Pattern of Haemopoietic Progenitor Cells Differentiation and Differentiation Enhancing Effect of *Telfairia occidentalis* Extract in Irradiated Guinea-pig.

[1,2]L.O. Olatunbosun, , [3]E.A.G. Khalil, [4]S.A. Atunwa, [1]F.D. Olalere, [4,5]S.A. Lawal, [5]S.A. Biliaminu, [6]K.A.T. Ogunwale, [1]K. Omokanye, [1]A.S. Babatunde, [7]O.J. AbdulRaheem, [8]O.O. Afolabi, [9]A. Bashir, [10]I.K. Lawal.

[1]Department of Haematology and Blood Transfusion, University of Ilorin Teaching Hospital, Ilorin, Nigeria.
[2]Department of Medical Laboratory Science, Al-Hikmah University, Ilorin, Nigeria
[3]Department of Clinical Pathology & Immunology, Institute of Endemic Diseases, University of Khartoum, Sudan
[4]Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Ilorin, Ilorin, Nigeria
[5]Department of Haematology, Faculty of Basic Medical Sciences, University of Ilorin, Ilorin, Nigeria
[6]Department of Chemical Pathology and Immunology, University of Ilorin Teaching Hospital, Ilorin, Nigeria
[7]Department of Medical Microbiology and Parasitology, University of Ilorin Teaching Hospital, Ilorin, Nigeria
[8]Department of Histopathology, Faculty of Basic Medical Sciences, University of Ilorin, Ilorin, Nigeria
[9]Department of Immunology, Faculty of Medical Laboratory Science, Usmanu Danfodiyo University, Sokoto, Nigeria
[10] Department of Physiology, Ladoke Akintola University of Technology, Ogbomosho, Nigeria/Dept. of Science Laboratory, Esa-Oke Polytechnic, Esa-Oke, Osun State.

Abstract

Ionizing radiation is essentially considered a viable treatment modality for human malignancies. Acute and chronic toxicities with severe morbidity and mortality are well documented. Experimental and clinical approaches to correcting radiation haemopoietic syndrome deficiencies have focused on cytokine activity with same documented toxicities. Limited data is available on differentiation enhancing effects of plant extracts on haemopoietic progenitor cells following radiation injury. This study aimed to determine the potential haemopoietic progenitor cells differentiation effects of *Telfairia occidentalis* in irradiated guinea pigs. Bone marrow cells from irradiated guinea pigs were harvested and treated with varying concentrations of 0.313-100% of the extracts of the *Telfairia occidentalis* and the degree of differentiation of the cultured guinea-pig bone marrow haemopoietic stem cells determined. The concentration of the extract ranges from 0.313-100%. This showed Mean ± SD of 3.54±0.30, 0.18±0.01 and 0.88±0.05 for the extract treated, non-extract treated (-ve control) and GM-CSF (+ve control) treated culture plates respectively [*p=0.000*].

Extract concentrations corrected positively with differentiation of the cultured guinea-pig bone marrow haemopoietic stem cells at 'r' 0.835 [*r= 0.812; p=0.001*]. Conclusively, *Telfairia occidentalis* extract has positive differentiation enhancing effects on haemopoietic progenitor cells with deflection in favour of erythroid lineage.

Key words: Differentiation, Erythroid, lineage, *Telfairia occidentalis* (TO), Haemopoietic, Progenitor Cells, Irradiated Guinea Pigs.

Correspondence to:

Olatunbosun, Luqman Olayinka,
Department of Haematology and Blood Transfusion,
University of Ilorin Teaching Hospital,
Ilorin, P. M. B. 1459, Kwara State, Nigeria
E-mail address:deluy008@yahoo.com,

Introduction

Damage to biological systems by ionizing radiation is caused primarily by the macromolecular lesions (particularly the genetic material, DNA). The damage is due to both direct interaction of radiation with the DNA and indirect action through reactive oxygen species (ROS) and reactive nitrogen species (RNS), which is amplified by cellular oxygen. At moderate doses, damage to hematopoietic and gastrointestinal systems mainly contribute to the acute effects due to mitotic and interphase deaths of constituent cells.[1] Radiation produces observable breaks in chromosomes, termed aberrations. Aberrations are further specified as either chromosomes or chromatid aberrations depending on whether they occur before or after DNA synthesis. DNA and chromosomal changes can give rise to malignancies either by activation of oncogenes or by the loss of suppressor genes.[1]

