Flow cytometry and microscopy method comparison for *in vivo* micronucleus studies in rat bone marrow and peripheral blood

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Abstract

With the adoption of the ICH S2(R1) the limitations associated with using rat peripheral blood have been overcome by using flow cytometry. Among the advantages of this method are: low blood volume requirements, a high number of cells scored, scoring of very young immature erythrocytes, and the ability to sample without sacrificing the animals. Here we compared the relative strengths and limitations of microscopic and flow cytometric methods for micronuclei scoring in rat bone marrow and peripheral blood.

Five animal replicates were treated independently at 24 hours intervals. In each replicate one group was treated with Cyclophosphamide at 20 mg/kg via intraperitoneal (i.p.) injection and a second group was either treated i.p. with 0.9% sterile saline solution or with purified water via oral gavage as vehicle controls. Bone marrow was collected 24 hours post-treatment and evaluated by fluorescence microscopy. Peripheral blood was collected 48 hours post-treatment and evaluated by fluorescence microscopy using supra-vital staining and by flow cytometry. At least 2,000 erythrocytes were evaluated by manual method and over 10,000 by flow cytometry. Overall the results were as expected with all three methods. There were low micronuclei frequencies with the vehicle controls and a significant increase in micronuclei frequencies in cyclophosphamide treated groups. However, the results percentages obtained with the bone marrow scored manually and peripheral blood scored automatically were closer: (0.13%, 1.99%), (0.13%, 1.57%), with vehicle and cyclophosphamide, respectively. The results obtained with peripheral blood stained supra-vitally were less evident (0.08%, 0.67%).

When using the supra-vital method it was difficult to select enough young immature erythrocytes for scoring. The flow cytometry is clearly superior for analysis of peripheral blood while the standard fluorescent microscopy remains a reliable method for analysing bone marrow preparations.

Introduction

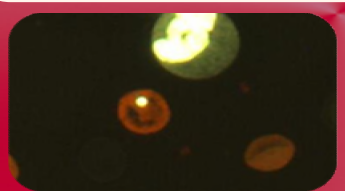
The micronucleus assay is a widely accepted test used in genetic toxicology to detect clastogens and aneugens. It is tested mostly in rodents bone marrow and mouse peripheral blood. The micronucleus in rats peripheral blood was less used because the spleen cleans the micronucleated cells and only newly formed erythrocytes were considered acceptable for the micronucleus assessment which was challenging to score. To overcome this limitation supravital method was developed in which only the very young erythrocytes are scored but this method is time consuming which reduces the number of slides and animal samples. More recently, a flow cytometry method was developed and applied to micronucleus testing. This method allows for accelerated data acquisition, analysis of large numbers of samples/cells therefore increase the statistical power, exclusion of subjective judgment and individual scoring skills, low blood volume requirements, and the ability to sample without sacrificing the animals.

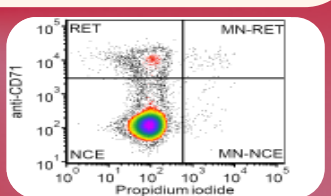
Objective

The objective of the study was to develop the micronucleus assay at ITR Laboratories and to compare results from different scoring methods and different blood compartments.

Experimental Design

During this study, 5 replicates of animals were treated independently at 24 hours interval. Each replicate contained one negative and one positive control group with three animals each group. The negative controls were treated with either 0.9% Saline sterile solution or purified water and the positive control groups were treated with cyclophosphamide monohydrate at 20 mg/kg.
Bone marrow and peripheral blood, were collected to evaluate the presence of micronucleus using microscopic and flow cytometric methods. The micronuclei frequencies were scored in bone marrow by using the standard microscopic method and in peripheral blood by using both microscopic and flow cytometric methods.
Bone marrow cells were collected washed, smeared on the slides and fixed with methanol. The cells were wet stained with Acridine Orange and evaluated for the presence of micronuclei within immature erythrocytes.
Sample of peripheral blood was taken from each animal and separated into two aliquots. First aliquot was used to prepare slides and the other aliquot was processed for flow cytometry analysis. The first sample was diluted in the FBS and few drops of this suspension was smeared on acridine orange pre-coated slides and read under fluorescent microscope [4]. The second sample was processed by using MicroFlow Plus kit from Litron Laboratories. The sample was diluted in anticoagulant, fixed in methanol, transferred to long term storage solution, labelled with antibody, stained with DNA staining then analyzed with flow cytometer.





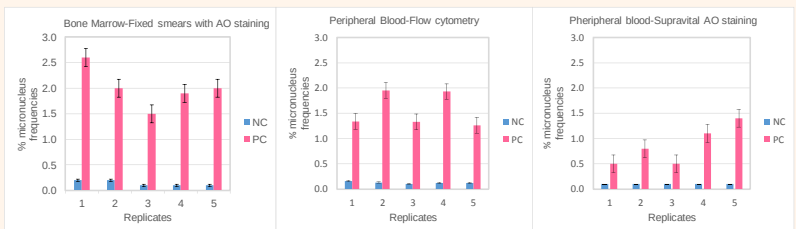


Figure 1: The frequencies of micronucleus in bone marrow and peripheral blood scored either manually or automatically.
Number of replicates is 5 and each replicate contains 2 groups with 3 animals in each group. NC: negative controls (water or saline), PC: positive controls (Cyclophosphamide), AO: acridine orange.

Results and Discussions

The results obtained with the two methods (microscopic and flow cytometric) were analyzed statistically using anova ($p \leq 0.01$) to determine variability between independent replicates, scoring method and the difference in the micronucleus frequency in bone marrow and peripheral blood.
The vehicle controls used in all replicates did not induce any increase in the micronucleus frequencies scored with both methods. The results were within the laboratory historical control data and consistent with values reported in the literature [1-3]. The positive controls induced, as expected, an increase in the number of micronucleated immature erythrocytes in the five replicates in bone marrow and peripheral blood ($p \leq 0.01$). However, in some experiments such 1 and 3, in peripheral blood, the detection of micronucleus was weak in either negative and positive controls scored with supravital method (0.08 %, 0.67%) compared to flow cytometry method (0.13%, 1.57%) or bone marrow scored manually (0.13%, 1.99%).

Results presented in figure 1 showed overall consistency between the 5 replicates regardless of the tissue analyzed or scoring methods suggesting that the manual and automated methods are comparable.

Conclusion

It was concluded that both bone marrow and peripheral blood could be used for rat micronucleus assay. For practical reasons, the bone marrow still could be evaluated using the standard fluorescent microscopic method however, it is more advantageous to score micronucleus in the peripheral blood with flow cytometry.

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Abstract Number: 1418 Poster Board: P147

Abstract Title: Flow Cytometry and Microscopy Method Comparison for in vivo Micronucleus Studies in Rat Bone Marrow and Peripheral Blood

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Session Title: Genetic Toxicology II

Presentation Date & Time: March 14, 2016 from 1:15 PM to 4:30 PM