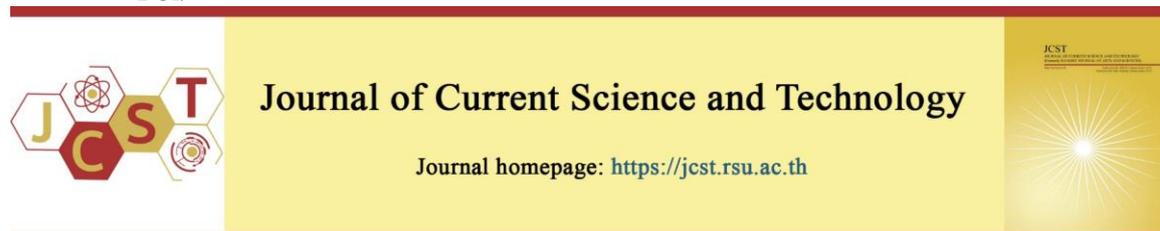


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Effect of X-ray diagnostic energy to peripheral blood mononuclear cells and CD34⁺/CD133⁺ expression: an *in vitro* study

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Abstract

X-rays are high-energy waves that have a great ability to penetrate other materials. For that reason, X-rays are often used in medical applications to diagnose and treat cancer. There are many benefits for patients from medical imaging but X-rays can damage the cells in human body. The aim of this research was to study the effects of diagnostic X-rays on peripheral blood mononuclear cells (PBMCs) and CD34⁺/CD133⁺ populations in healthy volunteers *in vitro* and determine the ratio of CD34⁺/CD133⁺ in PBMCs. The PBMCs were isolated by ficoll-centrifugation technique. The morphology and CD34⁺/CD133⁺ expression of PBMCs were observed by inverted microscope and flow cytometry at 1 hr, 1, 5, 10, and 15 days after irradiation based on plain film x-ray technique of 70-110 kVp, 5-40 mAs, and radiation dose of 0.47-2.30 mGy. Freshly isolated PBMCs were spherical and after X-ray irradiation at day 15 revealed that the morphology was similar in both groups and the CD34⁺/CD133⁺ expression showed no difference from control when using the lowest radiation dose at 0.47 mGy. The overall results indicated that increasing the radiation dose had significant effects on PBMCs and the CD34⁺/CD133⁺ population of cells. Despite these negative effects, the benefits of radiation to both workers and patients outweigh the drawbacks.

Keywords: CD34⁺; CD133⁺; ionizing radiation; peripheral blood; peripheral blood mononuclear cells; X-ray diagnostic.

1. Introduction

In recent years, there has been growing evidence that even ionizing radiation in diagnostic X-rays may have profound effects on cellular function. Individuals may be exposed to low doses of radiation either intentionally for medical purposes or accidentally, such as those exposed to radiological terrorism or those who live near illegal radioactive waste dumpsites (Alessio et al., 2015). Exposure to ionizing radiation is known to have lethal effects in cell biology especially peripheral blood mononuclear cells (PBMCs) and stem cells in blood circulation (Beer et al., 2017).

Peripheral blood is a large accessible source of PBMCs. Especially, the blood mononuclear fraction obtained by gradient-centrifugation (Lund, Joø, Westvik, Øvstebø, & Kierulf, 2000). PBMCs can differentiate into various cells such as blood cells, endothelial cells, hepatocytes, cardiomyocytes cells, smooth muscle cells, osteoblasts, osteoclasts, epithelial cells, neural cells, and myofibroblasts (Zhang, & Huang, 2012).

But the various behaviors and abilities of cells depend on the microenvironment and the many factors that are present at that time (Kantapan et al., 2016; Moonkum et al., 2018). It is known that the proteins expressed on the surface of stem cells, CD34 and CD133, were obtained simultaneously from the bone marrow and peripheral blood from recipients (Jaime-Pérez et al., 2016).

CD34 is predominantly regarded as a marker of hematopoietic stem cells (HSC) and hematopoietic progenitor cells (Sidney, Branch, Dunphy, Dua, & Hopkinson, 2014). Hematopoietic stem cells (HSCs) are rare cells that are maintained throughout life (self-renewing). They produce hematopoietic progenitor cells that differentiate into every type of mature blood cell within a well-defined hierarchy. Among hematopoietic stem/progenitor cell (HSPC) markers, CD34 is well known for its unique expression on HSPCs (AbuSamra et al., 2017). CD133 is another stem cell marker with this fraction containing mesenchymal stem cells (MSCs) with high proliferative potential. MSCs isolated from peripheral blood and umbilical cord blood show the characteristic pattern of mesenchymal surface markers and express Oct4 (Octamer-binding Transcription Factor 4), a marker of pluripotent stem cells (Pochampally, Smith, Ylostalo, & Prockop, 2004).