Radiation-induced DNA lesions are fundamental to investigating and understanding radiation-induced cell killing, cell transformation and

carcinogenesis, through induction of gene mutation and chromosome aberration.[2] Regarding early side effects of radiation, apoptosis can be extremely important even if its impact on late toxicity is limited. Apoptosis is important for acute effects because it is a rapid process that can dramatically reduce cell number. Among the most studied tissues undergoing brisk apoptosis are the bone marrow progenitor cells, lymphocytes, and endothelial cells.[3] Radiation damages some cells without actually killing them. This causes the stimulation of pathways involved in repopulation, maturation, angiogenesis, and inflammation.[5] It damages vasculature leading to perfusion dysfunction and hypoxia. Radiation-induced cell killing includes apoptosis, necrosis, and reproductive inactivation.[6]

Bone marrow failure and potentially lethal hemorrhage or infections are the important manifestations of biological effects of radiation on haematopoietic system.[7] The time course of the bone marrow failure (neutropenia, thrombopenia, anaemia, etc) is an excellent marker for evaluating potential countermeasures. Haemopoiesis is a process regulated by a complex network of soluble factors that stimulate the growth and differentiation of haemopoietic progenitor cells (HPC).[8] HPC have two major characteristics: self-renewal ability and the capacity to differentiate into different lineages of haemopoietic cells.[9] The proliferation and differentiation of HPC are influenced to a large extent by interactions among various cell types in the haemopoietic compartment and by haemopoietic cytokines produced by stromal cells and lymphocytes.[10] *In vitro* haemopoietic cell cultures are important experimental tools in biomedical research for understanding and analyzing the mechanisms underlying various cell functions.[10] These can be easily manipulated, and hence, help in the systematic studies of haemopoietic system. Therefore, they are widely used as important experimental models to evaluate treatment strategies of diseases with their efficiency and toxicity.[12]

The plant *Telfairia occidentalis (Hook F. J.)* is commonly known as fluted pumpkin, fluted gourd, Costillada (Spanish), Krobonko (Ghana), and Gonugbe (Sierra Leone). The plant belongs to the family *of Cucurbitaceae* and is cultivated across lowland humid tropics of West Africa.[13] *Telfairia occidentalis* leaves extract is used traditionally to treat malaria and anaemia.[14] The leaves are eaten as vegetables while the seeds are either roasted or ground for other food preparations. Apart from the nutritional[15], agricultural and industrial importance[16], the plant is also medicinally useful. It is claimed to possess anti-inflammatory, antibacterial, erythropoietin, anti-cholesterolemic and anti-diabetic activities.[17,18,19,20,21,22]

The ripe fruit contains up to 13% oil. The leaves and the young shoots of the plant are frequently eaten as a potherb.[15,23,24] The seeds of the plant are also popular items of diet and are cooked whole and ground up into soups. The root and leaves have been shown to contain highly toxic alkaloids and saponins.[18] The leaves also contain protein, vitamins, and flavours.[14,23] In Nigeria, the herbal preparation of the plant has been employed in the treatment of sudden attack of convulsion, malaria and anaemia.[14] Despite its widespread usage as food and medication, information on the biological activity of the plant is very scanty.

Materials and Methods

Ethical Approval for Animal Studies

Ethical clearance and approval for the animal study was given by the University of Ilorin Ethical Review Committee (UERC), University of Ilorin, Ilorin, Nigeria (UERC/ASN/2018/1109).

