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NMR based metabolomics reveals acute hippocampal metabolic fluctuations during cranial irradiation in murine model

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ABSTRACT

Cranial irradiation is widely used as a treatment modality or prophylactic treatment in cancer patients, but it is frequently related to neurocognitive impairment in cancer survivors. Though most of radiation-induced changes occur during early and late delayed phase of radiation sickness, recent reports have supported the evidence of impaired neurogenesis within 24–48 h of radiation exposure that may implicate changes in acute phase as well. Inspection of these acute changes could be considered important as they may have long lasting effect on cognitive development and functions. In the present study, ^{1}H NMR spectroscopy based metabolomic approach was used to obtain comprehensive information of hippocampus metabolic physiology during acute phase of radiation sickness in a mouse model for single dose 8 Gy cranial irradiation. The analysis demonstrated reduced metabolic activity in irradiated animals compared to controls, typically evident in citric acid cycle intermediates, glutamine/glutamate and ketone bodies metabolism thus providing strong indication that the hippocampus is metabolically responsive to radiation exposure. The data suggested reduced glucose utilization, altered intermediary and neurotransmitter metabolism in hippocampus tissue extract. To the best of our knowledge this is the first metabolomic study to document cranial irradiation induced acute metabolic changes using *in vitro* ^{1}H NMR spectroscopy.

1. Introduction

Radiation-induced brain injury has always remained an area of interest for radiation oncologists and biologists. In general, the brain is exposed to radiation either during radiation therapy or accidentally during nuclear incidents. Brain, a complex, highly regulated and networked organ which was earlier known to be radioresistant, has now been considered as radiosensitive and radiation exposure affects the functional and behavioral capabilities of an individual (Rola et al., 2004). Although severe functional and structural injuries occur after relatively higher doses, lower doses can lead to mild to moderate cognitive dysfunction without inducing significant morphological changes (Fike and Gobbel, 1991; Tofilon and Fike, 2000; Abayomi, 1996; Kramer et al., 1992; Mizumatsu et al., 2003). In the last two decades, extensive work predominantly in the field of cranial irradiation has been carried out to investigate the post radiation effects on the structure and function of the brain. Clinically, radiation-induced brain injury is classified as acute (days to weeks) early delayed (1–6 months) and late delayed (>6 months) following irradiation (Tofilon and Fike, 2000). Acute radiation injury is considered to be rare with current radiation therapy techniques and is normally reversible and solves spontaneously (Greene-Schloesser et al., 2012). On the other hand, most of the studies suggest vascular-glial hypothesis based irreversible early and late delayed changes post irradiation (van der Maazen et al., 1993; Calvo et al., 1988). Of all the brain regions, hippocampus is known to be very sensitive to radiation exposure particularly CA1 (*Cornu ammonis 1*) and subgranular zone regions (SGZ). Hippocampus-associated functions; learning and memory are significantly affected due to suppression of hippocampal neurogenesis by various stressors including radiation and oxidative stress (Mizumatsu et al., 2003; Monje et al., 2002). Predominantly, learning and memory impairments are reported during early delayed and late delayed phase of radiation-induced

Abbreviations: MR, magnetic resonance; PR, pattern recognition; PCA, principal component analysis; PLS-DA, partial least square discrimination analysis; PC, principal component; VIP, variance of importance; ROS, reactive oxygen species; GPE, glycerophosphoethanolamine; CA1, *cornu ammonis 1*.

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injury due to persistent oxidative stress induced impaired neurogenesis (Rola et al., 2007; Raber et al., 2004). But few recent reports have supported the evidence of impaired neurogenesis, increased hippocampal neuronal apoptosis and reduced growth hormone secretion within 24–48 h of radiation exposure (Rola et al., 2004; Achanta et al., 2007). Recently, radiation-induced oxidative stress led to altered redox balance in hippocampal microenvironment has been considered to play a major role in suppressing hippocampal neurogenesis (Zou et al., 2012). The redox system is essential in maintaining cellular homeostasis. Impaired redox balance not only disturbs cellular environment but also leads to defective cell death or apoptosis. The disturbed cellular environment might have an impact on the metabolic response as well. There is a need for holistic investigation of radiation-induced changes in hippocampus during early acute phase. Since, these acute changes could be considered important as they might play a significant role in early and late delayed effects. Current knowledge of the early metabolic response of hippocampus for radiation exposure is still fragmented. To attain comprehensive metabolic information of hippocampus post irradiation, high-throughput metabolomic technique such as NMR spectroscopy would be very supportive. Metabolomics, that mainly focuses on primary metabolism refers to the quantification of the free metabolites using high throughput analytical techniques and provides a complementary metabolic fingerprint of biological system (Lindon et al., 2004; Shockcor and Holmes, 2002).