Ionizing radiation such as X-rays can cause DNA damage and the development of cancer, yet people are constantly exposed to X-rays and other forms of radiation from many different sources (Jones, Mills, Mogensen, & Lee, 2012). X-rays and gamma rays can penetrate into living cells resulting in the transfer of radiation energy to the biological material. The absorbed energy can induce the formation of reactive oxygen species, break chemical bonds and ionize different biologically essential macromolecules, such as DNA, membrane lipids and proteins. The research on the effects of low-dose radiation on cells has a wide range of results due to the variation in cell types, radiation source, and doses (Chen et al., 2014). Some studies have shown no effect of low-dose (<0.1 Gy) radiation on cells (Jiang et al., 2008) but others have demonstrated that low dose X-rays affected DNA double-strand break repair after radiation doses as low as 1 mGy (Rothkamm, & Löbrich, 2003).

Recently, it was reported that radiation can damage living tissue by changing cellular structure and damaging DNA. The amount of damage depends on a number of variables, including the type and quantity of radiation absorbed and its energy (Stewart et al., 2012). The reports demonstrated that radiation (up to 2 Gy) induced hematopoietic stem cell death and found significant generation of mitochondrial superoxide production between 12 hr and 24 hr after X-ray irradiation (Ishikawa, Hayashi, Yamaguchi, Monzen, & Kashiwakura, 2015). In addition, following gamma irradiation endogenous and induced DNA double stranded breaks (DSBs) were evaluated in CD133[±] human umbilical cord blood cells (UCBC) and peripheral blood lymphocytes (PBLs).

However, the study of low radiation dose effects on cellular changes and the risk from diagnostic X-ray machines on the PBMCs and surface markers CD34 and CD133 stem cells during medical examination remain poorly understood and harmful effects of medical radiation remain controversial. Based on our research data, we have studied the effects of diagnostic radiation and analyzed characteristics of the surface markers CD34 and CD13 and documented a change in expression patterns before and after irradiation.

2. Objectives

To study the effects of medical diagnostic radiation using plain film x-ray exposure techniques performed in radiography on the characteristics of PBMCs and the ratio of CD34[±]/CD133[±] expression in healthy volunteers before and after different intervals of irradiation.

3. Materials and methods

3.1 PBMCs from healthy volunteers

The research was approved by the ethical review committee for research in human subjects, Faculty of Radiological Technology, Rangsit University (RSU-ERB2019/026). Four healthy volunteers, both male and female, 20-40 years old, comprised the cohort for the PBMCs used in this study. They did not have disqualifying diseases including diabetes, hypertension, heart disease, cerebrovascular diseases, cancer, or HIV. They were not overweight and did not smoke or consume alcohol. All donors signed informed consent documents prior to blood collection.

3.2 Collection of human peripheral blood mononuclear cells (PBMC) from volunteers and expansion in conventional culture

Whole blood was collected in sterile Vacutainer tubes containing heparin lithium, centrifuged at 4,000 rpm for 20 min and the buffy coat separated and transferred into a sterile conical tube. The cells were adjusted to a final volume of 5 ml with an isotonic solution and then gently mixed. Ficoll-Hypaque (5 ml) was carefully injected at the bottom of the tube prior to centrifugation at 4,000 rpm for 20 min. The PBMCs fraction was isolated and washed once using 5 ml of PBS and resuspended in 5 ml of fresh RPMI 1640 supplemented with 10% FBS and 1% penicillin / streptomycin (BioMedia) and placed in an incubator at 37°C at 5% CO₂ and 95% humidity. Cells (10⁶ cell/mL) were cultured in 24-well plates. The cell morphology was examined under an inverted light microscope and stained for CD34⁺ and CD133⁺ by using a flow cytometer at 1 hr, 24 hr, day 5, day 10, and day 15.

3.3 Enumeration of PBMC and CD34⁺, CD133⁺

The expression of CD34⁺ and CD133⁺ cells was analyzed by immunostaining with FITC-conjugated anti- CD34⁺ and PE- conjugated anti-CD133⁺ antibodies (US Biological Life Sciences). Briefly, cells were suspended with buffer solution and directly stained with fluorescence-conjugated antibodies. The reaction was incubated at 25°C for 30 minutes in the dark and analyzed by Flow cytometer (Beckman Coulter, CA, USA).

