ORIGINAL ARTICLE

Bone Marrow and Peripheral Blood Cells Toxicity of a Single 2.0 Gy Cobalt^{60} Ionizing Radiation: An Animal Model

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OPEN ACCESS

Citation: Shittu Akeem, Olatunbosun Lukman, Khalil Eltahir, Olalere Fatai, Babatunde Abiola, Omokanye Khadijat. Bone Marrow and Peripheral Blood Cells Toxicity of a Single 2.0 Gy Cobalt^{60} Ionizing Radiation: An Animal Model. Ethiop J Health Sci. 2018;29(2):195. doi:http://dx.doi.org/10.4314/ ejhs.v29i2.6

Received: September 26, 2018

Accepted: November 5, 2018

Published: March 1, 2019

Funding: Nil

Competing Interests: The authors declare that this manuscript was approved by all authors in its form and that no competing interest exists.

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ABSTRACT

BACKGROUND: Bone marrow is extremely vulnerable to damage caused by radiation therapy. Hence, bone marrow suppression is an important side effect of radiotherapy. Effective use of radiotherapy is therefore compromised by radiation-related injuries.

MATERIAL AND METHODS: Six Guinea-pigs were recruited for the study of which three were subjected to total body irradiation with Co^{60} while the other three served as controls. Bone marrow and peripheral blood samples were collected before and at days 9, 14 and 21, post irradiation. Manual and automated counts were performed for bone marrow nucleated cells and peripheral blood cells respectively.

RESULTS: Declining bone marrow cellularity was evident immediately post irradiation. Mean ± SD of marrow cell counted per mm^3 were 121,924±281, 87,603±772, 121,367±375 and 122,750±1000 pre-irradiation and days 9, 14 and 21, post-irradiation (p-values 0.10, 0.27 and 0.29 respectively). Significant drops in counts were noticed on day 9 post-irradiation for all red cell parameters (p-values <0.05), for Total White Blood Cell Count and Neutrophil count (p-values <0.05) and also on days 14 and 21 for Lymphocytes (p-values <0.05) and on day 21 for Eosinophil/Basophil/Monocytes (p-value <0.05). A significant drop in platelets counts was also noticed on day 9 (p-value <0.05) which significantly increased above pre-irradiation value on day 21.

CONCLUSION: Total body irrradiation with Co^{60} significantly affects the bone marrow with maximum reductions in marrow nucleated cells and peripheral blood cells counts on day 9 post irradiation.

KEYWORDS: Ionizing, Radiation, Marrow, Animal, Model

INTRODUCTION

Ionizing Radiation Therapy or Radiotherapy is the use of Ionizing Radiation (IR) for the treatment of radio-sensitive malignancies. It is an indispensable treatment modality for cancer, alone or supported with surgery and or chemotherapy. It consists of electrons and gamma rays or as alternative radioisotope. Commonly used isotopes include Iodine^{125}, Iridium^{192}, Strontium^{89}, Iodine^{131}, Ytrium^{90} and Cobalt^{60}.

IR produces free radicals which specifically damage DNA. For curative use, the dose of IR delivered to tumour cells is optimized while minimizing the dose delivered to the normal surrounding tissues. However, the effective use of IR is compromised by radiation-related injuries or side effects. The important role of radiation-damaged bone marrow, skin and gastrointestinal tract epithelium in the severity of the acute radiation illness as well as those of the central nervous system, lungs, heart, liver, kidney and gonads has been well elucidated (1-4). Bone marrow is extremely vulnerable to damage caused by radiation therapy (5). Hence, bone marrow suppression is one of the most important side effects of radiotherapy. This has adverse effects on the success of cancer treatment (5). However, the mechanisms through which IR induces BM injury remains poorly understood (6), nor has it an effective treatment been developed to ameliorate the injury (7). Long term exposure to even low doses of radiation can affect proliferating cells (8).