In vivo Magnetic Resonance (MR) spectroscopy is routinely used in clinical set up to obtain metabolites information non-invasively from a particular region of interest. Though nowadays, under normal physiological conditions a high magnetic field 7T and above, can provide a neurochemical profile of more than 15 metabolites including most of neurotransmitters non invasively (Duarte et al., 2012). However, due to the presence of significant spectral overlap in *in vivo* spectroscopy, sometimes metabolic fluctuations in response to the stimulus are missed out. These limitations can be overcome by using high resolution *in vitro* NMR spectroscopy based metabolomics on tissue extracts. *In vitro* ^{1}H NMR spectroscopy can currently detect numerous unique metabolites in cerebral tissues compared to metabolites identified using *in vivo* spectroscopy. Brain extracts are often used to obtain or supplement information not available from *in vivo* methodology as small molecules involved in biochemical processes provide information on the status and functioning of a living system caused by changes in gene expression. Recently, we observed acute significant microstructural changes on animal model for cranial radiation of 8 Gy, but metabolic changes could not be detected using *in vivo* MR spectroscopy (Gupta et al., 2013). However, we anticipated radiation-induced metabolic changes. Therefore, the present study was designed to probe the metabolic changes, if any, in response to a single dose acute cranial irradiation using high resolution NMR spectroscopy based metabolomics approach.

2. Materials and methods

2.1. Animal handling and radiation exposure

Eighteen Swiss albino strain 'A' male mice (8–10 weeks old) weighing between 25 and 35 g were taken and acclimatized for 48 h in polypropylene cages under standard temperature, humidity conditions prior to group allocation and treatment. During the study, room temperature and relative humidity was maintained within a range of 19–23 °C and 45–65% respectively. The fluorescent lighting was provided (6 a.m. to 6 p.m.) for 12 h light/12 h dark cycle. Animals were randomly divided into two groups. To look for the effect of single dose of cranial irradiation, twelve animals in the first group were exposed to 8 Gy single dose cranial radiation using Tele ^{60}Co gamma irradiation facility (Bhabhatron II, Panacea, India) with source operating at 2.496 Gy/min. We opted a model for a single dose of cranial radiation on 8 Gy as this radiation dose in mouse approximate a clinical relevant dose (Monje et al., 2002; Marks et al., 1981). Only cranium was irradiated with a total field of view of 2×2 cm^2 covering the cranium at a surface to source distance (SSD) of 80 cm. Rest of the animals ($n = 6$) served as sham irradiated controls. During irradiation and sham radiation, mice were mildly sedated using a mixture of ketamine/xylazine (80/10 mg per kg body weight) and placed on the table individually in prone position.

2.2. Dissection and extraction of hippocampal tissue

Based on our recent diffusion tensor imaging (DTI) finding of cranial radiation-induced microstructural changes, at days 5 and 10 post irradiation; these time points were chosen to look for metabolic changes in hippocampus (Gupta et al., 2013). Therefore, hippocampus tissue was collected on days 5 and 10 post irradiation, between 8:00 A.M and 10:00 A.M following the sacrifice of animals ($n = 6$ at each time point) by cervical dislocation. Hippocampal tissue was collected from control animals ($n = 6$) only once. After sacrificing the animals, whole brain was removed and kept on a sterile ice cold glass plate placed over the ice in an ice bucket. Hippocampus of each animal was immediately and carefully isolated. After that, each tissue sample was rapidly weighed, frozen in liquid nitrogen and stored at −80 °C until further analysis. The entire process from removal of brain to dissecting out hippocampus was completed within 2–3 min. Animal handling and experimental protocols were performed and approved (INM/IAEC/2012/10) in strict accordance with the guidelines of the institutional animal ethical committee.

The polar metabolite extracts of dissected hippocampus (50–70 mg) were obtained based on the acetonitrile extraction method as explained by Becknort et al. (2007). In short, tissue was homogenized with 5 mL of acetonitrile/water mixture (1:1, v/v) per g of frozen tissue using handheld Homogenizer (IKA T-10, Germany). The homogenized mixture was then centrifuged at 12,000 g for 10 min at 4 °C. The supernatant was taken out, lyophilized and stored at −80 °C until NMR analysis.

2.3. ^{1}H NMR spectroscopy of hippocampus tissue extracts

The tissue extracts were reconstituted in 480 μl of deuterated water (D_2O) containing 1 mM trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP) as an internal NMR chemical shift standard and transferred to 5 mm NMR tubes for NMR spectroscopy. ^{1}H NMR spectra were acquired for each sample at 400.13 MHz on a Bruker 400 MHz spectrometer (Bruker Biospin, Germany) at 300 K using standard 1D ^{1}H NMR pulse sequence with water suppression (ZGPR). For each sample, 128 transients were collected into 32 K data points with a relaxation delay of 2 s, flip angle of 90° and a mixing period of 100 ms. A spectral width of 6410.256 Hz and an acquisition time per scan of 5.11 s were used. The free induction decay (FID) signal was multiplied by an exponential weighting function corresponding to line broadening (0.3 Hz) before Fourier transform. The metabolites were assigned on the basis of their chemical shifts and signal multiplicity as described by Lindon et al. (1999).

2.4. Data Reduction and Pattern Recognition (PR) analysis of ^{1}H NMR spectra

NMR spectra were manually corrected for phase and baseline distortions using Bruker TOPSPIN 2.1. Each ^{1}H NMR spectrum from