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Effects of low-dose X-rays on the oxidative state, lipid peroxidation and membrane fluidity of human peripheral blood mononuclear cells

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ABSTRACT

Background: There is a concern about the effects of low-dose radiation used in medical applications due to risk of biological effects, as these effects have not been fully evaluated.

Objectives: This study aimed to evaluate the effects of low-dose X-ray on the intracellular reactive oxygen species (ROS), lipid peroxidation, membrane fluidity, and cell viability of human peripheral blood mononuclear cells (PBMCs).

Materials and methods: Cells were irradiated using an X-ray generator at the radiation energy of 120 kVp to obtain the absorption dose of 0.05, 0.1 and 0.2 Gy. Fluorescent probe 2'7'-dichlorofluorescein diacetate (DCFH-DA) was used to evaluate intracellular reactive oxygen species of PBMCs. Thiobarbituric acid reactive substances assay (TBARS) was applied to determine malondialdehyde (MDA) level which is an indicative of lipid peroxidation. Membrane fluidity was also determined by fluorescence anisotropy of the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH). Finally, cell viability was determined by resazurin assay.

Results: The instant effects of low-dose X-rays show a significant decrease in ROS level at 0.1 and 0.2 Gy. MDA level per cell of non-irradiated PBMCs was 6.12±1.67 (SD) fmole per cell. There are no significant alteration of MDA level and membrane fluidity from the effects of X-ray at doses up to 0.2 Gy. The cell viability at 72 hours after irradiation at 0.2 Gy shows a significant decreased.

Conclusion: Low-dose of X-rays on human peripheral blood mononuclear cells (PBMCs) shows a significant decrease in the intracellular reactive oxygen species (ROS) (0.1 and 0.2 Gy) and cell dead (0.2 Gy).

Introduction

Due to risk of biological effects from low-dose ionizing radiation used in medical imaging, radiation effects at the subcellular level need to be better evaluated. Intracellular biochemical changes such as damage of DNA, lipids, and proteins can be provoked from ionizing radiation due to direct energy transfer and by free radicals from water radiolysis. High linear energy transfer (LET) radiation can directly damage DNA, while the low LET radiations (i.e. γ-rays, X-rays) can cause damage by oxidative production.[1-6] LET value of diagnostic X-ray is about 3 keV.μm^{-1}.[7] Ionizing radiation is the most important physical stimulus that causes intracellular reactive oxygen species (ROS) formation. In radiation therapy, ROS induces cancer cell death by biochemical changes not only from DNA damage, but also from oxidative membrane damage that causes defects in the cellular mechanism. Membrane damage by ROS occurs as the lipid peroxidation of membrane leads to an altering fluidity and permeability.[2] In high dose X-rays (20-100 Gy), double strand DNA breakage

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that causes cell damage can be determined.[8] High dose radiation also induces lipid peroxidation and increases the membrane fluidity at the interface between proteins and lipids in cell membranes. The lipid peroxidation can be acquired from unsaturated fatty acid interactions with reactive oxygen species to produce lipid hydroperoxides (LOOH), which then form secondary products such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). MDA can induce cellular damage or tissue injury.[9, 10] A study done on high dose radiation (up to 50 Gy) revealed the death of PBMC cells and the increase of membrane fluidity.[11, 12] Despite the fact that radiation induces oxidative stress leading to lipid peroxidation, at very low-doses radiation (0.1 mGy), is contradictory effects on MDA values of different tissues, For example, there are decreases in brain tissue, but increases in lung tissue in experiments done using mice models.[13] Diagnostic radiation doses for general procedures are in range of 0.001-1.5 mGy, while, more special procedures reach up to 10 mGy. Radiation dose of the organ in the beam during CT scanning ranges from 10-100 mGy.[14] In fact, late effects of low-dose radiation of γ-rays were studied by our team in animal models which showed that the effect on genomic instability depended on radiation dose, duration after irradiation and animal type. Our previous data showed no evidence of genomic instability being induced by low-doses.[15-17] DNA is considered to be a principle cellular target for stochastic effects of radiation on cells that occur either directly or indirectly. During irradiation, free radicals are always generated and act as an indirect effect on cellular targets. While DNA damage leads to chromosomal aberrations, gene mutations, and then cell death, defects in other organelles or cellular mechanisms such as mitochondria, endoplasmic reticulum, or plasma membrane can occur, as well. Additionally, reactive oxygen species can also cause cell malfunction.[18-21] Indeed, the interaction of radiation with cell organelles is not a selected target. Aside from DNA, other intracellular targets for determining the early responses to radiation effects should be investigated. Among cellular organelles, plasma membrane is a cellular frontier that involves many cellular mechanisms such as transport controllers, cellular identified markers, immune responses, etc. Therefore, damage to plasma membrane should be a major concern. To clarify effects of radiation in diagnostic procedures, this work focuses on the instant effects occurring at the cellular level of low-dose X-ray (up to 0.2 Gy) that induce reactive oxygen species, lipid peroxidation, membrane fluidity, and cell viability of human peripheral blood mononuclear cells (PBMCs).