IR destroys both mature blood cells and hematopoietic progenitor/stem cells in the bone marrow. While loss of mature blood cells is a key factor in radiation morbidity, mortality is believed to occur due to prolonged myelo-suppression due to loss of Hematopoietic Progenitor Cells (HPC) and primitive Hematopoietic Stem Cells (HSC) (5,9).

The impacts of exposure to IR on human and animal bone marrow have been studied by various researchers. Unlike the recent studies that centred on human bone marrow, most of the early studies were conducted on laboratory animals. The assay in which much of the experimental data is based is the spleen colony method of Till and McCulloch in which survival fraction of irradiated mouse cells is inversely proportional to radiation dose (10,11). IR has also been shown to significantly reduce hematopoietic cellularity, megakaryocytes counts and raised myeloid/ erythroid ratio due to erythroid hypoplasia in irradiated mice.

Radiation induced human bone marrow injury from Magnetic Resonance Imaging (MRI) was noticed 6 to 12 months after radiotherapy and most evident 5 to 6 years after. The authors of this study also reported partial and complete recovery 2 to 9 years and 10 to 23 years after exposure respectively (12).

Sahid *et al*. (13) noticed a decline in most haematological parameters when radiation-exposed workers were compared with non radiation-exposed workers. In their study, MCH was mostly affected. Also, statistically significant differences were noticed in the MCHC and lymphocytes, with a decline in platelets, hematocrit and lymphocytes, whereas neutrophil was found to be elevated in association with Annual Average Effective Dose (AAED) (0.29-1.91) mSv.

Clinical applications of experimental data for bone marrow kinetics in animal models have not been realy successful. Future strategies in which radiotherapy will be used for treatment of human malignancies require more studies both in animals and human. These studies will further highlight among other things, bone marrow toxicity effects of radiotherapy. In modern practice, radiation doses as low as 2.0Gy are used in non-myeloablative bone marrow transplantation because of the believe that this dose does not destroy host bone marrow but causes sufficient immune suppression to promote graft engraftment. This study was conducted on guinea pigs because of several biological similarities to humans and also because they are validated experimental animal models (14-16). Hence, for this study, we decided to evaluate the impact of a single 2.0Gy dose of $Cobalt^{60}$ IR on the bone marrow and peripheral blood cells counts of guinea pigs.

MATERIAL AND METHODS

Animal source: Six young male Guinea-pigs, approximately 450 gram in weight, obtained from the animal house, Ladoke Akintola University of Technology College of Medicine Osogbo, Osun-State, Nigeria were recruited for the study. They were labeled A, B, C, D, E and F and kept in the animal house of the Department of Anatomy, University of Ilorin in a temperature and

DOI: http://dx.doi.org/10.4314/ejhs.v29i2.6

humidity-controlled room that was maintained on a 12-hour light and dark cycle. Food and water were available *ad libitum* throughout the experiment. Animals were treated according to the National and European Union directive 2010/63/EU guidelines for handling animals used for scientific purposes. Guniea-pigs labeled A, B and C were subjected to lethal dose (2.0Gy at 98.56cGy/minute) of Total Body Irradiation with Co^{60} and returned to the animal house. Guinea pigs D, E and F served as controls.

Method of irradiation of the Guinea-pig: Irradiation was performed at University College Hospital Ibadan, one of the few centers in Nigeria that have facility for radiotherapy. The method described by Harris (17) was adopted. After general anesthesia using intra-muscular ketamine 5mg/kg body weight plus 1 mg Atropine, each Guinea-pig was placed in a cotton-gauze bag and positioned lying on the side. Each animal was given 200r (2.0Gy) whole-body gamma-irradiation under general anaesthetic, using a Co^{60} therapy unit as a source, at a dose rate of 98.560cGy/minute.

After-care of the irradiated animals: To minimize the two hazards enumerated by Harris (17), i.e. the danger of internal haemorrhage from minor trauma and the risk of infection, resulting from the effects of irradiation on haemopoietic tissues, each irradiated animal was kept in a separate cage and excessive handling avoided until it was due for sacrifice. Each animal was adequately fed and given adequate supply of water.

