JOURNAL OF RECENT ADVANCES IN APPLIED SCIENCES (JRAAS) 28:96-99, 2013
ISSN 0970 1990, ST CODE 08 ,www.jraas.org. E MAIL: mhcdr@rediff.com
DISPLAYED ON LINE ON JUME 2012

TOXIC ASSESSMENT OF POTASSIUM NITRATE BY USING CFU-GM ASSAY

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Communication Number 82901
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ABSTRACT

CFU -GM assay is used to predict myelosuppression of cells caused by chemical induction as well as to find the haematotoxic potential of chemical compounds and the harmful effects of xenobiotics on the proliferative ability of the progenitors. CFU–GM was assessed on different concentrations ($4x10^{-8}$ mg/ml to $4x10^{-1}$ mg/ml) of potassium nitrate in the bone marrow taken from Charles Foster (CF) rats under inverted microscope at 25X magnification. No such effect of the potassium nitrate was seen which could be related to the molecular mechanism of toxic effect of potassium nitrate used in the study.

KEY WORDS: CFU-GM assay, Potassium nitrate, Charles Foster rats

INTRODUCTION

Haematotoxicity can be caused by xenobiotics by interfering with committed progenitors, mature blood cells or stem cells following a cytotoxic insult, which make the haematopoietic system to be the limiting factor. *In vitro* assays are used as screens, as investigative tools, and in the clinical setting of bone marrow transplantation. The screening of drugs leading for low haematotoxicant potentiality is one of the main applications. The *in vitro* CFU-GM assay is used to predict the haematotoxic potential of chemical compounds, such as myelosuppression of cells, direct harmful effects and the exposure level of xenobiotics on the proliferative ability of the progenitors (1,2). It is also used to study the synergistic and antagonistic effects of many compounds (3). Bone marrow failure can be described by myelotoxicity as a result of adverse effect of xenobiotics on haematopoiesis. Three types of haematopoietic progenitor clonogenic assays: CFU GM assay, BFU-E assay and CFU-MK assay can be used to detect myelotoxicity induced by chemicals, drugs, food and environmental contaminants (4), which can be estimated from the number of surviving progenitors as a function of exposure level on administration of maximum concentration of cytokines (5). Pessina and Bonomi had developed the standard method of CFU-GM assay for evaluation of myelotoxicity of drug (6,7) which is certified by the ECVAM Scientific Advisory Committee (ESAC) for knowing the acute neutropenia in humans as a substitute to using a second species (8,9,10). The assay has many uses in development of antineoplastics and therapy measurments. The frozen cryopreserved bone marrow cells of rat were refined and improved for prediction of haematotoxicity (11) and its variability was evaluated in an international study[3]. The use of this technique was reported by Gribaldo and his colleagues (1). The assay provides a lot of information about number of colonies, its potential sensitivity and linearity (January 1997-July 1998). The assays are used to choose the most appropriate animal species for use in preclinical evaluation of compound and therapeutic index-based screening to identify less-myelosupressive analogues for deriving structure-toxicity relationship. As investigative tools they are used for studying mechanisms of haematotoxicity of radiation, chemicals and biological agents. For clinical trials these are used to identify the dependency of myelotoxic effects which provides information about mechanisms of action, plan of cytokine support and estimate dose escalation effect (12). The present study was conducted to check the *in vitro* haematotoxicity of different concentrations of potassium nitrate on the rat bone marrow cells.

MATERIALS AND METHOD

Chemicals:

A number of chemicals were required to conduct the experiment which was purchased from different companies. Iscove's Modified Dulbeccco Media (IMDM), Bovine Serum Albumin (BSA), Trypan blue and Penicillin-Streptomycin solution were purchased from Sigma-Aldrich Chemical Private Ltd., New Delhi while methyl cellulose , L-glutamine and colony stimulating factor (GM-CSF) were purchased from Bio Basic Inc., India.

Preparation of Methyl Cellulose Culture Media:

For murine bone marrow cells methyl cellulose culture media type A was prepared. It contained 1% methylcellulose in IMDM, 30% FBS, 1% BSA, 2Mm L–glutamine and 10ng/ml granulocyte-macrophage colony stimulating factor (GM-CSF). The bottle containing MCM was thawed overnight at 4^0C and homogenized by inverting the bottle several times and aliquots were prepared in sterilized tubes.