Materials and methods

Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were collected from buffy coat of healthy donors (age 20-30 years old, blood group O) who had no record of thalassemia and glucose-6-phosphate dehydrogenase deficiency. PBMCs were separated by a gradient density centrifugation technique using ficoll hypaque solution (Lymphoprep™, Norway).[11] Cells were cultivated in an RPMI-1640 medium (Capricorn-scientific, German) supplemented with 10% fetal bovine serum (Capricorn-scientific, German) and 1% penicillin-streptomycin (Capricorn-scientific, German) and they were incubated at 37 °C humidified with 5% CO_2 until time of irradiation.

This study was approved by the Ethical Committee of the Faculty of Associated Medical Sciences, Chiang Mai University (Reference number: AMSEC -62EM-007).

Radiation exposure

Accuracy and linearity of radiation output of X-ray machine (Shimadzu, Collimator type R-20J, Japan) were evaluated first, using a Scintillation detector (Radcal, AGMS-D+, USA). For irradiation, an appropriate amount of PBMCs for each experiment was collected from a culture medium and kept in a volume of 100 µL of phosphate buffer solution (PBS) pH 7.4, then was exposed at 120 kVp and 320 mA.

Measurement of intracellular reactive oxygen species levels

Intracellular reactive oxygen species level in PBMCs was determined by using 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Sigma-Aldrich, USA) as a molecular probe. Irradiated PBMCs (1×10^6 cells) were incubated in 900 µL PBS containing 2 µM of DCFH-DA for 30 minutes. Dichlorofluorescein (DCF) fluorescence intensity representing reactive oxygen species level was measured using a spectrofluorometer (Perkin Elmer, model LS55, USA) at 523 nm when excited at 502 nm.[22]

Measurement of lipid peroxidation

Lipid peroxidation level was measured by using thiobarbituric acid reactive substances assay (TBARS). This method directly measures malondialdehyde (MDA) that is a degradation product of fats as lipid peroxidation. Irradiated PBMCs ($4x10^6$ cells) were incubated at 80 °C for 1 hr in a solution containing 400 µL PBS and 500 µL of working reagent consisting of 2 $mg.mL^{-1}$ of 2-thiobarbituric acid (TBA; Sigma-Aldrich, Japan) solubilized in a mixture of 50 mM NaOH (RCl labscan, Thailand) and glacial acetic acid (Fisher Scientific, UK) with a ratio of 1:1. Next, this solution was completed by cooling down at 25 °C for 10 minutes. An absorption spectrum of MDA-TBA reaction product was determined by spectrophotometer (Agilent, model 8453, China).[23, 24] MDA standard curve was generated using an MDA standard range of 1 to 100 µM.

Membrane fluidity

Membrane fluidity of PBMCs was observed using fluorescence probe 1,6-diphenyl-1,3,5-hexatriene (DPH; Sigma-Aldrich, Japan). Irradiated cells ($5x10^5$ cells) in 100 µL of PBS were added into 0.1 µM DPH solution (1,900 µL), and incubated for 10 minutes. Next, the fluorescence intensity at 430 nm (when excited at 350 nm) was determined using a spectrofluorometer. Fluorescence anisotropy (r) value was calculated as follows:[25, 26]

$$r = \frac{I_{VV} - G.I_{VH}}{I_{VV} + 2G.I_{VH}}$$

Where I_{VV} : fluorescence intensity of components for vertical/vertical (parallel),

I_{VH} : fluorescence intensity of components for vertical/horizontal (perpendicular),

Correction factor G : ratio of I_{HV}/I_{HH}

Cell viability

Cell viability was determined by fluorescent resazurin sodium salt (Sigma-Aldrich, Japan). Irradiated PBMCs (5×10^4 cells) were cultivated in a 900 µL RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C humidified with 5% CO_2 for 72 hrs. To investigate cell viability, 100 µL of resazurin solution (0.1 $mg.mL^{-1}$) was added and cells were incubated at 37 °C and humidified with 5% CO_2 for 4 hrs. Resazurin fluorescence intensity at 590 nm (exited at 570 nm) that is an indicator of living cells was measured on a spectrofluorometer using well-plate reader.[27] Cell viability was quantified by resazurin fluorescence intensity of samples in terms of the percentage of control.