Plant Collection and Preparation

Fresh samples of leaves of *Telfairia occidentalis* were obtained from the he Plant Garden of African Centre for Herbal Research Institute, University of Ilorin, Ilorin. The plant was identified by carrying out macroscopic examination as stipulated by Dalziel[25] and authenticated by Mr Bolu-Ajayi of the Herbarium Unit, Department of Plant Biology, University of Ilorin, Ilorin, Nigeria. The sample of *Telfairia occidentalis* was given Serial Number 959 and Ledger Number 150. Thereafter, collected samples were deposited in the herbarium of the institution for future references. The sample was dried in an incubator at 37^{o}C (Uniscope, USA). Plant extraction was carried out by the method of Olowosolu and Ibrahim.[26] Plant material was macerated in pestle and mortar with 100ml distilled water at room temperature and then filtered using muslin cloth. Filtrate obtained was subsequently passed through Whatman's No.1 Filter paper under aseptic conditions and the filtrate was collected in fresh sterilized glass tubes and used within 24 hours for the research work.[13] The final concentration of 1.0 g/mL was obtained as aqueous extract which served as the stock solution for dilutions needed during the course of the work.

Animal Source

Twelve young male guinea-pigs of approximately 450 g were obtained from the animal house of College of Medicine, Ladoke Akintola University of Technology (LAUTECH), Osogbo, Osun State, Nigeria. Animals used for the experiments were housed in the animal house of the Department of Anatomy, Faculty of Basic Medical Sciences, College of Medicine, University of Ilorin in a temperature and humidity-controlled room that was maintained on a 12-hour light/dark cycle. Food and water were available *ad*

libitum throughout the period of experiment.

Method of Irradiation of the Guinea-Pigs

The type, dose and method of irradiation as well as the after-care of the irradiated guinea-pigs were all based on the procedures adopted by Harris.[27] Each guinea-pig was separately irradiated under general anesthesia (intramuscular ketamine 5 mg/kg body weight plus 1.0 mg Atropine). The animal was placed in a cotton-gauze bag and positioned lying on its side. Irradiation was given to each flank, the irradiation time being divided equally between each side, i.e the animal was turned over onto its opposite side half-way through the procedure. Each animal was given 200r (2.0Gy) whole-body gamma-irradiation under general anaesthesia, using a Co^{60} therapy unit as source at University College Hospital (UCH), Ibadan, Nigeria at a dose rate of 98.560cGy/min.

After-Care of the Irradiated Guinea-Pigs

To minimize the two hazards enumerated by Harris[2], 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 animal after irradiation 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 clean water.

Bone Marrow Harvest

Bone marrow cells from treated guinea pigs were harvested by the method of Galvin colleagues.[28] The animals were sacrificed by cervical dislocation and the Femurs were carefully located and removed aseptically. Adherent soft tissue and cartilage were stripped from the bones and the tip of each bone was removed with a rongeur. The marrow was harvested by inserting a syringe needle (27-gauge) into the proximal end of the bone and flushing with phosphate buffer saline (PBS) into a universal bottle containing phosphate buffer saline, 200 unit/ml heparin, Hanks balanced salt solution (HBSS), supplemented with 2% fetal calf serum (FCS). The suspended marrow cells were further diluted 1 in 20 in PBS and the cells were counted to achieve a cell count of 1.0×10^9/L.

Microscopic Examination of Harvested Bone Marrow

Harris has given a report of the haemopoietic events occurring in guinea-pig bone-marrow following sub-lethal whole-body gamma irradiation at 15 days after irradiation. This stage of haemopoietic recovery was described by Harris.[27] The initial phase of final haematopoietic recovery was confirmed by cyto-chemical reactivities as described by Caxton-Martins using May-Grunwald's staining technique.[29] The harvested cells stained with May-Grunwald's staining technique showed cells of high nuclear: cytoplasmic ratio, lepto-chromatic nucleus, basophilic cytoplasm and presence of a nuclear hof. Cyto-chemically, cells were negative for myeloperoxidase, specific and Non-specific esterase, leucocyte alkaline phosphatase and acid phosphatase, Sudan Black B, and Periodic acid Schiff.

