group I, II and III from control. To compare the regions of the spectra contributing to the classification, PC loading versus chemical shift was plotted in all groups and control (Figure 3a–c). The PC loading plots also showed the consistent characterisation with visual inspection of serum 1H NMR spectra. For example, the loading plots for irradiated mice and control group suggested that the separation was attributed to the NMR signals at δ 0.86, 0.9, 0.94, 1.34, 1.48, 2.06, 3.22, 3.54 ppm corresponding to LDL, VLDL, BCA, lactate, alanine, unsaturated lipid, choline and glucose which confirmed the visual comparison of the NMR spectra from irradiated and control mice (Figure 3).

Discussion

A number of biological compounds become altered after radiation exposure. These changes occur as a result of the radiation damage, or in response to mobilisation for repair, regeneration and cell proliferation (Walden and Farzaneh 1990). Radiation-induced changes could be reflected as biochemical perturbations in biofluids. Earlier studies on biochemical and physiological assays have yielded some important insights into the complex response
to ionising radiation, (Altman 1971, Roy et al. 2006). Recently, NMR-based metabonomic methods have been utilised as a rapid analytical technique for the study of biochemical variation in biological fluid (Lindon et al. 2004, Ala-Korpela 2008). In the present study, results of $^1$HN M R spectroscopy of serum provide strong evidence of biochemical variation due to moderate radiation exposure.

Radiation-induced energy metabolism disturbance and oxidative stress

In the present study, a number of metabolites involved in energy generation were perturbed as a result of gamma irradiation. After radiation exposure, decrease in serum sugar and an increase in lactate was evident in all three irradiated groups. The observed decrease in glucose and the concomitant increase in lactate indicates enhancement in anaerobic metabolism. These changes resulted from increased energy demand and were suggestive of changes in carbohydrate and energy metabolism after radiation exposure (Lerman et al. 1962). Increased alanine in serum also suggests a metabolic switch towards energy conservation and gluconeogenesis, which is activated in radiation diseases (Borovikova et al. 1985). Our observations are in concurrence with a recent GC-MS based study that has reported down regulation of energy metabolites, citrate, 2 oxo glutarate in urine samples in rats post irradiation (Lanz et al. 2009).

Table I. Summary of proton NMR spectroscopy based-radiation-induced variations in serum metabolites in irradiated mice as compared to controls.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Chemical shift with multiplicity (bracket)</th>
<th>3 Gy (Group I)</th>
<th>5 Gy (Group II)</th>
<th>8 Gy (Group III)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 1</td>
<td>Day 3</td>
<td>Day 5</td>
</tr>
<tr>
<td>Lipids (LDLs/VLDLs)</td>
<td>0.86, 1.30</td>
<td>–</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Branched chain amino acid</td>
<td>0.94 (m)</td>
<td>–</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.33 (d)</td>
<td>–</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.48 (d)</td>
<td>–</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Acetate</td>
<td>1.92 (s)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Glutamine/glutamate</td>
<td>2.46 (m)</td>
<td>–</td>
<td>–</td>
<td>↑</td>
</tr>
<tr>
<td>Choline</td>
<td>3.21 (s)</td>
<td>–</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Phosphoethanolamine</td>
<td>3.23 (t)</td>
<td>–</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Betaine</td>
<td>3.25 (s)</td>
<td>–</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Glucose</td>
<td>3.54 (m)</td>
<td>–</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

↑ indicates relative increase in signal; ↓ relative decrease in signal; – no change. Keys: s: singlet; d: doublet; t: triplet; m: multiplet.

Figure 1. Representative NMR spectra of serum from 3, 5 and 8 Gy irradiated group and control at day 5 post irradiation.
Ionising radiation generates reactive oxygen species (R/OS) as a result of water radiolysis that induce oxidative stress (Rousselot et al. 1997). These ROS induce oxidative damage to vital cellular molecules and structures including lipids, proteins and membranes (Cadet et al. 2004). The increase in amount of LDL, VLDL, lactate and choline in serum from irradiated mice was commonly observed in ^1H NMR spectral and pattern recognition analysis. The rise in the level of serum lipids and lipoproteins on day 5 in all radiation dose denoted disturbance in lipid metabolism. Lipoproteins (LDL, VLDL) are important carrier proteins of triacylglycerol, cholesterol and cholesteryl esters. Ionising radiation may affect plasma lipoproteins by several possible mechanisms. Radiation exposure could modify the interaction of lipoproteins with their corresponding cell membrane receptors (Feurgard et al. 1999). This reduced activity for LDL clearance by the liver may contribute to the marked accumulation of LDL in sera of irradiated animals. Some earlier studies have shown increased triacylglycerol levels in plasma due to inhibition of lipoprotein lipase in small animals irradiated with γ rays (Dousset et al. 1984, Sedlakova et al. 1986). Many earlier studies have shown initiation of lipid peroxidation followed by radiation exposure (Leyko and Bartosz 1986, Pathak et al. 2007). Radiation-induced lipid peroxidation of cell membrane modifies membrane fluidity (Berroud et al. 1996) and the activities of some membrane enzymes (Yukawa et al. 1983). Membranal damage results in release of phospholipids and choline compounds as choline is the major head of phospholipids. In our study, an increase in choline and phosphocholine levels reflects an up-regulation of synthesis of structural phospholipids to cope with the demand of membrane resynthesis. Recently, a LC-MS-based study has reported increased membranous structure phospholipids and suggested phospholipid profiles as a promising way to assess the radiation exposure (Wang et al. 2009). Our results illustrate changes in membrane metabolites, choline, and lipoproteins due to radiation-induced oxidative stress. Similarly, Lanz et al. (2009) have observed GC-MS-based distinct radiation metabolomic phenotype derived from oxidative stress.

