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# Early antioxidant responses via the concerted activation of NF-κB and Nrf2 characterize the gamma-radiation-induced adaptive response in quiescent human peripheral blood mononuclear cells

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ABSTRACT

The radiation-induced adaptive response (RI-AR) is a non-targeted effect which is outside the scope of the classical Linear-No-Threshold (LNT) dose-response paradigm. However, the mechanisms of the RI-AR are not well understood. We have studied the RI-AR in quiescent human peripheral blood mononuclear cells (PBMCs). PBMCs in $G_0$ phase were 'primed' with a low dose (100 mGy gamma radiation) and then, after an 'adaptive window' of 4 h, 'challenged' with a high dose (2 Gy). A small (5.7%) increase in viability and a decrease in DNA strand breaks were seen in primed cells, compared to non-primed cells. This was consistent with lower levels of reactive oxygen species, higher mitochondrial membrane potential, and increased activity of antioxidant enzymes such as catalase, superoxide dismutase, thioredoxin reductase, and glutathione peroxidase, in the primed cells. Reduced oxidative stress in primed PBMCs correlated with greater nuclear translocation of the redox-sensitive transcription factors Nuclear factor kappa B (NF-κB) and Nuclear factor E2-related factor 2 (Nrf2). Distinct differences in responses were seen in PBMCs irradiated with low dose (100 mGy) and high dose (2 Gy). These findings provide insight into the mechanisms of radioadaptation in human cells.

## 1. Introduction

The effect of low-dose radiation (LDR) on human cells is a relevant public health issue. Humans are continually exposed to LDR from cosmic rays, terrestrial sources, and medical sources. At present, health risks of exposure to LDR are usually estimated by extrapolating from data obtained with exposure to high-dose radiation (HDR) under the linear no-threshold (LNT) hypothesis [1]. However, evidence for non-targeted responses, such as the radiation-induced adaptive response (RI-AR), bystander effects, genomic instability, and low dose hypersensitivity, indicates that cellular responses might differ with LDR as compared to HDR, casting doubt on the basic assumptions of LNT [2].

RI-AR occurs when a prior low "priming" dose (PD) decreases the biological effectiveness of a subsequent high "challenge" dose (CD). RI-AR does not involve direct irradiation of a defined target in the cell and, thus, challenges the traditional target theory of radiation biology. Since the seminal report of Olivieri et al. indicating the existence of RI-AR in mammalian cells [3], adaptive responses have been demonstrated *in vitro* and *in vivo* using endpoints such as chromosome aberrations, micronuclei, mutations, neoplastic transformation, and induction of immune response and gene expression [4–8].

The mechanisms of RI-AR remain poorly defined because of challenges such as variability between individuals, cell types and cell-cycle phases. There have been conflicting reports on the existence of an adaptive response (AR) in quiescent human cells. Many groups, such as Shadley et al. and Wang et al., did not observe AR in pre-irradiated lymphocytes [9,10], while others showed even a synergistic response [11,12]. In the human body, some cells are constantly dividing while others remain in a non-dividing state ($G_0$). Thus, data from *in vitro* studies on exponentially dividing cells may be of limited relevance to the effects of radiation on tissues *in vivo*.

We have used $G_0$ human peripheral blood mononuclear cells (PBMCs), derived from freshly drawn venous blood, to study RI-AR. The use of primary human PBMCs allows us to capture early cellular events without the bias associated with transformed cell lines, which may present altered repair mechanisms and cell-cycle progression. Since PBMCs are non-dividing, variations in radiosensitivity as cells move through the cell cycle were averted. Thus, human PBMCs, which exist in a state of quiescence, but can be disengaged when necessary, effectively mimic *in vivo* conditions. We used DNA strand breaks to establish the

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existence of AR in quiescent cells. Several hypothesized mechanisms of RI-AR, including the roles of DNA damage and repair, reactive oxygen species (ROS), antioxidant defence systems, and the stress-responsive transcription factors Nuclear factor kappa B (NF-κB) and Nuclear factor E2-related factor 2 (Nrf2), were simultaneously tested in the same sample set to minimize variability between individuals and provide a holistic view of the process.

## 2. Materials and methods

### 2.1. Collection of samples

The work was approved by the institutional Medical Ethics Committee and informed consent was obtained from each donor. Peripheral blood was drawn from seven healthy volunteers (non-smokers; age 22–35 y) by venepuncture into EDTA vacutainer tubes (BD™ Vacutainers, NJ, USA) and processed within 30 min of withdrawal. For repeated collection of blood from the same individual, a minimum 15-d gap between two withdrawals was maintained.

### 2.2. Isolation of PBMCs and irradiation

PBMCs were isolated by density gradient centrifugation using Histopaque 1077 (Sigma-Aldrich Corp, MO, USA) according to the manufacturer's instructions and washed twice with phosphate-buffered saline (PBS) before further processing. Cells were counted in a hemocytometer and used immediately.

