



Research Paper

Haemopoietic multi-stem cells differentiation enhancing effect of *Telfairia occidentalis* extract

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ABSTRACT

Ionizing radiation is one of the most important modalities for the treatment of human malignancies. Acute and chronic toxicities with severe morbidity and mortality are well documented because it is not tumor specific. This disability also limits the role of radiotherapy in cancer treatment. Experimental and clinical approaches to correcting radiation haemopoietic syndrome deficiencies have focused on cytokine activity. Such treatments are not without certain risks. There has been an upsurge of interest on the effects of various dietary insufficiencies on haemopoietic and immune responses in recent years. Limited data is available on differentiation enhancing effects of plant extracts on haemopoietic stem cells following radiation injury. This study aimed to determine the potential differentiation effects of *Telfairia occidentalis* on guinea pig marrow haemopoietic multi-stem cells following irradiation. Bone marrow cells from irradiated guinea pigs were harvested and treated with varying concentrations of 0.313 to 100% of the extracts of the *T. occidentalis* and the degree of differentiation of the cultured guinea-pig bone marrow haemopoietic stem cells determined. The extract concentration ranges of 0.313 to 100% showed significant statistical difference in differentiation [$p=0.000$] when compared to the control. There was also strong positive correlation [$r= 0.812$; $p=0.001$] between the concentration of the extracts of the *T. occidentalis* and the degree of differentiation of bone marrow haemopoietic stem cell. Analysis of the cell expression though uncoordinated during cell differentiation is more in favour of erythroid lineage. *T. occidentalis* extract has positive differentiation enhancing effects on haemopoietic multi-potent stem cells. The differentiation effect correlates with the concentration of the plant extract and deflect in favour of erythroid lineage.

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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 direct interaction of radiation with the DNA as well as, indirectly through reactive oxygen species (ROS) and reactive nitrogen species (RNS), amplified by cellular oxygen. At moderate doses, damage to hematopoietic and gastrointestinal systems mainly contributes to the acute effects due to mitotic and interphase deaths of constituent cells (Dwarakanath et al, 2009).

Radiation produces breaks in chromosomes, termed aberrations, which can be observed. 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 (Dwarakanath et al, 2009).

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 (Grover and Kumar, 2002). 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 (Paul et al., 2008).

Molecular markers for radiation effects on normal tissues are either predictive markers or prognostic markers or diagnostic markers or dosimetric markers. They include metabolites, proteins, RNA, DNA, and even complicated physiological states. Many genotoxicity markers are proven biodosimeters, most prominently dicentric chromosome aberrations which are essentially unique to irradiation. Genotoxicity markers including chromosome aberrations, micronuclei, and DNA complexes to phosphorylated histone H2AX may also be prognostic markers of oncogenesis (Al Rashid et al., 2005; Yu et al., 2006; Klokov et al., 2006).

Radiation damages some cells without killing them, causing the stimulation of pathways involved in repopulation, maturation, angiogenesis and inflammation. It damages vasculature leading to perfusion dysfunction and hypoxia. Radiation-induced cell killing includes apoptosis, necrosis and reproductive inactivation.

Bone marrow failure and potentially lethal hemorrhage or infections are the important manifestation of biological effects of radiation on hematopoietic system (Dainiak, 2002). The time course of the bone marrow failure (neutropenia, thrombopenia and anemia, 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) (Metcalf, 1989). HPC have two major characteristics: self-renewal ability and the capacity to differentiate into different lineages of haemopoietic cells (Ogawa, 1993). 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 (Jacobsen, 1996).

Cytokines modulate haemopoiesis by maintaining the self-renewal of stem cells and stimulating the proliferation and maturation of committed progenitor cells required for the continuous replacement of mature blood cells. Various combinations of cytokines including interleukin-1 (IL-1), IL-3, IL-6, stem cell factor (SCF), and erythropoietin (EPO) have been found to support the growth of multi-potent progenitor cells *in vitro*. Clinically, granulocyte-colony-stimulating factor (G-CSF) and erythropoietin (EPO) provide effective treatments for neutropenia and anaemia and are used to enhance peripheral blood progenitors as an alternative to bone marrow transplantation for cancer patients. In haemopoietic tissue reconstitution after chemo-radiotherapy, thrombopoietin (TPO) was initially considered to be a selective stimulator in

megakaryocytopoiesis and platelet production. However, Grossmann et al. (1996) reported that treatment with TPO accelerates platelet, red blood cell and neutrophil recovery in myelosuppressed mice, indicating *in vivo* effects of TPO on multiple cell lineages. They also showed that the combined use of TPO and G-CSF further improves neutropenia associated with intensive chemotherapy in mice (Grossmann et al., 1996).

In vitro haemopoietic cell cultures are important experimental tools in biomedical research for understanding and analyzing the mechanisms underlying various cell functions. These can be easily manipulated, and hence, help in the systematic studies of haemopoietic system. Therefore, these are widely used as important experimental models to evaluate treatment strategies of diseases with their efficiency and toxicity (Larson et al., 2003).