Figure 2. Score plots (PC1 vs. PC2) from PCA analysis of proton NMR spectra of serum from control (○), 3 (▲), 5 (■) and 8 (●) Gy irradiated groups at day 1 (a), 3 (b) and 5 (c) post irradiation. Ellipse indicates schematic representation to differentiate the cluster of groups.
Radiation-induced amino acid metabolism perturbations

The present study shows increased levels of BCA in serum of irradiated mice as compared to controls. Holecek et al. (2002a) have also reported increased amino acid metabolism at 48 h time point post irradiation. γ-radiation induces degeneration of skeletal muscle that results in the release of amino acids from irradiated muscle into blood stream (Schwenen et al. 1989). As a result of activated protein breakdown induced by irradiation, BCA are released from body proteins and extensively catabolised. The amino group released during transamination of BCA is used primarily for synthesis of alanine and glutamine (Chang and Goldberg 1978). Alanine is an important precursor for gluconeogenesis in liver during increased energy demand. To maintain the levels of alanine for gluconeogenesis, BCA levels were released in blood from liver for their catabolism in skeletal muscle. The observed increase in amino acid (BCA, alanine) on day 3 and 5 following irradiation in all three groups reflected the consequences of radiation-induced protein breakdown. Glutamine acts as a nitrogen shuttle among organs, an important fuel for rapidly dividing cells such as erythrocytes and cells of the immune system and a precursor for the synthesis of nucleotides (Holecek 2002b). However, glutamine was found to be increased only on day five post irradiation. It shows that glutamine resynthesis took little more time for their release in blood stream.

Pattern recognition analysis provides a novel and powerful method to analyse the data obtained from NMR spectra after giving respective treatment (Serkova and Niemann 2006). For classification of data, PCA has been widely chosen as a preliminary unsupervised pattern recognition tool to reduce data dimensions originating from 1H NMR spectra. In our study, PCA analysis showed both dose and time dependent changes in the endogenous serum metabolites. Mice treated with higher dose (8 Gy) showed symptoms at day one post irradiation and could easily be separated from controls through PCA analysis. Group I started showing metabolic changes only at day 3 post irradiation, whereas, group II could be separated only at day 5 which could be dose dependent. Similarly, changes observed in irradiated animals were time dependent. Maximum changes in all three groups were observed at day 5 post irradiation, where all three irradiated groups could be separated from controls at score plot.

Conclusions

From the above findings, it is demonstrated that 1H NMR spectra of serum samples show metabolic perturbations in mice after irradiation. In this study, it is implied that 3, 5 and 8 Gy radiation doses induce changes in body lipid, protein and membrane metabolism. Based on these metabolic alterations, irradiated group could be separated from controls by NMR-PR analysis. Although the biochemical assays could not be performed on our samples which could have correlated better with our results, the study has identified many earlier known radiation-induced biochemical changes all together in one spectrum. This is a basic and first study as per our knowledge to explore the application of NMR spectroscopy for studying radiation induced metabolic perturbations.
in serum. NMR based metabolomics could be used for development of metabolic markers and further be extended for multiparametric approach for mass screening and evaluation of radiation damage.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References


NMR spectroscopy based metabolic profiling of urine and serum for investigation of physiological perturbations during radiation sickness