3.4 Irradiation system

The cells from the volunteers were counted and divided into two groups for the control non-irradiation and X-ray irradiation. Irradiations were done at 1 hr after the initiation of PBMC. At irradiation, the 24 well plate was placed in the center of an X-ray beam at 100 cm from the X-ray tube and setting the exposure technique from the plain film x-ray. The experiment was performed in triplicate by a diagnostic X-ray machine (Quantum medical imaging, Quest HF series, Carestream, NY, USA), and the radiation dose was measured by Radcal – Accu-Gold Touch Testing Devices and the parameters of irradiation are shown in Table 1.

Table 1 The parameters obtained by the medical X-ray machine operated at 70-110 kVp, 5-40 mAs and absorbed radiation dose

Exposure technique	kVp	mAs	mGy
Technique for PA chest X-ray	110	5	0.47
Technique for pelvis X-ray	70	20	0.71
Technique for abdomen X-ray	90	20	1.16
Technique for lumbar spine X-ray	90	40	2.30

kVp = kilovolt peak, mAs = milliampere-seconds, mA = milliampere, sec = seconds, mGy = milligray

3.5 Statistical analysis

Statistical analysis was performed by OriginPro8 Software. All data were expressed as the mean ± SD (standard deviation). For all statistical analyses, values of *p* < 0.05 were considered significant. Statistical analysis was done by T-test.

4. Results

4.1 Radiation dose from exposure technique

The radiation dose from X-ray output was 70- 110 kVp and 5-40 mAs. The PBMCs for each experiment were exposed at doses of 0.47, 0.71, 1.16, and 2.30 mGy from exposure techniques for the chest, pelvis, abdomen and lumbar spine. All studies were operated in parallel comparison with the non-irradiated group.

4.2 Effect of X-ray irradiation on PBMCs culture

After isolation, the freshly harvested PBMCs were cultured in RPMI media containing 10% fetal bovine serum (FBS). The morphology of these cells was characterized using an inverted microscope, comparing the control group and the irradiated group.

As seen in Figure 1, the PMBCs were only suspension cells in 1 hr and had a round oval shape throughout the well, keeping this morphology throughout culturing until day 5; the colonies of PBMCs were observed in both groups. At days 5, 10, and 15 we found cell differentiation with most cells having undergone a shape change such as mesenchymal cells and the PBMCs which can similarly undergo differentiation in day 15 for both groups.

4.3 Expression of CD34 and CD133 stained in PBMCs after irradiation

PBMCs cells were stained with anti-CD34-FITC and anti-CD133-PE and compared

expression of cells stained between the non-irradiated and the irradiated group by various radiation doses from exposure techniques. The data are shown in Table 2.