The plant *T. occidentalis* is commonly known as fluted pumpkin, fluted gourd, Costillada (Spanish), Krobonko (Ghana), and Gonugbe (Sierra Leone). The plant belongs to the *Cucurbitaceae* family and is cultivated across lowland humid tropics of West Africa (Burkett, 1968). *T. occidentalis* leaves extract are used traditionally to treat malaria and anaemia (Gbile, 1986). The leaves are eaten as vegetables while the seeds are either roasted or ground for other food preparations. Apart from the nutritional (Okoli and Mgbeogu, 1983), agricultural and industrial importance (Akoroda, 1990), the plant is also medicinally useful. It is claimed to possess anti-inflammatory, antibacterial, erythropoietin, anti-cholesterolemic and anti-diabetic activities (Odoemena, 1995; Ajayi et al., 2000; Eseyin et al., 2000; Oluwole et al., 2003; Eseyin et al., 2005a, 2005b). The ripe fruit contain up to 13% oil. The leaves and the young shoots of the plant are frequently eaten as a potherb (Okoli and Mgbeogu, 1983; Tindal, 1968; Okigbo, 1977). The seeds of the plant are also popular items of diet and are cooked whole and ground into soups. The root and leaves have been shown to contain highly toxic alkaloids and saponins (Ajayi et al., 2000). The leaves also contain protein, vitamins and flavours (Gbile, 1986; Tindal, 1968). In Nigeria, the herbal preparation of the plant has been employed in the treatment of sudden attack of convulsion, malaria and anaemia (Gbile, 1986). Despite its widespread usage as food and medication information on the biological activity of the plant it is very scanty.

It is highly desirable and beneficial to identify certain natural compounds that can promote differentiation of haemopoietic stem cells or other stem cells individually or synergistically. This manuscript aims to establish the differentiation activities of the leave extracts of *T. occidentalis* on haemopoietic stem cell, since it has been widely used to treat anaemia in folk medicine in West Africa.

MATERIALS AND METHODS

Telfairia occidentalis

Fresh samples of the plant were obtained within Ilorin metropolis. The plant was identified by carrying out macroscopic examination as stipulated by Dalziel (1968) and confirmed and authenticated by staffs in the herbarium of Department of Plant Science, University of Ilorin, Nigeria.

T. occidentalis plant was given Serial Number 959 and Ledger Number 150. The sample was dried in an incubator at 37°C (Uniscop, USA). Plant extraction was carried out by the method of Olowosulu et al. (2006). Plant material was macerated in pestle and mortar with 100 ml 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 h for the research work (Burkett, 1968). 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 in weight were obtained from the animal house, LAUTECH College of Medicine Osogbo, Osun-State, Nigeria. Animals used for experiments were housed in the animal house of the Department of Anatomy, University of Ilorin in a temperature and humidity-controlled room that was maintained on a 12-h light/dark cycle. Food and water were available *ad libitum* throughout the experiment.

Method of irradiation of the guinea-pig

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 (1967). Each guinea-pig was separately irradiated under general anesthesia (i.m. 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, that is, 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 Co⁶⁰ therapy unit as source at University College Hospital, Ibadan, Nigeria at a dose rate of 98.560cGy/min.

After-care of the irradiated animals

To minimize the two hazards enumerated by Harris (1967), that is, 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 water.

Bone marrow harvest

Bone marrow cells from study guinea pigs were harvested

by the method of Galvin et al. (1996). 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 units/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 $1.0 \times 10^9/L$.

Microscopic examination of harvested bone marrow

Harris (1967) gave 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 (1967). The initial phase of final haematopoietic recovery was confirmed by cyto-chemical reactivities as described by Caxton-Martins using May-Grunwald's staining technique (Caxton-Martins, 1973). The harvested cells stained with May-Grunwald's staining technique shows cells of high nuclear: cytoplasmic ratio, leptochromatic nucleus, basophilic cytoplasm and presence of a nuclear hof. Cytochemically, cells were negative for myeloperoxidase, specific and non-specific esterase, leucocyte alkaline phosphatase and acid phosphatase, Sudan Black B, 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 exposed and incised and about 5 ml of blood collected in a centrifuge tube through a glass funnel. After clotting, the blood was centrifuged for 10 min 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 (1973). A similar technique was used by Harris (1967) and Yoffey (1956). This technique was used to keep the damaged cells in the marrow smears to the minimum.

May-Grunwald staining technique

May-Grunwald staining techniques are described as:

- Air dried films were fixed by immersing in a jar of methanol for 20 to 25 min. The slides were transferred to a staining jar containing May-Grunwald's stain freshly diluted with an equal volume of buffered water for 15 min;
- 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 to 15 min;
- The slides were transferred to a jar containing buffered water pH 6.8, rapidly washed in 3 to 4 changes of water and finally allowed to stand undisturbed in water for 2 to 5 min for differentiation to take place;
- Keep the slides in upright position to dry and report using X100 objective lens.