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Abstract Radiation accidents are rare events that induce radiation syndrome, a complex pathology which is difficult to treat. In medical management of radiation victims, life threatening damage to different physiological systems should be taken into consideration. The present study was proposed to identify metabolic and physiological perturbations in biofluids of mice during different phases of radiation sickness using $^1$H nuclear magnetic resonance ($^1$H NMR) spectroscopy and pattern recognition (PR) technique. The $^1$H NMR spectra of the biofluids collected from mice irradiated with 5 Gray (Gy) at different time points during radiation sickness were analysed visually and by principal components analysis. Urine and serum spectral profile clearly showed altered metabolic profiles during different phases of radiation sickness. Increased concentration of urine metabolites viz. citrate, α-ketoglutarate, succinate, hippurate, and trimethylamine during prodromal and clinical manifestation phase of radiation sickness shows altered gut microflora and energy metabolism. On the other hand, serum nuclear magnetic resonance (NMR) spectra reflected changes associated with lipid, energy and membrane metabolism during radiation sickness. The metabonomic time trajectory based on PR analysis of $^1$H NMR spectra of urine illustrates clear separation of irradiated mice group at different time points from pre dose. The difference in NMR spectral profiles depicts the pathophysiological changes and metabolic disturbances observed during different phases of radiation sickness, that in turn, demonstrate involvement of multiple organ dysfunction. This could further be useful in development of multiparametric approach for better evaluation of radiation damage as well as for medical management during radiation sickness.

Keywords Radiation sickness · $^1$H NMR spectroscopy · Serum · Urine · Metabonomics

1 Introduction

In the present scenario, increasing burden of nuclear arsenal and scramble of nuclear power leads to a big threat of radiation exposure to the population at large. In all potential radiation disasters, we are likely to encounter whole body or partial body radiation exposure. Radiation sickness occurs after whole body or significant partial body irradiation of greater than 1 Gray (Gy) radiation dose (Waselenko et al. 2004). It is a sequence of phased symptoms beginning with prodromal phase of non specific clinical response spanning from few hours to a few days, followed by symptom free latent phase lasting a few days or weeks depending upon the radiation exposure dose. Clinical symptoms reappear during illness manifestation phase which may last for weeks and survivability of a radiation exposed person depends on the recovery in this phase. A dose dependent latent period occurs between the end of the prodromal phase and the onset of later haemopoietic or gastrointestinal complications (AFFRI 2003). Therefore, regular monitoring in illness phase is essential for clinical management. During management of...
victims of radiological accidents, besides evaluating global radiation dose received by biological dosimetry, radiation induced damage to different physiological systems should also be taken into consideration. In the last few years, new concept of radiation pathophysiology has been proposed that involves radiation induced multi-organ involvement (RIMOI) and radiation induced multi-organ failure (RIMOF). Both RIMOI and RIMOF contribute to the clinical outcome and prognosis of radiation accident victim (Fliedner et al. 2005; GenYao and Changlin 2005). Few bio indicators for radiation induced damage to the physiological system (Becciolini et al. 1984; Prat et al. 2006; Lutgens et al. 2004) are available in literature. Thus there is a crucial need to obtain information from various metabolic, cellular and molecular levels of radiation damage during all phases of radiation sickness. In this scenario, high-throughput non-invasive studies are required to develop multiparametric approach with strong prognostic capabilities which could provide adequate information for subsequent medical management and treatment decisions (Reeves et al. 1996).

$^1$H nuclear magnetic resonance ($^1$H NMR) spectroscopy is rapidly evolving as a tool in system biology for monitoring metabolic fluxes in toxicological studies in small animals. (Liao et al. 2007; Wei et al. 2008; Wang et al. 2006; Waters et al. 2006). It is well established that nuclear magnetic resonance (NMR) technique, coupled with pattern recognition (PR) methods, can provide valuable information on biochemical perturbations due to both exogenous and endogenous factors by analysis of biofluids (Waters et al. 2006; Holmes et al. 2008). Serum and urine samples provide a well established surrogate medium for the study of metabolic physiological functions of not only liver and kidney, but also for other vital organs. Earlier studies in the field of radiation metabolomics have shown time and dose dependent changes due to acute radiation stress in animal model and cell system (Tyburski et al. 2008; Lanz et al. 2009; Patterson et al. 2009; Wang et al. 2009; Khan et al. 2010). However, metabolic changes during different phases of radiation sickness are yet to be elucidated. The present study has been designed to assess physiological perturbations in urine and serum during different phases of radiation sickness using $^1$H NMR spectroscopy based metabolomics.