Fig 1: Control (Very Dense Cells)

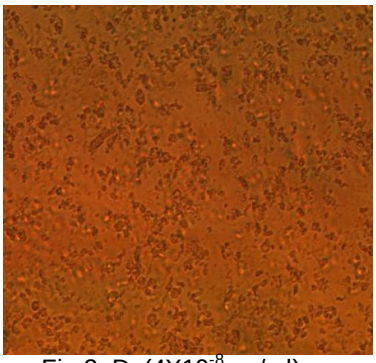

Fig 2: D_1 ($4X10^{-8}$mg/ml) 80,000 Cells/ Dish

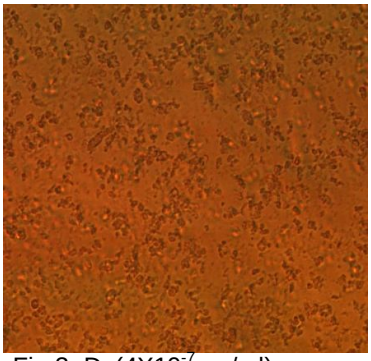

Fig 3: D_2 ($4X10^{-7}$mg/ml) Dense Cells 75,000 Cells/Dish

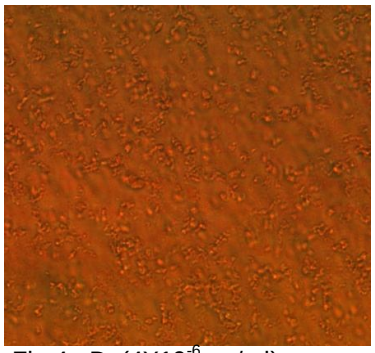

Fig 4: D_3 ($4X10^{-6}$mg/ml) Diffuse Spread Cells

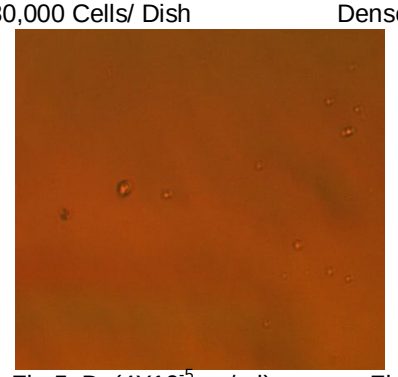

Fig 5: D_4 ($4X10^{-5}$mg/ml)

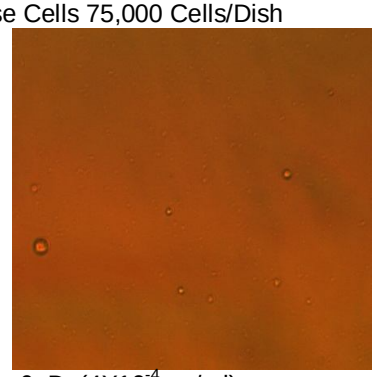

Fig 6: D_5 ($4X10^{-4}$mg/ml)

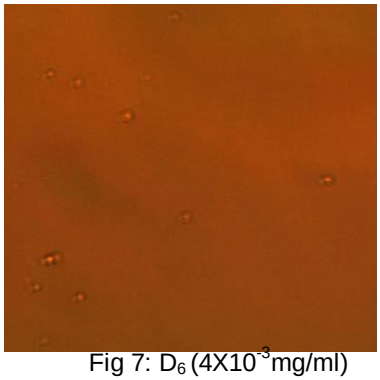

Fig 7: D_6 ($4X10^{-3}$mg/ml)

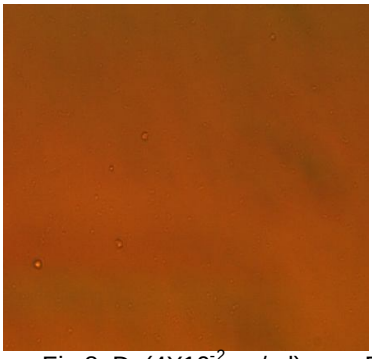

Fig 8: D_7 ($4X10^{-2}$mg/ml)