Bone marrow harvest and peripheral blood collection, pre and 9th, 14th and 21st days post irradiation: Bone marrow and peripheral blood cells counts were performed pre irradiation and on days 9, 14 and 21 post irradiation. Previous studies reported that bone marrow appears to be in the initial stage of final recovery of haemopoiesis on day 14 post irradiation and will be immediately followed by the reappearance of definitive precursor cells. Hence, days 14 and few days before (day 9) and few days after (day 21) were selected for bone marrow harvest and peripheral blood cells counts.

Bone marrow harvest: The method described by Arthur *et al*. (18) was employed. Each animal was strapped to the table with dorsum presenting and extremities extended. The hair over sacral area was shaved and the skin sterilized with 100% alcohol. The antero-superior border of iliac crest was identified. A sterile 20 gauge Quincke spinal tap needle about one and half inches long was inserted through the skin and muscle close to the iliac crest. Upon reaching the periosteum, the needle was rotated and with boring movement pushed until embedded in the bone. The stylet was removed and another 20 ml syringe used to aspirate about 0.2 ml of marrow. A drop was immediately placed on a clean glass slide to make thin film while the rest was aspirated into a bottle containing EDTA and immediately processed. The needle and the attached syringe were removed from the animal and discarded.

Peripheral blood collection: Two milliliters of venous blood were collected aseptically from the Lateral Saphenous vein from each animal using the method described by Malene, MB et al (19) according to protocols approved by the Danish Animal Experimentation Inspectorate under the Ministry of Food, Agriculture and Fisheries. The samples were dispensed into bottles containing EDTA and analyzed immediately.

Bone marrow staining and counting: Two films were made separately and stained with May Grunwald and Giemsa stains following the method described by John V. Dacie and S.M. Lewis (20). Nucleated cells and megakaryocytes counts were performed similarly.

Peripheral blood cell counting: The collected blood was mixed well and inserted into Sysmex Automated Analyser Xp-300 by Sysmex Corporation 1-5-1 Wakinohama, Kaignodori, Chuo-ku, Kobe, 651-0073, Japan. Haemoglobin concentration (HB) in g/dl, Packed Cell Volume in % (PCV), Total Red Cell count (TRBC) x 10^9/L, Mean Corpuscular Volume (MCV) in fl, Mean Corpuscular Haemoglobin (MCH) in pg, Mean Corpuscular Haemoglobin Concentration (MCHC) in g/dl, Total White Blood Cell counts (TWBC) x10^9/l and its differentials in % [Neutrophils (NEUT), Lymphocytes (LYMP) and Eo sinophil/Basophil/Monocytes (EOS/BAS/MON), Platelets (PLT) x 10^9/l counts

DOI: http://dx.doi.org/10.4314/ejhs.v29i2.6

and also Mean Platelet Volume (MPV) were measured.

Statistical analysis: The results were expressed as mean ± standard deviation. Statistical differences between tests and controls and between pre- and post-irradiation values were calculated using student paired sample t-test. Data analysis was performed using Statistical Package for the Social Sciences, version 20. A p-value of <0.05 was considered significant.

RESULTS

Microscopic observation of harvested bone marrow and marrow cells counting: Prior to irradiation, marrows were normocellular with normal myelopoiesis, erythropoiesis, lymphopoiesis and megakaryopoiesis and Myeloid: Erythroid ratio of 3:1. Mean±SD of marrow cell counted was 121,924±281/mm^3 (Table 1).

A decline in bone marrow cellularity was noticed immediately post-irradiation up till day 9, but it started to rise again till day 21. Mean ± SD of marrow cell counted was 87,603±772, 121,367±375 and 122,750±1000/mm^3 on days 9, 14 and 21 with p-values <0.01, 0.27 and 0.29 respectively, (Table 1).

Table 1: Effects of Ionizing Radiation on the Marrow Cell Counts.