2 Materials and methods

2.1 Animal handling, radiation exposure and sample collection

Twenty strain ‘A’ male mice of 10 weeks of age were housed individually in metabolic cages in a well-ventilated room with controlled conditions. During the study, room temperature was maintained within 19–23°C, relative humidity within 45–65%, and fluorescent lighting was provided (6 a.m. to 6 p.m.) for 12 h light/12 h dark cycle. Mice kept in pi cage were irradiated with 5 Gy of radiation through gamma irradiation facility at our institute employing $^{60}$Co [Gamma Cell-220, Atomic Energy of Canada Limited (AECL), Ottawa, Ontario, Canada] with source operating at 0.20 Gy/min. A dose of 7 Gy in mice is generally considered to be equivalent to 4 Gy on humans (Hall and Giaccia 2006). Thus the dose we used was roughly equivalent to 2.5 Gy on humans and this dose is considered to be associated with haemopoietic syndrome with some gastrointestinal symptoms. A summary of symptoms appearing at different phases of acute radiation syndrome are given in Table 1.

Urine samples from mice were collected in metabolic cages. To reduce contamination, mice were placed in clean cages and 0.1% sodium azide was added in urine collection tube. To look for metabolic changes during different phases of radiation sickness, several time points were selected for sample collection viz. 6 h and day 5 post irradiation for prodromal phase, day 10 and 15 as latent phase and day 20 and 25 as clinical manifestation phase. For blood collection, only one time point from each phase of radiation sickness was selected. Animals ($n = 5$ at each time point) were sacrificed and blood was collected through heart puncture at pre dose, day 5, 10 and 25 post irradiation. Serum was separated by centrifugation at $2,665 	imes g$ for 10 min. All samples were stored at $-80°C$ until $^1$H NMR spectroscopic analysis was carried out.

2.2 Sample preparation and NMR spectroscopy

All chemicals, NMR solvents, trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP) and deuterium oxide (D$_2$O) used in the study were obtained from Sigma-Aldrich (St Louis, MO, USA). Animal handling and experimental protocols adopted in this study were approved by the Animal Ethical Committee of Institute.

2.2.1 $^1$H NMR spectroscopy measurement of urine

Deuterated phosphate buffer solution (400 µl; 0.2 M Na$_2$HPO$_4$/0.2 M NaH$_2$PO$_4$, pH 7.4 containing 1 mM TSP prepared in D$_2$O) was mixed with 200 µl of urine to minimize variations in pH of the urine samples. TSP acted as chemical shift reference ($0.0$ ppm) and D$_2$O provided a lock signal. Samples were then centrifuged at $4,722 	imes g$ for 5 min to remove particulate matter and then transferred to 5 mm NMR tube. NMR spectral data were acquired on a Bruker-Av400 spectrometer (Bruker, Rheinstetten,
Karlsruhe Germany) with Broad Band Observe (BBO)
probe operating at a frequency of 400.13 Hz and a tem-
perature of 298 K. Water signal was suppressed using
water presaturation pulse; nuclear overhauser enhanced
spectroscopy (NOESYPR1D) pulse sequence (RD-90°-t-
90°-tm-90°-acq) with relaxation delay (RD) of 2.0 s and an
acquisition time (acq) of 2.5 s was used. Typically, 64 free
induction decay (FID) were collected into 32 K data points
over a spectral width of 6410 Hz.

2.2.2 $^1$H NMR spectroscopy measurement of serum

Serum sample of 200 μl was mixed with 400 μl of D$_2$O
and transferred to 5 mm NMR tubes containing 1 mM TSP
as reference in closed capillary (Wilmad, SP Industries, NJ,
USA). All NMR spectra were acquired at 400.13 MHz on
Bruker-AV400 spectrometer (Bruker, Rheinstetten, Kar-
lsruhe, Germany) at 298 K. Water suppressed Carr–Pur-
cell–Meiboom–Gill (CPMG) spin echo pulse sequence
(RD-90°-t-180°-t-90°-tn-acq) with a total spin echo (2nτ) of
200 ms was used to attenuate broad signals from proteins
and lipoproteins. Sixty-four FID were collected into 32 K
data points over a spectral width of 8223.68 Hz, with a RD
of 2 s and acq of 3.98 s.

Each FID was weighted by an exponential function with
a 0.3-Hz line broadening factor prior to Fourier transfor-
mation and peaks were assigned on the basis of chemical
shifts according to previously reported literature (Lindon
et al. 1999).

2.3 Data reduction and PR analysis of $^1$H NMR spectra

All the spectra were phase and baseline corrected and
 calibrated (TSP at 0.0 ppm, 1 mM) using TopspinTM
(Bruker, Karlsruhe, Germany). Each $^1$H NMR spectrum
from urine and serum over the range (δ0.2–10.0 ppm) was
reduced to 245 regions of equal width (0.04 ppm) and
signal intensity in each region was integrated using AMIX
software (Bruker, Karlsruhe, Germany). The region
between δ4.5 and 5.0 ppm was removed prior to any sta-
tistical analysis in order to elude any spurious effects of
variability in the suppression of water resonance. For urine
spectra, the region containing urea (δ5.0–6.0) was also
excluded to eliminate any cross-relaxation effects of urea.
Following removal of these regions, data was normalized
in AMIX by dividing each integrated segment by the total
area of the spectrum to reduce any significant concentration
difference. Output data in ASCII data format was imported
to Microsoft Excel (Microsoft Office 2003), mean centered
and then exported to Matlab 7.1 (MathWorks, MA, USA)
for PR analysis.