Preparation of Marrow Suspension

The marrow suspension was prepared using fresh autologous serum. The abdomen of the anaesthetized animal was opened up and the inferior vena-cava was exposed and incised. About 5mL of blood was collected in a centrifuge tube through a glass funnel. After clotting, the blood was centrifuged for 10 minutes at 3000 rpm and the serum was withdrawn with a Pasteur pipette into clean, small glass tubes. The isolated bone marrow cells were placed into the autologous serum contained in a clean glass tube fitted with a rubber stopper. This marrow suspension was used for the cyto-chemical studies to further establish the relative incidence of transitional cells as enumerated by Caxton-Martins.[29] A similar technique was used by Harris and Yoffey.[27,30] This technique was used to keep the damaged cells in the marrow smears to the minimum.

May-Grunwald Staining Technique:

Air dried films were fixed by immersing in a jar of methanol for 20-25 minutes. The slides were transferred to a staining jar containing May-Grunwald's stain freshly diluted with an equal volume of buffered water for 15 minutes. The slides were transferred directly without washing to a jar containing Giemsa's stain freshly diluted with 9 volumes of buffered water, pH 6.8 for 10–15minutes.The slides were transferred to a jar containing buffered water pH 6.8, rapidly washed in 3-4 changes of water and finally allowed to stand undisturbed in water for 2-5 minutes for differentiation to take place. The slides were kept in upright position to dry and reported using X100 objective lens.

Culture Media Preparation:

Eagles MEM (Minimum Essential Media) is to prepare [1000 mls of Growth and maintenance Eagles MEM media] as follows: One bottle of Eagles MEM powder containing 9.4 grams was dissolved completely in 1000 mls of sterile double distilled de-ionized water. The mixture was autoclaved at 121°C for 15 minutes with cap slightly loose and allowed to cool to room temperature. The pH of the autoclaved Eagles MEM was ensured to be between (4.3 – 4.5). The reagents below were dispensed into 2 empty sterile 500 ml bottles.7.5% Sodium bicarbonate(12.5mls), L-Glutamine (5mls), HEPES IM (5mls), Penicillin Streptomycin (5mls), FBS (10mls).The autoclaved

Eagles MEM was added to each of the 500 ml bottles containing the reagents above to reach the mark of 500 ml on the bottle. The pH was adjusted between (7.2 – 7.4).An aliquot of 250 ml of each prepared bottle of medium was added to tissue culture tubes and label appropriately for sterility testing. The aliquots were incubated at 36°C for 5 – 7 days, inoculate into Thioglycollate broth. The prepared media was stored at +4°C until use.[31]

Cell Culture Technique:

At 13th day Post-irradiation, the bone marrow committed cells were harvested and cultured with the extract of the plant at concentration ranges of 0.313-100%. A5 µl of suspended guinea-pig bone marrow cells harvested were cultured in a Laminar Flow Cabinet at a concentration of 1.0 X 10^9/L in 20 µl of Growth Eagles Minimum Essential Media (MEM). Then, 10 µl of the plant extract of *Telfairia occidentalis* at concentration ranges of 0.313-100% was added to enhance differentiation. Another set of 24 Wells were also set alongside with the test to serve as control in which no extract was added. The culture plates (48 wells) were incubated at 37°C for 72 hours. After the treatment, the cells were prepared for 3-[4, 5-Dimethylthiazol-2-YL]-2, 5-Diphenyltetrazolium Bromide (MTT) analysis of cell differentiation.

Cell Differentiation Assay:

A 20 µl of dimethylthiazo 2 yl)-2,5,-diaphenyl tetrazolium bromide (MTT) Solution was added to each well 5 hours before the end of the treatment in the culture plate. The plates were then incubated in a CO_2 incubator for 5 hours and the culture media removed with needle and syringe. Then, 200 µl of DMSO was added to each well with pipetting up and down to dissolve crystals. Plates were re-incubated in a CO_2 incubator for 5 minutes, transferred to a micro-plate reader and the absorbance measured at 550 nM (S).

Differentiated Cell Morphology

Cells harvested on day 13 were cyto-centrifuged on a glass slide, fixed for 5 minutes with acetone-methanol (9:1, v/v) at room and stained with benzidinedihydrochloride stain, myeloperoxidase, acid phosphatase, Sudan Black B and Periodic acid Schiff.