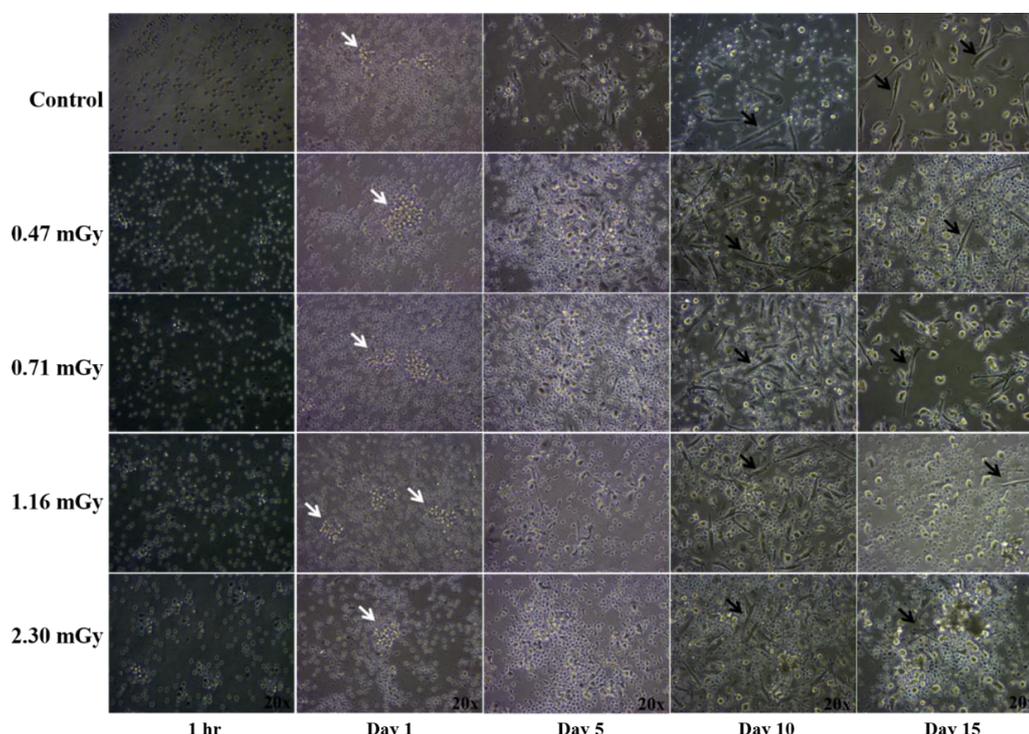


Figure 1 Micrographs of PBMCs at 1 hr, day 1, day 5, day 10, and day 15 (20x). The white arrows show the colonies of PBMCs and black arrows show cell differentiation.

Table 2 The percentage CD34⁺ and CD133⁺ expression in PBMCs

Times	Techniques	Doses (mGy)	% positive cells stained (CD34 ⁺)	% positive cells stained (CD133 ⁺)
1 hr	Control	0	84.35	95.67
	Technique for chest X-ray	0.47	84.99	96.04
	Technique for pelvis X-ray	0.71	84.52	96.89
	Technique for abdomen X-ray	1.16	84.49	95.75
	Technique for lumbar spine X-ray	2.30	85.71	96.39
24 hr	Control	0	85.63	78.35
	Technique for chest X-ray	0.47	91.11	97.37
	Technique for pelvis X-ray	0.71	85.75	95.45
	Technique for abdomen X-ray	1.16	91.35	97.38
	Technique for lumbar spine X-ray	2.30	91.37	97.37
Day 5	Control	0	99.62	95.47
	Technique for chest X-ray	0.47	89.76	96.64
	Technique for pelvis X-ray	0.71	89.86	96.42
	Technique for abdomen X-ray	1.16	88.73	97.16
	Technique for lumbar spine X-ray	2.30	91.88	97.73
Day 10	Control	0	95.65	96.20
	Technique for chest X-ray	0.47	92.94	96.94
	Technique for pelvis X-ray	0.71	91.51	96.03
	Technique for abdomen X-ray	1.16	93.14	96.79
	Technique for lumbar spine X-ray	2.30	92.29	95.44
Day 15	Control	0	93.85	72.31
	Technique for chest X-ray	0.47	96.77	96.27
	Technique for pelvis X-ray	0.71	97.90	84.54
	Technique for abdomen X-ray	1.16	75.39	64.60
	Technique for lumbar spine X-ray	2.30	96.37	90.43

In the analysis, cells stained cell by anti-CD34-FITC and anti-CD133-PE found that the percentage of CD34⁺ was less than CD133⁺ in both the control and irradiated groups during the first period but when the experiment continued until day 15, we found that the percentage CD34⁺ cells were more than CD133⁺ in both groups. The percentage of cells stained in the irradiated group were treated by various radiation doses 0.47, 0.71, 1.16, and 2.3 mGy. The expression of CD34⁺ cells ranged between 84.35-85.71 while the levels of CD133⁺ ranged from 95.75-96. At 1 hr, Figure 2a shows that the percentage of cell stained for CD34⁺ was less than CD133⁺ in both groups. But at 24 hr and 5

days, it was found that the percentage of cells stained for CD34⁺ were more than CD133⁺ in the control group but in the irradiated group various radiation doses were found were to have caused more of a reduction in cells stained for CD34⁺ than CD133⁺ in Figures 2b and 2c.

At day 10 we found that the percentage of cell stained for CD34⁺ (91.51 to 65.95%) was less than CD133⁺ (95.4 to 96.94%) in both groups. The results were similar to the experiments at 1 hr after being irradiated (Figure 2D). Samples taken at day 15 were found to stain for CD34⁺ more than CD133⁺ in both groups as seen in Figure 2e.