Statistical analysis

Descriptive data were informed as mean±standard error (SE). Statistical analysis was performed by OrigingPro8 Software. All obtained parameters from a variety of radiation doses were analyzed by One Way ANOVA (Tukey procedure). Significance level was denoted at the alpha value of 0.05.

Results

X-rays output showed a good quality either accuracy or linearity with a dose rate of 11.5 $Gy.min^{-1}$ at 120 kVp and 320 mA. The PBMCs for each experiment was exposed at 0.05, 0.1, and 0.2 Gy. All studies were operated in parallel comparison with the non-irradiated group.

The intracellular reactive oxygen species of PBMCs

The fluorescence intensity levles of DCF revealing intracellular reactive oxygen species (ROS) of irradiated PBMCs at 0.05, 0.1, and 0.2 Gy were evaluated and the percentage of non-irradiated cells was reported. The results showed a significant decrease in ROS level at 0.1 and 0.2 Gy. (Figure 1)

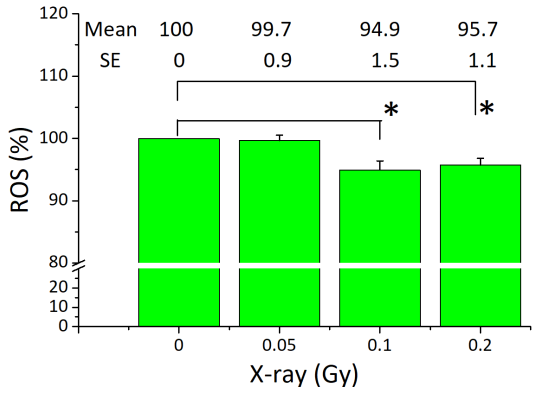

Figure 1. *Percentage of intracellular reactive oxygen species level (ROS) of PBMCs (mean±SE) after irradiation to X-rays dose of 0.05, 0.1 and 0.2 Gy (*p<0.05).*

Lipid peroxidation of PBMCs

Lipid peroxidation of PBMCs was quantitatively measured from optical density of MDA-TBA production at 532 nm. The results demonstrated that intrinsic MDA levels of non-irradiated PBMCs was equal to 6.12±1.67 (SD) fmole per cell. Lipid peroxidation of PBMCs was slightly diminished by the effects of low-dose X-rays, but this was not a significant difference. (Table 1).

Table 1 Malondialdehyde (MDA) levels of PBMCs after the irradiation to X-rays dose of 0.05, 0.1 and 0.2 Gy.

X-ray (Gy)	Mean of MDA (fmole per cell)	SE
0	6.12	0.48
0.05	5.85	0.35
0.1	5.92	0.33
0.2	5.90	0.40

Membrane fluidity of PBMCs

Membrane fluidity of PBMCs taken as an indicator of mobility of membrane lipids was explored by measuring fluorescence anisotropy at 37 °C. Our results showed that anisotropy value at a dose of 0.2 Gy was not significantly altered after irradiation. (Figure 2)

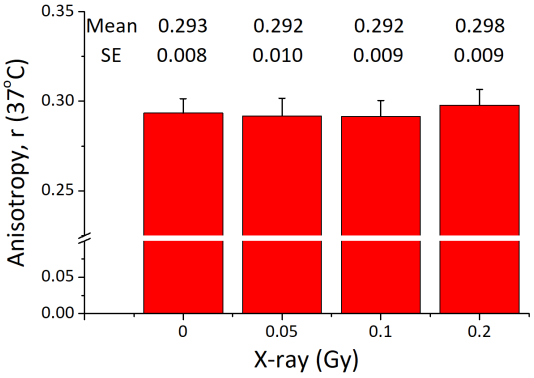

Figure 2. *Fluorescence anisotropy (r) of PBMCs determined at 37 °C after irradiation to X-rays dose of 0.05, 0.1, and 0.2 Gy.*

Cell viability

Effect of low-dose X-rays on cell viability was assessed at 72 hrs after irradiation. Data showed a significant decrease as a function of the increasing absorbed dose. (Figure 3)