Results

Differentiated Cell Morphology

Analysis of the cell expression showed that expression of erythropoietic and leukopoietic markers were uncoordinated during cell differentiation. With 0.2% benzidine-dihydrochloride in 0.5 M acetic acid 30% hydrogen peroxide (superoxide), the slides were graded for the preponderance of colonies which were uniformly benzidine-reactive (blue). Morphologically, giant cells with indentation, convolution, and segmentation of nuclei were observed. The majority of the cells resembled polychmomatophilic erythroblasts with azurophilic granules of varying dimensions.

Discussion

Hematopoiesis is a tightly regulated process where decisions have to be made on whether the HSC should self-renew, proliferate, differentiate or enter the apoptotic pathway. Haemopoietic stem cell differentiation plays a central role in recovery process of marrow depression consequents to radiotherapy or chemotherapy. Several studies have illustrated the

Table 1: A Comparison of Mean ± SD of the plant extract 13th Day Post-Irradiation at Concentration ranging between 0.313% - 100%.

Protocol	Culture Wells	Mean±SD	p-Value
Telfairia occidentalis treated culture plates	24	3.54 ± 0.30	0.000
Non-extract treated culture plates	24	0.18 ± 0.01	
GM-CSF treated culture plates	24	0.88 ± 0.05	

Table 2: Correlation between concentrations of the extract of the *Telfairia occidentalis* and degree of differentiation.

Protocol	Culture Wells	Mean ± SD Deviation	Correlation Coefficient 'r'	p-Value
Telfairia occidentalis treated culture plates	24	2.94 ± 0.94	0.835	0.001

Table 3:**A Comparison of Mean ± SD of the Differentiated Cell Morphology expression Of the plant extract 13th Day Post-irradiation.**

Cell Lines	Benzidene-Mixed-Nonuniform Reactive	Myelo-peroxidase	Sudan Black B	Acid Phos-phatase	Periodic Acid Schiff
Telfairia occidentalis (TO) treated Cells	3.8333 ± 0.38	1.7083 ± 0.46	1.9583 ± 0.46	1.8750 ± 0.45	2.8333 ± 0.56
Non-extract treated Cells	2.7083 ± 0.46	1.2083 ± 0.51	1.2083 ± 0.51	1.2917 ± 0.55	1.1667 ± 0.38
p-Value	0.000	0.000	0.000	0.000	0.000

Grading Scheme:
0 = No reaction
1 = 10% of the cell population reactive
2 = 30% of the cell population reactive
3 = 50% of the cell population reactive
4 = 100% of the cell population reactive

multi-potentiality of leaf extract of *Telfairia occidentalis* and its usefulness as sources of enhancing haemopoiesis locally in the treatment of malaria and anaemia.[14,18]

In this study, haemopoietic stem cells from irradiated guinea pigs were cultured and subsequently treated the culture plates with or without aqueous leave extracts of *Telfairia occidentalis*. The comparison of the means of concentration between the treated plates (3.54±0.30) and non-treated plates (0.18±0.01; GM-CSF treated controls (0.88±0.05) showed a statistical significance difference (Table 1). The study further tested the differentiation strength of various concentrations from 0.313% to 100% and found that the differentiation increases as concentration increases with correlation pattern of 2.94±0.94 and calculated "r" 0.835 (Table 2). Analysis of the cell expression showed that expression of erythropoietic and leukopoietic markers were uncoordinated during cell differentiation. However, it was more in favour of erythroid lineage. This was in keeping with anti-anaemic and erythropoietic reports of Ajayi*et. al*., 2000 and Gbile, 1986.

Conclusion

This study confirmed the differentiation enhancing effects of the plant leaves extract on haemopoietic multi-potent stem cells. The differentiation effect correlates with the concentration of the plant extract and deflect more in favour of erythroid cell lineage. Further studies are required to determine the potential radio-protective efficacy of this plant extract and how it might influence various endpoints of haemopoietic radiation damage during radiotherapy for cancer.

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