Principal component analysis (PCA) was performed for
pre dose and all time points post irradiation urine and
serum samples. PCA plots of first two components
allowed visualisation of the data and to establish whether
there were any time dependent changes in the metabolic
profile of urine or serum post irradiation. Variation
between samples can be detected in the score plots,
whereas spectral region responsible for the differences
can be viewed in the corresponding loading plots. These
spectral regions were examined and potential metabolites
were identified and integrated. Additionally, to calculate
relative changes in identified metabolites during different
phases of radiation sickness, relative concentration of
each identified metabolite in relation to the total spectral
integral area, subsequent to removal of water resonance,
was determined and one way analysis of variance
(ANOVA) and multi comparison test was performed in
MATLAB. The score values from PCA were subjected to
ANOVA to test significant difference between pre and
post irradiation samples.

Mean values of principal component (PC) were calcu-
lated for urine samples at each time point and plots of PC1
versus PC2 for the mean data were constructed. These
maps gave information about the metabolic trajectory
through different phases of radiation sickness.

3 Results and discussion

Radiation incidents are relatively rare events; however, the
consequence of accidental radiation exposure can be

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Symptoms of acute radiation syndrome during exposure of whole body radiation in the range of 2–6 Gy of radiation dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prodromal phase</td>
<td>Haemopoietic changes: Development of lymphopenia, granulocytopenia</td>
</tr>
<tr>
<td>Latent phase</td>
<td>Gastrointestinal symptoms: Headache, nausea vomiting and diarrhea</td>
</tr>
<tr>
<td>Manifest illness phase</td>
<td>Neuromuscular symptoms: Easy fatigability, fever, headache</td>
</tr>
</tbody>
</table>

Different phases of radiation sickness
Symptoms

Prodromal phase
Haemopoietic changes: Development of lymphopenia, granulocytopenia
Gastrointestinal symptoms: Headache, nausea vomiting and diarrhea
Neuromuscular symptoms: Easy fatigability, fever, headache

Latent phase
Pancytopenia and predisposition to infection
State of intense immunosuppression

Manifest illness phase
Lymphopenia, opportunistic infections
Haemopoietic and mucosal system affected

Study of radiation sickness by NMR spectroscopy
extensive, both physically and physiologically. New approaches and technologies are available for the study of health impairments as a function of dose of whole body irradiation at different organ levels. In this study, we have used radiation metabonomics based on $^1$H NMR spectroscopy to demonstrate radiation induced metabolic or physiological perturbations during different phases of radiation sickness.

3.1 $^1$H NMR spectroscopic analysis of urine and serum

Typical $^1$H NMR spectra of urine samples obtained from irradiated mice at various time points are illustrated in Figs. 1 and 2. Both types of biofluids contained lactate, alanine, glutamine, glutamate and alanine. Metabolites like hippurate, taurine, trimethyl amine (TMA), $\alpha$-ketoglutarate ($\alpha$-KG) and 2-ketoisocaprate that are unique to urine metabolic profile, were present in urine $^1$H NMR spectra amongst others. NMR spectral profile of serum was characterised by predominance of various lipids along with resonances from creatine, several amino acids and organic acids.

A number of perturbations in endogenous metabolites were observed in the $^1$H NMR spectra of urine samples collected at various time points post irradiation (Table 2). Changes were observed mainly in metabolites associated with energy metabolism (succinate, $\alpha$-KG, citrate), amino acids (branched chain amino acids, isoleucine, phenylalanine, taurine), gut flora metabolites [hippurate, TMA], osmolytes (betaine, sarcosine) and creatinine in urine samples. Figure 3 summarises the relative changes in different metabolites through different phases of radiation sickness. A dramatic change in metabolic profile occurred during 6 h post irradiation with significant increase in almost all metabolites. Continued increase of most of the metabolites was observed in $^1$H NMR spectra of urine samples at day 5. The shift in metabolite changes in irradiated animals then turned back to pre dose values at day 10, post irradiation. Thereafter, levels of $\alpha$-ketoglutarate, citrate, succinate, trimethylamine and other associated metabolites of gut flora increased again during day 15, 20 and 25 post irradiation. On the other hand, changes observed in serum metabolic profile during prodromal and clinical manifestation illness phase was mainly in lipids, branched chain amino acid and lactate (Table 3). Moreover, membrane metabolites viz., choline and phosphoethanolamine was increased only during day 5 post irradiation (prodromal phase).