Irradiation	Day	Test counts/mm^3±SD	Control counts/mm^3±SD	p-value
Pre	0	121,924.67±281.12	122,460.33±491.67	0.21
Post	9	87,603.33±772.42	122,508.33±574.101	0.00
	p	0.00		
	14	121,367.33±375.75	122,032.33±60.48	0.09
	p	0.27		
	21	122,750±1000.61	122,699.67±834.17	0.95
	p	0.29		

PERIPHERAL BLOOD CELLS

Erythroid cells: A significant difference between test and control results was noticed on day 9 post-irradiation for all red cell parameters and also on days 14 and 21 MCV and on day 21 for HB. No significant differences between tests and controls were noticed in these parameters at other stages of the study (Tables 2 and 3). When pre-irradiation and post irradiation results were compared, a significant drop was noticed in all the erythroid parameters on day 9 (p-values 0.01, 0.01, 0.01, 0.02, 0.00 and <0.01, for HB, PCV, RBC, MCV, MCH and MCHC respectively). Subsequently, a significant increase above pre-irradiation values was noticed only in HB, p-value 0.02 (Table 2).

Myeloid cells: No significant differences was noticed in the pre-irradiation results for the tests and controlsfor TWBC, NEUT, LYMP, and EOS/BAS/MON, with p-values 0.12, 0.42, 0.42 and 0.84 respectively, (Table 4). Like the results for erythroid cells, a significant drop was noticed for TWBC and NEUT on day 9 post irradiation (p-values <0.01, 0.04 respectively) but for LYM on days 14 and 21 (p-value 0.05 and <0.01 respectively) and EOS/BAS/MON on day 21 (p-value 0.01) (Table 4).

When comparison was made between pre- and post-irradiation results, a significant reduction was noticed in the TWBC on day 9 (p-value <0.01) but this subsequently rose up to the pre-irradiation value. This significant reduction was noticed on days 9 and 14 for NEUT (p-values 0.05), on days 14 (p-value 0.03) and 21 (p-value 0.02) for the LYM and only on day 21 (p-value 0.02) for EOS/BAS/MON.

DOI: http://dx.doi.org/10.4314/ejhs.v29i2.6

Table 2: The effects of ionizing radiation on Erythroid cells (HB, PCV and Total RBC) for the tests and controls.

Irradiation	Day	ERYTHROID CELLS								
		HB (g/dl)			PCV(%)			TOTAL RBC(x 10^9/L)		
		T	C	p-value	T	C	p-value	T	C	p-value
Pre	0	14.1±0.3	14.5±0.46	0.183	45.0±2.0	46.33±2.08	0.06	6.36±0.29	6.36±0.64	1.04
Post	9	10.53±0.85	14.7±0.25	0.02	30±2.6	47.33±1.15	0.02	4.42±0.24	6.53±0.47	0.03
	p	0.01			0.01			0.01		
	14	15.43±0.55	14.7±0.46	0.3	47.6±1.53	46.0±2.0	0.42	6.88±0.39	7.09±0.12	0.47
	p	0.11			0.25			0.23		
	21	16.23±0.61	15.37±0.45	0.02	49.3±1.53	53.33±3.06	0.06	7.18±0.22	7.72±0.44	0.06
	p	0.02			0.12			0.08		

* T- Test, † C- Control, ‡ HB- Haemoglobin concentration, § PCV- PACKED Cell Volume, || RBC- Red Blood Cell count

Table 3: The effects of ionizing radiation on Erythroid cells (MCV, MCH and MCHC) for the tests and control