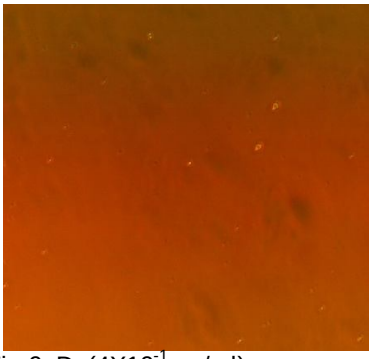

Fig 9: D_8 ($4X10^{-1}$mg/ml)

Figure 1-9: Growth of CFU-GM at different concentrations

Preparation of Drug:

Potassium nitrate was provided by Merck Specialities Pvt. Ltd., India. The dry powder was reconstituted with autoclaved distilled water and diluted to a concentrated stock solution. The different concentrations of drug dilutions ($4x10^{-8}$ mg/ml to $4x10^{-1}$ mg/ml) were freshly prepared from the stock prior to experiment to avoid degradation.

Preparation of Bone Marrow Cells (BMCs):

Fresh bone marrow cells were collected from the femur of young and healthy male Charles Foster rats and suspended in IMDM supplemented with 30% FBS. After evaluating the percentage of cell viability in a haemocytometer under microscope, the cell suspension was adjusted at $1.5x10^6$ viable cells/ml of suspension.

Colony Forming Assay:

The experiment was performed according to Pessina et al., 2001(8). Eight MCM tubes were taken for different concentrations of the compound and one for control. IMDM was added to each tube while toxicant dilution was added in their respective tubes. Immediately after that, equal amount of bone marrow cell suspension was added to each tube and moved gently to mix. Then 1ml of cell medium mixture from each tube was transferred into their respective plate and rotated gently to spread the mixture evenly. The

cultures were incubated at 37^0C with 5% $C0_2$ under saturated humidity for 7 days.

Scoring of Colonies:

Colonies were counted after 7 days of incubation. The CFU-GM colonies were scored by scanning the whole plate by using an inverted microscope under 25X magnification. The aggregates with 50 or more cells were defined as CFU-GM colonies while aggregates with 20-25 cells were defined as clusters and not counted as colonies.

RESULT AND DISCUSSION

No toxicity was observed at molecular level which could be related to the dose of potassium nitrate (Fig 1-9) (However, dose related insignificant inhibitions of CFU-GM colonies were observed: Fig 5-9). The scientific validation of this model was confirmed by the accuracy of pre-analysis and a broad range of SOP testing, which has many applications in assessment of haematotoxicity. CFU-GM assay enables us to find the results of screening phase which can score different colonies like Compact, Diffuse and spread, Multicentric and Burst forming units(13).

In a study conducted in our laboratory; dose levels of 0, 45, 90 and 135 mg/kg of body weight of potassium nitrate was given to Charles Foster rats for assessment of *in vivo* toxicity which showed no such adverse effect on vital organs (14). In vitro tests minimize the chances of uncertainties of toxicological investigation as a result of exploitation of animals and human and hence give a clearer picture for calculating clinical dosages (10). The CFU-GM assay lack to predict xenobiotics that are non toxic to haematopoiesis and fail to inhibit neutrophil progenitor as it specifically detects myelosupressive agents (15), mentioned earlier. An algorithm is developed which is capable of recognizing and scoring the CFU-GM colonies by data fusion (16). Recently a non-clonogenic fluorometric microculture cytotoxicity assay (FMCA) has been established as an alternative to CFU-GM assay in preclinical haematotoxicity studies (17).

Another similar study was conducted in our laboratory with Charles Foster rats in which chloramphenicol was administered for 14 days toxicity which caused significant haematotoxicity and hepatotoxicity, which was reversed with the use of coconut water (20ml/kg) in 14 days establishing that coconut water has potential for amelioration of chloramphenicol toxicity (18). Dose related reversible bone marrow depression and fatal aplastic anaemia is caused by Chloramphenicol (19). Earlier researchers suggested that bone marrow showed vacuolation, erythroid maturation arrest, hypocellular marrow and erythroid depletion in chloramphenicol treated rats at different dose level (20,21,22). We will soon conduct the *in vitro* toxicity of chloramphenicol by using CFU-GM assay in our laboratory.

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