![Fig. 1 Comparative $^1$H NMR urine spectra from irradiated mice at different time points](image)
3.2 PCA of urine and serum

All urine and serum samples collected at all the time points of the study were assessed using PCA analysis to investigate time dependent effect of radiation over 25 days of experimental duration. PCA proved to be a useful and rapid means of establishing whether the urine spectra obtained from irradiated mice were different and also identifying at which time point the maximum biochemical changes have occurred. A pair wise day to day comparison by PCA can provide more detailed information on the onset of different phases of radiation sickness. Score plot of NMR spectra of urine samples collected during different post irradiation time point revealed clear separation between pre-dose and post dose time points (Fig. 4a–f). However, on day 10, post irradiation, overlapping of score values with pre dose values suggested that there were no significant changes in the urine metabolic profile at this time point. PCA score values calculated at different time points were used to plot metabolic trajectory, a method of visualising changes in metabolic profiles with time. PCA analysis based time trajectory was able to differentiate amongst various phases of radiation sickness based on physiological perturbations observed in NMR spectra of urine samples from irradiated animals. The mean score plots for the first two PCs at each time point has been shown in Fig. 4g. The trajectory of irradiated sample moved away from the control or pre dose position along PC1 axis as early as 6 h and continued till day 5. Irradiated animals turned back towards pre dose time point that could be visualised by the close proximity of day 10 score values to pre dose score. From day 10 onwards, trajectory moved away from pre dose point and attained maximum shift by the end of the experimental duration (day 25). In case of serum, aliphatic region (0.5–4.5 ppm) containing most endogenous metabolite signals in serum were chosen for PCA analysis. Figure 5 represents score plots on first two PCs of $^1$H NMR spectra from pre dose serum samples and samples from irradiated mice collected on day 5, 10 and 25 post irradiation. Clear separation of day 5 and day 25 serum samples from pre dose samples showed radiation induced changes during prodromal and manifest illness phase respectively. In the score plot of NMR spectra of serum samples, overlapping of score values of NMR spectra from serum sample collected on day 10 with pre dose time point sample values suggest latent phase of radiation sickness. PCA was repeated on group mean data in order to simplify the map and to establish a biochemical trajectory of effect. The time
course of radiation induced changes in serum is shown in Fig. 5. The metabolic perturbations during radiation sickness phase based on PC loading values and statistical analysis of spectral regions as described in methods were characterised by increasing branched amino acids, lactate, alanine and betaine at day 5 post irradiation. However, no

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Chemical shift with multiplicity (bracket)</th>
<th>Prodromal phase</th>
<th>Latent phase</th>
<th>Manifestation illness phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6 h</td>
<td>Day 5</td>
<td>Day 10</td>
</tr>
<tr>
<td>β-Hydroxybutyrate</td>
<td>1.20 (d), 2.31, 2.41 and 4.16 (ABX)</td>
<td>↑</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>2.45 (t), 3.01 (t)</td>
<td>↑↑</td>
<td>↑</td>
<td>–</td>
</tr>
<tr>
<td>Succinate</td>
<td>2.41 (s)</td>
<td>↑↑</td>
<td>↑</td>
<td>–</td>
</tr>
<tr>
<td>Citrate</td>
<td>2.55 (AB), 2.67 (AB)</td>
<td>↑↑</td>
<td>↑</td>
<td>–</td>
</tr>
<tr>
<td>Trimethylamine</td>
<td>2.88 (s)</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Creatinine</td>
<td>3.05 (s), 4.06 (s)</td>
<td>–</td>
<td>↑</td>
<td>–</td>
</tr>
<tr>
<td>Choline</td>
<td>3.21 (s), 3.52 (m)</td>
<td>↑</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Betaine</td>
<td>3.27 (s)</td>
<td>↓</td>
<td>↑</td>
<td>–</td>
</tr>
<tr>
<td>Taurine</td>
<td>3.25 (t), 3.43 (t)</td>
<td>–</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Acetoacetate</td>
<td>2.30 (t), 3.45 (s)</td>
<td>↑</td>
<td>↑</td>
<td>–</td>
</tr>
<tr>
<td>Sarcosine</td>
<td>2.74 (s), 3.60 (s)</td>
<td>–</td>
<td>↑</td>
<td>–</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>3.74 (d), 3.76 (d), 4.03 (m), 4.52 (d)</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Trans-aconitate</td>
<td>3.48 (s), 6.62 (s)</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.28 (m), 7.33 (m), 7.38 (m), 7.43 (m)</td>
<td>↑</td>
<td>↑</td>
<td>–</td>
</tr>
<tr>
<td>Hippurate</td>
<td>3.97 (d), 7.55 (t), 7.64 (t), 7.84 (d)</td>
<td>–</td>
<td>↑</td>
<td>–</td>
</tr>
</tbody>
</table>

†, indicates relative increase in integral value for the region containing the selected metabolite; –, no change in integral value for the region containing selected metabolite; †, indicates relative decrease in integral value for the region containing the selected metabolite. P values for the changing metabolites were assessed using ANOVA in MATLAB based on the integrals of the selected peaks and were all less than 0.05 levels s singlet, d doublet, t triplet, m multiplet.