Irradiation	Day	ERYTHROID CELLS								
		MCV (fl)			MCH (pg)			MCHC (g/dl)		
		T	C	p-value	T	C	p-value	T	C	p-value
Pre	0	71.33±1.52	70.3±0.6	0.47	21±1.0	21.7±2.08	0.74	30±1	32±1.7	0.32
Post	9	50±4.5	70.1±1.15	0.01	15.6±0.58	20.7±1.53	0.03	19.6±1.5	31±1.7	0.03
	p	0.02			0.00			0.00		
	14	61±1.0	74±2	0.01	21.3±3.2	24±1.7	0.41	32±1.7	31.3±1.5	0.18
	p	0.9			0.9			0.07		
	21	75.33±1.53	71.7±1.5	0.05	22.7±2.52	20.7±2.08	0.22	30.3±2.9	29.7±0.6	0.75
	p	0.12			0.49			0.9		

* T- Test, † C- Control, ‡ HB- Haemoglobin concentration, § PCV- PACKED Cell Volume, || RBC- Red Blood Cell count

DOI: http://dx.doi.org/10.4314/ejhs.v29i2.6

Table 4: Effects of Ionizing Radiation on Myeloid cells for the Tests and Control

Irradiation	Day	Myeloid Series											
		Total WBC (x 10^9/L)			NEUT (%)			LYMP (%)			EOS/BAS/MON (%)		
		T	C	p	T	C	p	T	C	P	T	C	p
Pre		6.9±0.3	6.1±o.3	0.12	10.3±0.6	10±0.0	0.42	89.3±1.2	90±0.0	0.42	0.3±0.6	0.0±0.0	0.84
Post	9	1.5±0.5	7.3±1.1	0.01	4.3±2.5	11±1	0.04	95.3±3.06	87.3±2.1	0.08	0.3±0.6	0.7±1.5	0.38
	p	0.00			0.05			0.09			0.57		
	14	7.5±0.7	6.9±0.3	0.38	24.7±5.8	10±0.0	0.06	73.3±5.8	91.3±2.3	0.05	1.7±0.6	0.0±0.0	0.84
	p	0.26			0.05			0.03			0.67		
	21	6.7±0.8	6.4±0.5	0.65	8.7±2.3	10.3±1.5	0.37	81.3±2.3	88.7±1.2	0.01	9.3±1.2	1.0±1.7	0.01
	P	0.63			0.29			0.02			0.02		

* T- Test, † C- Control, ‡ HB- Haemoglobin concentration, § PCV- PACKED Cell Volume, || RBC- Red Blood Cell count

Platelets: Similarly, there was no significant difference between the tests and controls in the Total Platelets count and MPV during the pre-irradiation period, p-value 0.92 and 0.09 respectively. A significant difference between tests and controls was noticed only with platelets on day 9, p-value <0.01 (Table 5). Comparing the pre- and post-irradiation results, a significant drop was noticed in Total Platelets counts on day 9 (p-value 0.006) which significantly increased above pre-irradiation value on day 21 (p-value 0.01) (Table 5). There were 78.26%, 75% and 30.5% reductions in WBC, PLT and RBC counts from pre-irradiation to post-irradiation values with p-values <0.01, 0.01 and 0.01 respectively (Table 6).

Table 5: Effects of Ionizing Radiation on Platelets for Test and Control.

Irradiation	Day	Total Platelets (X 10^9/L)			MPV		
		T	C	p	T	C	p
Pre	0	874.7±110.3	865±49.9	0.92	9.3±0.3	10.2±0.2	0.09
Post	9	218.7±35.8	893±105.7	0.00	9.8±1.1	9.1±2.4	0.48
	p	0.01			0.59		
	14	812.7±41.6	853.3±75.6	0.47	7.7±2.3	9.9±0.3	0.21
	p	0.55			0.35		
	21	1190±161.9	977.3±146.7	0.20	10.3±0.6	10.1±0.2	0.65
	p	0.01			0.07		

* T- Test, † C- Control, ‡ MPV- Mean Platelet Volume

Table 6: Percentage Reductions RBC, WBC and PLT from Pre to Post Irradiation Values.