Cells were irradiated in PBS (500 µl) at room temperature using a $Co^{60}$ gamma-ray source (Blood irradiator, 2000, BRIT, India) at a dose rate of 0.417 Gy/min. For irradiation, four sets of cells were prepared. The first set was irradiated with a priming dose (PD) of 100 mGy followed by a challenge dose (CD) of 2 Gy after an adaptive window of 4 h (henceforth referred to as 'primed cells'). The second set was sham irradiated along with the first irradiation of set one and then irradiated with a 2 Gy dose after an adaptive window of 4 h, as in set one (henceforth referred to as 'non-primed' cells). The third set was irradiated with 100 mGy dose only, to understand the effects of LDR. The fourth set of cells was sham irradiated and served as controls. The cells were processed either within 5 min of irradiation (henceforth, referred to as 0 min time point) or were incubated in complete Roswell Park Memorial Institute (RPMI) – 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C in a humidified 5% $CO_2$ incubator (Eppendorf, Hamburg, Germany) for the required time, as specified for different assays. Fetal bovine serum was obtained from Gibco BRL (MD, USA). All other chemicals for cell culture were procured from Sigma-Aldrich Corp MO, USA.

### 2.3. Alkaline single-cell gel electrophoresis (comet) assay

The assay was performed as described earlier [13]. Briefly, $2.5 \times 10^4$ cells (100 µl) were mixed with warm 0.8% low-melting agarose (Sigma-Aldrich Corp MO, USA), 400 µl, prepared in 0.9% saline and applied to frosted slides precoated with 1% normal agarose. After lysis and alkaline unwinding, electrophoresis was carried out at 25 V, 300 mA for 30 min (~1.4 V/cm).

After neutralization in tris buffer (pH 7.4) for 5 min, comets were visualized using SYBR Green II (Sigma-Aldrich Corp MO, USA) using a dilution of 1:10,000. Fifty cells from each slide were examined using a fluorescence microscope (Carl Zeiss AG, Switzerland) equipped with a 3CCD camera (Sony Corp, Tokyo) at magnification 400X. Images were analysed using CASP software. The extent of DNA damage was expressed as % DNA in the comet tail.

### 2.4. Cell viability

Cell viability was assessed with the trypan blue dye-exclusion assay. A 1:1 dilution of cell suspension was made using a 0.4% trypan blue solution (in PBS, pH 7.2). Cell suspension (10 µl) was loaded onto a Neubauer hemocytometer immediately and counted under an inverted bright field microscope (Carl Zeiss AG, Switzerland). Viability index (VI) was calculated as (number of live cells in treatment/number of live cells in control) x 100.

### 2.5. Mitochondrial membrane potential (MMP)

Radiation induced changes in MMP ($\Delta\Psi m$) were measured using a dual emission fluorescent cationic dye, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1). In healthy cells with high $\Delta\Psi m$, JC-1 spontaneously forms J-aggregates which give intense red fluorescence. In unhealthy cells with membrane depolarization, JC-1 remains in the monomeric form and gives only green fluorescence. PBMCs ($1 \times 10^6$) were irradiated and incubated in complete RPMI-1640 media at 37 °C in a humidified 5% $CO_2$ atmosphere for 24 h. After incubation, PBMCs were loaded with 5 µM JC1 (Sigma-Aldrich Corp MO, USA) for 10 min at 37 °C and 5% $CO_2$ in the dark and 20,000 cells were acquired on a CyFlow Space flow cytometer (Partec, GmbH Germany). Data was analysed using FlowJo (Treestar Inc, Ashland, USA).

### 2.6. Assessment of apoptosis using Annexin V-FITC and Propidium Iodide dual staining

After irradiation, PBMCs ($1 \times 10^6$) from each dose group were incubated at 37 °C in a humidified 5% $CO_2$ atmosphere (Eppendorf, Hamburg, Germany) for 24 h. The cell pellet was then suspended in 100 µl labelling solution for 15 min in the dark, as per manufacturer's instructions (Annexin-V-FLUOS labelling kit, Roche Diagnostics GmbH, Germany). Fluorescence intensity was measured by flow cytometry (Partec, GmbH Germany) and 20,000 cells, in triplicate, were acquired. Cells without label and cells stained separately with only Annexin V-FITC and only PI were used as control for setting the gates. FlowJo (Treestar Inc, Ashland, USA) was used for data analysis.

### 2.7. Intracellular and mitochondrial (mt) ROS

Intracellular and mitochondrial ROS levels were measured using $H_2$DCF-DA (2′, 7′-dichlorodihydrofluorescein diacetate) and MitoSOX red [3,8-phenanthridinediamine, 5-(6′-triphenylphosphoniumhexyl)-5,6-dihydro-6-phenyl], respectively. $H_2$DCF-DA was purchased from Sigma-Aldrich Corp MO, USA and MitoSOX red was procured from Molecular Probes (Invitrogen, CA, USA). PBMCs ($1 \times 10^5$) were incubated either with 20 µM $H_2$DCF-DA or with 5 µM MitoSOX red indicator dye at 37 °C for 20 min before irradiation and analysed immediately after irradiation (0 min). Fluorescence of DCF was measured by acquiring 20,000 cells on a flow cytometer (Partec, GmbH Germany) and data was analysed using FlowJo software (Treestar Inc, Ashland, USA).

For direct visualization, PBMCs were adhered to poly L-lysine coverslips (BD Biosciences, NJ, USA), incubated with $H_2$DCF-DA (20 µM) and observed under 200X magnification on a fluorescence microscope (Carl Zeiss, AG, Switzerland). MitoSOX red fluorescence was measured at λex/em = 510 nm/580 nm using a multiwell plate reader (Synergy H1 Hybrid, Biotek, USA).

### 2.8. Total thiols and intracellular thiols

Total thiols and intracellular thiol levels were measured either immediately (0 min) or 60 min after irradiation. Total thiols were estimated using $2 \times 10^6$ PBMCs with a thiol detection kit procured from