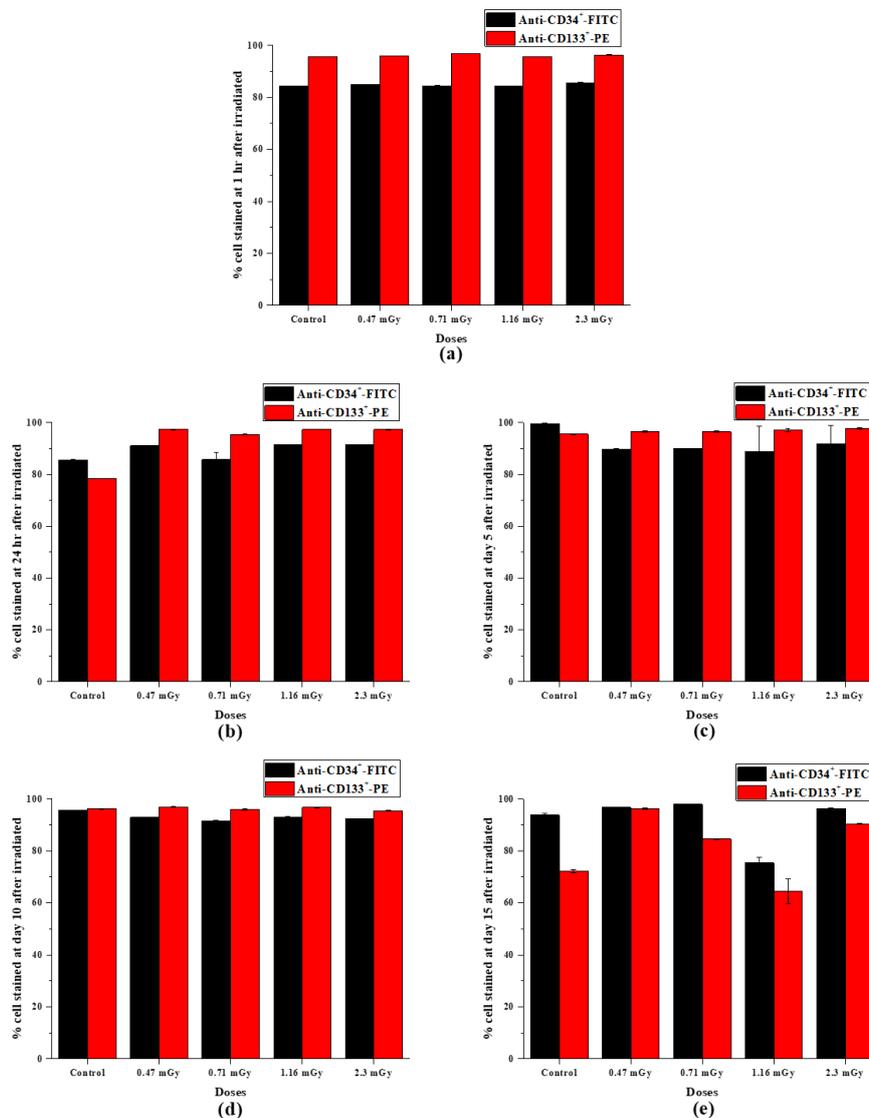


Figure 2 The percentage of cell stained for CD34⁺ and CD133⁺ after irradiation by X-ray (a) 1 hr, (b) 24 hr, (c) Day 5, (d) Day 10, and (e) Day 15.

Comparing the results of 1 hr post-treatment to 15 days post treatment, we noted that the expression of CD34⁺ had increased while CD133⁺ had decreased. There was a concomitant increase of CD34⁺ expression with the dosage and location of radiation (Figure 2).

4.4 Ratio of CD34⁺/CD133⁺ expression in PBMCs

The ratio of CD34⁺/CD133⁺ expression in PMBCs after irradiation by various radiation doses is shown in Figure 3. The results found that the ratio of CD34⁺/CD133⁺ expression in non-irradiated and irradiated groups were different in 0.71, 1.16, and 2.30 mGy ($p < 0.05$), but not different between control and radiation dose 0.47 mGy, which was the lowest amount of radiation.