Fig. 3 Relative changes in levels of selected metabolites in the spectra of urine (a–d) and serum (e–f) through different time points of radiation sickness. Data presented as relative levels with respect to total spectral area ± standard deviation, with overall differences between all groups (P < 0.05) and post hoc tests (P < 0.05) revealing a difference from pre dose level.
marked changes could be seen on day 10 post irradiation (latent phase). During manifest illness phase (day 25) of study, there was reappearance of increased levels of lipids, alanine and branched amino acid in NMR spectra of serum. Our findings corresponded well with different phases of radiation sickness.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Chemical shift with multiplicity (bracket)</th>
<th>Day 5</th>
<th>Day 10</th>
<th>Day 25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipids (LDLs/VLDLs)</td>
<td>0.86, 1.30</td>
<td>↑</td>
<td>–</td>
<td>↑↑</td>
</tr>
<tr>
<td>Branched chain amino acid</td>
<td>0.94 (m)</td>
<td>↑</td>
<td>–</td>
<td>↑</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.33 (d)</td>
<td>↑</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.48 (d)</td>
<td>↑</td>
<td>–</td>
<td>↑</td>
</tr>
<tr>
<td>Acetate</td>
<td>1.92 (s)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Choline</td>
<td>3.21 (s)</td>
<td>↑</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Phosphoethanolamine</td>
<td>3.23 (t)</td>
<td>↑</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Betaine</td>
<td>3.25 (s)</td>
<td>↑↑</td>
<td>–</td>
<td>↑</td>
</tr>
</tbody>
</table>

Fig. 4 PCA score plots (a-f) based on $^1$H NMR urine spectra at 6 h, day 5, 10, 15, 20 and 25 post irradiation, respectively (filled circle pre-dose, open circle post dose). PCA metabolic trajectory plot (g) summarising the changes in the NMR visible metabolome through different phases of radiation sickness.

Prodromal phase of radiation sickness is associated with immediate effect of radiation exposure and lasts for few hours to few days depending on absorbed radiation dose. During this acute phase of radiation sickness, most of the metabolites in serum and urine were found to be increased after sub lethal dose of whole body radiation. However,
most of the changes observed in urine were Kreb’s cycle intermediates. These and other energy metabolites represent bioenergetic status of the body and might have resulted from increased energy demands and were, therefore, suggestive of changes in energy metabolism after radiation exposure (Lerman et al. 1962). Dose dependent acute effects of radiation showing altered membrane and energy metabolism in serum have been reported in our earlier study (Khan et al. 2010). In this study, highest increase in the energy intermediates was observed at 6 h post dose in urine samples. Increased α-keto-glutarate, succinate and citric acid could also be due to the effect of radiation on renal tubular physiology. It has been earlier reported in the literature that whole body radiation causes depression of tubular secretion and reduces glomerular filtration in kidney cells (Judas et al. 1997), as a result of which these metabolites are released in more quantity in urine. Moreover, mitochondria have long been considered as direct intracellular target of ionizing radiation which in turn results in inhibition of citric acid cycle enzyme (Kergonou et al. 1981). Due to inhibition of these enzymes, these metabolites might be under utilised in renal tubular cells and this could have resulted in release of these metabolites in urine of irradiated mice. Inhibition of aerobic mitochondrial enzyme or metabolism is also supported with the presence of increased lactate in serum NMR spectra of irradiated mice. Therefore, changes observed in energy intermediates at 6 h post dose could be cumulative expression of both disturbed energy metabolism due to increased energy demands as well as altered renal tubular function.

1H NMR spectra of serum from irradiated mice showed radiation induced oxidative stress related changes in the form of increased choline compounds. Ionizing radiation generates reactive oxygen species (ROS) as a result of water radiolysis that induce oxidative stress (Rousselot et al. 1997). These ROS induce oxidative damage to vital cellular molecules and initiate lipid peroxidation (Cadet et al. 2004). Membranal damage due to lipid peroxidation results in release of phospholipids and choline compounds as choline is the major head of phospholipids. In our study, increase in choline and phosphocholine levels in NMR spectra of serum at day 5 reflects an up-regulation of synthesis of structural phospholipids to cope with the demands of membrane resynthesis. However, this altered membrane metabolism was restricted to only prodromal phase, since most of the changes occurring in this phase are due to radiation induced oxidative stress.