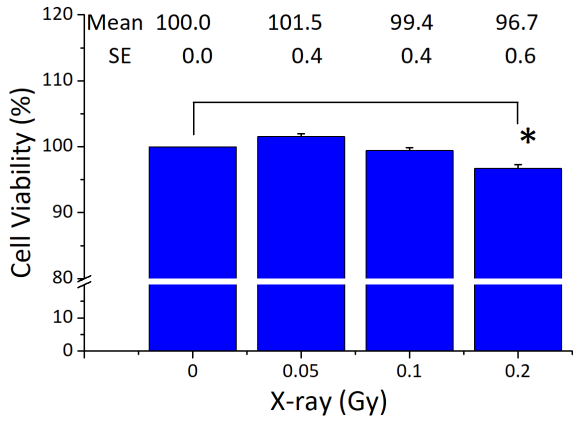

***Figure 3**. Percentage of cell viability of PBMCs after irradiation to X-rays dose of 0.05, 0.1, and 0.2 Gy (*p<0.05).*

Discussion

In this work, normal human peripheral mononuclear cells were used as the cellular model. Intracellular reactive oxygen species (ROS), lipid peroxidation in terms of malondialdehyde (MDA), and membrane fluidity of peripheral blood mononuclear cells (PBMCs) were instantly explored after irradiation by X-ray at 0.05, 0.1, and 0.2 Gy, whereas the cell viability was examined at 72 hours after irradiation. The results indicated a significant decrease in ROS and cell viability and no detectable effect on lipid peroxidation which is also consistent with the absence of influence on membrane fluidity in PBMCs exposed to low-dose X-rays. In normal condition, malondialdehyde is the main lipid peroxidation production that causes free radical such as superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). Superoxide radical ($O_2^{\bullet-}$) is catalyzed by superoxide dismutase (SOD) to be hydrogen peroxide (H_2O_2). Then hydrogen peroxide is further catalyzed to produce H_2O.[28, 29] Effects of radiation on oxidant state in living beings is spontaneously regulated depending on the oxidant species, antioxidant mechanism, tissue types, and time. In animal models, superoxide dismutase (SOD) regulate by decreasing its activity at very low-dose of 100 μGy, but continuously increases activity several hours after radiation at doses ranging from 0.05 Gy to 0.2 Gy. This is consistent with our observation in PBMCs and suggests that under low dose X-rays irradiation conditions either the activity or cell concentration of superoxide dismutase increases in order to decrease percentage of cellular irradiation-generated ROS. Alternative mechanisms of cell protection against ROS might be involved since apart from an increase of SOD activity, contradictory results were found in MDA levels.[13, 30] DCFH-DA is a free radical probe that mainly interacts with hydrogen peroxide species (H_2O_2) in intact cell, thus a diminishing of intracellular reactive oxygen species occurs after irradiation which might explain the consequent effects of SOD activity.[31] Similar effects were also observed on normal human lung epithelial cells (HBE135-E6E7) treated with irradiation doses in the range 0.02-0.1 Gy. Moreover, it was observed that X-rays stimulated cell proliferation before appearance of growth inhibition at higher doses of 0.2-3 Gy.[32] Concerning the membrane fluidity, fluorescence anisotropy (r) is observed by fluorescent probe (DPH) that is embedded in the lipophilic part of lipid bilayer. In this work, membrane fluidity did not significantly change from the effects of X-ray at doses up to 0.2 Gy which is in agreement with the results obtained at the same physiological temperature on a previous medical diagnostic X-rays study.[26] The effects of radiation might involve either lipids or membrane proteins that causes membrane rigidity through lipid-protein or protein-protein crosslink.[33, 34] Absence of significant effect on lipid peroxidation under our irradiation conditions is then correlated with the absence of detectable effect on membrane fluidity. This study showed at X-rays doses of 0.05-0.2 Gy no detectable deleterious effects could be observed on cellular membranes. Our results suggest that under the irradiation conditions described the integrity of cellular membranes of PBMCs was conserved.

Conclusion

In summary, low-dose X-ray (0.05-0.2 Gy) lead to decreases in the intracellular reactive oxygen species (ROS) and cell viability in human peripheral blood mononuclear cells (PBMCs). However, radiation doses at 0.05 and 0.1 Gy altered PBMCs by insignificantly diminishing lipid peroxidation (MDA) and increased membrane fluidity. At 0.2 Gy, contradictory evidence was observed. This might be caused by the regulation of cellular antioxidant mechanism.

Conflicts of interest statement

The authors declare none of conflict of interest.

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