Irradiation	RBC	WBC	PLT
Pre	6.36	6.9	874.7
Post	4.42	1.5	218.7
p	0.01	0.00	0.01
% reduction	30.5%	78.26%	75%

* RBC- Red Blood Cell count, † WBC- White Blood Cell count, ‡ PLT- Platelet count

DISCUSSION

Cells of haemopoietic system are highly sensitive to radiation (21). The haemopoietically active bone marrow, like other actively dividing tissues in the body, is an appropriate model to study radiation induced injuries. Injuries to the bone marrow can be studied directly by analyzing the bone marrow itself or indirectly by analyzing its effects on peripheral blood cells.

Several methods, though with different sensitivity, specificity and accuracy are available to evaluate bone marrow activity. Conventional radiography, scintigraphy, Computed Tomography, Magnetic Resonance Imaging and needle biopsy have all been used in the study of bone marrow disorders (22).

The status of the bone marrow at every stage of treatment is of utmost importance in the follow-up of

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cancer patients on radiotherapy. In fact, the amount of viable red bone marrow after each exposure will determine whether patients can be further treated with radiotherapy or alternatively chemotherapy.

In this study, we evaluated the impact of Co^{60} ionizing irradiation on bone marrow and peripheral blood cells through needle bone marrow biopsy and automated peripheral blood cells analyzer using Sysmex Automated Analyser Xp-300 respectively on days 9, 14 and 21after exposure. Bone marrow suppression post-rradiation was unequivocal as evidenced by decline in cellularity (as evidenced by declining cells counts), replacement of the cellular component of marrow with fat as well as reduction in all the cell lines in the peripheral circulation. This is similar to the findings in most studies (both in animals and humans) (21-24). The maximum reduction effects of Co^{60} ionizing radiation on the bone marrow and peripheral blood cells were noticed on day 9 post-irradiation, similar to the findings of Deguan Li *et al.* (25), except lymphocytes and eosinophil/basophil. These values subsequently increased to the pre-irradiation values with complete recovery from days 9 to 21 post-irradiation. Lymphocyte and MPV initially rose above pre-irradiation level untill day 9 and subsequently reduced and reached values close to pre-irradiation values.

WBC counts are mostly affected with 78% reduction from pre-irradiation values of 6.9 x 10^9/l to 1.5 x 10^9/l with a p-value <0.01. This was was followed by PLT and then RBC with percentage reduction of 75% and 30.5% and p-values 0.01 and 0.01 respectively. Theoretically rapidly proliferating and differentiating cells of the bone marrow are more affected by irradiation than the more mature cells. The results of our study revealed that myelopoiesis is most affected by irradiation, followed by lymphopoiesis and then erythropoiesis. Literature on the degree of affectation of each of the marrow cell line by radiotherapy is sparse for us to make comparison with our findings. Few studies in which findings were close to ours was the one in which Granulocyte Colony-Stimulating Factor (G-CSF) was found to moderately reduce the severity and duration of IR- and/or chemotherapy-induced BM injury in experimental animals and cancer patients (26, 27), and the one in which leukocytes and lymphocytes are said to be the most highly radiosensitive (28-30). Currently, G-CSF is the only treatment recommended to be given to radiation victims soon after accidental exposure (31-33). The ASCO Update Committee agreed unanimously that reduction in febrile neutropenia was an important clinical outcome that justifies the use of CSFs, regardless of impacts on other factors (34).

In conclusion, this study demonstrated that total body ionizing radiation with Co^{60} significantly affects the bone marrow with maximum reductions in peripheral blood cells counts on day 9 post irradiation. Myelopoiesis is mostly affected by the radiation, followed by megakaryopoiesis and then erythropoiesis. Injuries to bone marrow are one of the most important side effects of conventional radiotherapy. This limits the success of cancer treatment and adversely affects the quality of life of cancer patients. Although this study was conducted on Guinea pigs, the findings were similar to what was found in human experiments. Oncologists and radiation therapists should be aware of these findings when spacing radiotherapy courses for cancer patients.

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DOI: http://dx.doi.org/10.4314/ejhs.v29i2.6