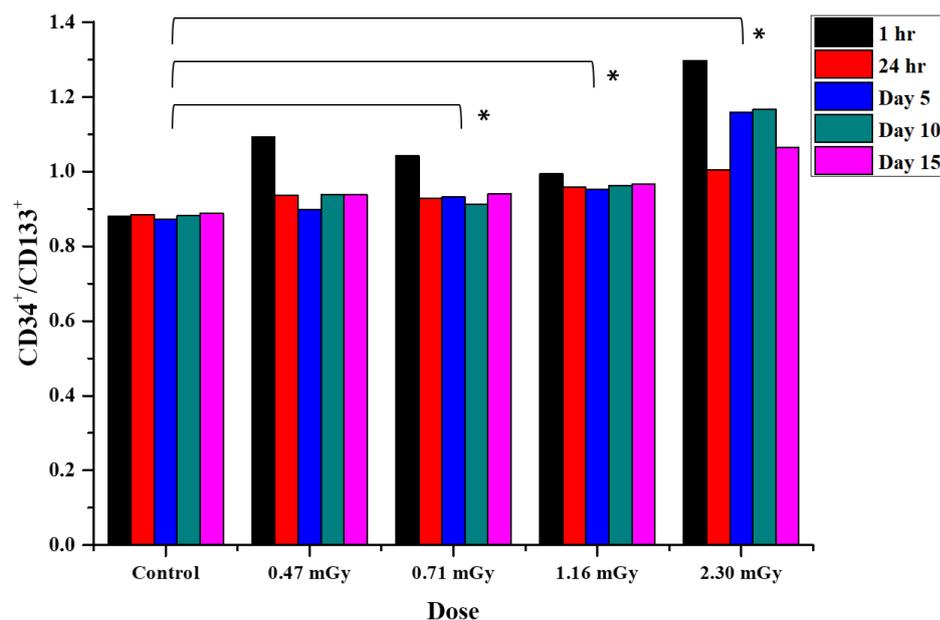


Figure 3 Ratio of CD34⁺/CD133⁺ in various radiation dose. *Statistically significant from the control ($p < 0.05$)

The results indicated that the radiation dose from X-ray exposure technique such as chest, pelvis, abdomen and lumbar spine (0.47, 0.71, 1.16, and 2.30 mGy) showed that the higher radiation doses were related to the ratio of CD34⁺/CD133⁺ seen in Figure 3. The higher radiation dose altered the ratio of cells stained in PBMCs differently between the control group at all periods in the experimental range and the percentage of CD34⁺ was less than CD133⁺ at 1 hr to 10 days. But on the other hand, the results showed that CD34⁺ stained more than CD133⁺ stained at day 15.

5. Discussion

The research focused primarily on the number and characteristic of hematopoietic stem cells. The number of cells collected from four healthy volunteers could be analyzed statistically. In this experiment studying the effects of radiation

on cells in cell culture conditions with supplemental protein serum, comparing the previous research of fetal bovine serum (FBS) or free serum, showed that the condition of cell culture may have an effect on the results, and the protein supplement in the culture medium may be contributing to the potency of cell differentiation.

Previous research therefore corresponds to this research, which found that cell morphology was similar in both groups. But when assessing cell morphology at day 20, Moonkum et al. (2020) showed the presence of large adherent cells in the control group, which may indicate that the various radiation doses may affect the cell differentiation to adherent cells. Including the low dose radiation in radiological diagnostic effect on the proportion of CD34⁺ and CD133⁺ expression which was different from the control group, when using the radiation doses 0.71 mGy, 1.16 mGy, and 2.30 mGy from the

results, it was found that there was a difference when increasing the radiation dose and no difference when using the smallest radiation dose at 0.47 mGy. The morphology of the PBMCs changed due to radiation response.

This study utilized an *in vitro* model to assess CD34⁺ and CD133⁺ expression following exposure to X-rays. Flow cytometry and light microscopy are simple yet powerful techniques that are able to quickly and accurately assess damage at a cellular level. In particular, the radiation technologist and patients should be protected by a radiation blocking barrier, especially children and pregnant women. Moreover, the researchers chose to study PBMCs because the cells can be easily stored and used as a biomarkers following irradiation.

6. Conclusion

The radiation from X-ray exposure techniques, which are low dose radiation and used in the hospital for medical imaging and diagnostic have many positive potentials, but the effects of radiation in cells and at the molecular level are still in need of research especially in the *in vitro* method. In conclusion, the effect of diagnostic radiation such as radiation doses from plain film may affect PBMCs, which are normal cells in the human body and CD34 and CD133 expression on cells surface membrane which are markers for hematopoietic stem cells and mesenchymal stem cells (Jaime-Pérez et al., 2016; Takahashi et al., 2014). This research provides a scientifically fundamental basis of cell response to diagnostic radiation such as PBMCs and also provides insight into the medical X-ray machines on the potential of PBMC and awareness that is necessary for the use of radiation for the benefit and safety for humans.

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