Culture media preparation

Eagles MEM is to prepare [1000 ml of Growth and maintenance Eagles MEM media] as follows:

- One bottle of Eagles MEM powder containing 9.4 g was dissolved completely in 1000 ml of sterile double distilled de-ionized water;
- The mixture was autoclaved at 121°C for 15 min 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 to 4.5);
- The reagents below were dispensed into two empty sterile 500 ml bottles;
- 7.5% Sodium bicarbonate (12.5 ml), L-Glutamine (5 ml), HEPES IM (5 ml), Penicillin Streptomycin (5 ml), FBS (10 ml).

The autoclaved Eagles MEM was added to each of the 500 ml bottles containing the reagents aforementioned to reach the mark of 500 ml on the bottle:

- The pH was adjusted between (7.2 to 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 to 7 days and inoculate into Thioglycollate broth;
- The prepared media was stored at +4°C until use (WHO, 2004).

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 to 100%. 5 µl of suspended guinea-pig bone marrow cells harvested were cultured in a Laminar Flow Cabinet at a concentration of 1.0×10^9 /L in 20 µl of Growth Eagles Minimum Essential Media (MEM) and 10 µl of the plant extract of *T. occidentalis* at

concentration ranges of 0.313 to 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 h. 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

Twenty µl of MTT Solution were added to each well 5 h before the end of the treatment in the culture plate. The plates were then incubated in a CO₂ incubator for 5 h and the culture media removed with needle and syringe. 200 µl of DMSO was added to each well with pipetting up and down to dissolve crystals. Plates were re-incubated in a CO₂ incubator for 5 min, transferred to a micro-plate reader and the absorbance measured at 550 nm (S).

Differentiated cell morphology

Cells harvested on day 13 were cytocentrifuged on a glass slide, fixed for 5 min with acetone-methanol (9:1, vol/vol) at room and stained with benzidine dihydrochloride stain, myeloperoxidase, acid phosphatase, Sudan Black B and Periodic acid Schiff.

RESULTS

Analysis of the cell expression showed that expression of erythropoietic and leukopoietic markers were uncoordinated during cell differentiation (Table 1). 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 polychromatophilic 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 groups illustrated the multipotentiality of leaf extract of *T. occidentalis* plant and its usefulness as sources of enhancing

Table 1: A comparison of mean $\pm$ Sd of the differentiated cell morphology expression of the plant extract 13th day post irradiation.

Plant extract	Benzidine-mixed-non-uniform reactive	Myelo-peroxidase	Sudan Black B.	Acid phosphatase	Periodic acid Schiff.
<i>Telfairia occidentalis</i> (TO) treated Cells	3.8333 $\pm$ 0.38	1.7083 $\pm$ 0.46	1.9583 $\pm$ 0.46	1.8750 $\pm$ 0.45	2.8333 $\pm$ 0.56
Non-extract treated cells	2.7083 $\pm$ 0.46	1.2083 $\pm$ 0.51	1.2083 $\pm$ 0.51	1.2917 $\pm$ 0.55	1.1667 $\pm$ 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.

Table 2: A Comparison of mean $\pm$ Sd of the plant extract 13th day post irradiation at concentration ranges 0.313 to 100%.

Plant extract	Variable	Mean $\pm$ standard deviation	p-value
<i>Telfaria occidentalis</i> treated culture plates	24	3.54 $\pm$ 0.30	0.000
Non-extract treated culture plates	24	0.18 $\pm$ 0.01	
GM-CSF treated culture plates	24	0.88 $\pm$ 0.05	

Table 3: Correlation between concentrations of the extract of the *Telfaria occidentalis* and degree of differentiation.

Plant extract	Variable	Mean $\pm$ standard deviation	Variable	P-value
<i>Telfairia occidentalis</i> treated culture plates	24	2.94 $\pm$ 0.94	2.94 $\pm$ 0.94	0.001

haemopoiesis locally in the treatment of malaria and anaemia (Gbile, 1986; Ajayi et al., 2000).

In this study, we cultured haemopoietic stem cell and subsequently treated the culture plates with or without aqueous leaf extracts of *T. occidentalis*. The comparison of the mean $\pm$ standard deviation between the treated plate (Mean $\pm$ Standard deviation; 3.54 $\pm$ 0.30) and non-treated plate (Mean $\pm$ Standard deviation; (0.18 $\pm$ 0.01; GM-CSF treated control (0.88 $\pm$ 0.05)] shown a statistical significance difference at p-value=0.000 (p-value < 0.05)(Table 2). 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 Mean $\pm$ standard deviation (2.94 $\pm$ 0.94) and calculated "r"(0.835) at statistically significance p-value(0.001)(p-value<0.05)(Table 3). Analysis of the cell expression showed that expression of erythropoietic and leukopoietic markers were uncoordinated during cell differentiation but more in favour of erythroid lineage. This is in keeping with antianaemic and erythropoietic reports of Ajayi et al. (2000) and Gbile (1986).

Conclusion

This study investigated and 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 lineage. Further studies are required to determine the potential radioprotective efficacy of this plant extract and how it might influence various endpoints of haemopoietic radiation damage during radiotherapy for cancer.

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