Ionizing radiation is said to cause oxidative damage to tissues and a few earlier studies have reported radiation induced liver and kidney injury (Jindal et al. 2006; Sharma et al. 2006; Rana et al. 2009). In our study, radiation induced changes in lipid metabolism were also observed in the form of increased lipoproteins viz. low density lipoprotein and very low density lipoprotein (LDL and VLDL respectively) in serum 1H NMR spectral analysis. Significant increase in the levels of serum lipid profile and LDL are demonstrated 5 days post irradiation possibly as a result of liver injury which is in agreement with previous studies in Syrian hamster (Feurgard et al. 1999) and mice (Agrawal et al. 2001). This indicates that ionizing radiation induced oxidative stress might alter hepatic lipid metabolism and serum lipoproteins. Radiation induced liver injury has also been reflected as increased taurine signals in urine metabolic profile in our study. Taurine is considered as urinary biomarker for liver injury (Waterfield et al. 1993; Beckwith-Hall et al. 1998; Clayton et al. 2003) and its level was increased during prodromal phase which continued to increase till day 15. Increased urinary taurine is generally observed in fatty liver and in our study liver steatosis was observed in histological studies on liver tissue obtained on day 5 from mice irradiated with a radiation dose of 5 Gy (unpublished observations).

Acute radiation sickness involves gastrointestinal symptoms that include diarrhoea (Table 1). Gastrointestinal symptoms during radiation sickness could be due to radiation induced dysmotility of intestine, denudation of villi and loss of epithelial lining that could be associated with bacterial overgrowth (Macnaughton 2000; Somsoy et al. 2002). Bacterial overgrowth during radiation infection might be related with disturbed gut flora metabolism. Gut microbiota extensively catabolise proteins and aromatic amino acids such as hippurate, phenylacetylglucine, phenylalanine, tyrosine and tryptophan. These species are present in urine as products of intestinal microflora.
metabolism. Disturbed gut flora metabolism is directly reflected in urine NMR spectral profile as increased TMA, hippurate, phenylalanine and other aromatic amino acid signals. TMA is an indicator of methylamine metabolism. Methylamines are important osmo-regulatory compounds and are produced via degradation of dietary choline to TMA and its di and mono amine metabolites by gut flora (Martin et al. 2008). In our study, increased levels of TMA might be attributed to altered intestinal bacterial metabolism. The increased levels of urinary phenylalanine and phenylacetylglucose related compounds and hippurate also suggest radiation induced effects on the gut flora because their precursors, benzoic acid and phenylacetic acid are produced by bacterial metabolism.

Maximum radiation induced changes appeared during prodromal phase as radiation induced oxidative stress lasts mainly in this phase. In latent phase, reversal of most of the changes occurring during prodromal phase to normal could be due to repair mechanism occurring simultaneously in the body. However, changes started reappearing from day 15 onwards in manifest illness phase and reappearance of these changes during clinical manifestation illness phase could be associated with immunosuppressed state of the body during lymphocytopenia or other changes in haemo-poietic and gastrointestinal system during prodromal phase. Although, these changes in metabolic profile were not as high as observed in prodromal phase of radiation sickness. In our study, most of the changes during late phase pertain mainly to gut flora and energy metabolism suggesting disturbed gastrointestinal symptoms and opportunistic infection due to immunosuppression of the body.

Our NMR based spectral profiling of biofluids has identified changes in organal and physiological functioning at different phases of radiation syndromes. In recent years, new concept of radiation pathophysiology has been proposed that involves multi organ injury, multi organ dysfunction or multi organ failure and marked involvement of lung, kidney, liver and other organs in addition to acute radiation syndrome (Fliedner et al. 2005; Waselenko et al. 2004). Although we have not observed clinical parameters of liver, kidney or other organ dysfunction, our results suggest patho-physiological changes leading to multi organ dysfunction syndrome. Consequently, it could be proposed that an underlying radiation induced multi organ dysfunction syndrome should be evaluated irrespective of radiation dose exposure.

4 Conclusions

The application of metabolomics in radiation casualty management and monitoring is still in its infancy. However, information provided using metabolomics will nonetheless aid in medical diagnostic practices during radiation accident. Results of the present study clearly showed metabolic changes during different phases of radiation sickness. These changes could be the consequence of radiation induced damage to physiological system leading to multi organ dysfunction. Such studies will not only help in medical management during radiation accident but also beneficial for patients undergoing cancer treatment using ionizing radiation.

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References


