Brief Communication

Application of Feulgen-Light Green Staining Method to Show the Micronuclei in Fetal Rat Blood

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A micronucleus is a small spherical body in the cytoplasm of a cell. It is not associated with the nucleus, but it contains a chromatin mass (DNA).[1] Therefore, it has a similar staining quality as the main nucleus. In a nucleated cell, its diameter is smaller than 1/3 of the diameter of the main nucleus.[2] Micronuclei arise when replicating cell populations are subjected to chromosomal breakage by clastogens (agents causing chromosomal breakage) or to chromosome loss by mitotic spindle dysfunction.[3] Therefore, the micronucleus test can be used to assess the cytogenetic damage caused by genotoxic agents, and is claimed to be as sensitive as the laborious cytogenetic analysis. Moreover, this test is now widely used to test the mutagenicity of various agents, due to its simplicity and rapidity. The in vivo micronucleus test using fetal mice is claimed to be more sensitive to test a premutagen that needs activation, because blood cells develop in the fetal liver, alongside hepatocytes which metabolise premutagens to the active form.[4] However, the fetal blood screened for micronucleus contains RNA positive young erythrocytes (reticulocytes).[3] The micronucleus staining methods widely used are the modifications of the Romanowsky staining method (eg. Wright, Giemsa), which also stain the RNA. RNA aggregates can resemble a micronucleus, thus causing difficulty in screening.[1] To overcome this problem, fluorescent stains which could differentiate the DNA from RNA were introduced.[1,3,5] However, the use of fluorescent stain needs fluorescent microscopy, and the screening must be done quickly, due to the limited fluorescent time. The Feulgen stain is a specific stain that stains DNA, but it is usually used to stain the nuclei in paraffin sections. Recently, the Feulgen-Congo red (FCR) staining method was developed to stain micronuclei in blood smears, but the FCR stained cells were less intensively stained than the Giemsa stained cells, and the FCR stained micronuclei were less well pronounced.[6] Consequently, it is difficult to score manually, though the result is reliable. The aim of this study is to develop a staining method that only stains the micronuclei intensively. We used the Feulgen stain which stains only the DNA (micronuclei), and light green as a counter stain, in the expectation of well pronounced micronuclei.

The pregnant rats of the Lembaga Makanan Rakyat (LMR) strain were sacrificed on day 17th and 18th of gestation, the fetuses were severed to get the fetal blood, and fetal blood smears were made. The slides were fixed in absolute methanol for 1 min.[1] and stained using the Feulgen and light green stain.[7] The reagents used in the Feulgen staining method are N/1 hydrochloric acid, bisulphite solution and de Tomasi Schiff reagent,[7] and 1 % (W/V) light green solution as counter stain.[7] Staining method: the slides were rinsed in water, and N/1 HCl (1 min.) consecutively, then placed in N/1 HCl at 60 C (hydrolization), and rinsed in N/1 HCl at room temperature (1 min.); after that, the slides were tranfered to Schiff's reagent for a certain time and rinsed 3 times consecutively in bisulphite solution (2 min. each); finally the slides were rinsed well in distilled water, counterstained using light green solution, rinsed in water, dehydrated through graded alcohols to xylene and mounted.[7] In this investigation, several hydrolization (using N/1 hydrochloric acid) times (5, 10, 15, 25 min.), Schiff reagent (30, 45, 90 min.) and light green (2min., 10, 2, 1 sec.) staining time were tried; each were done in duplo. The slides were examined to determine the shortest time which gave the best result in hydrolization, Schiff and light green staining.

The best result was obtained at hydrolization time of 10 and 15 minutes, Schiff staining time of 45 and 90 minutes, and light green time of 1 and 2 seconds. Using the Feulgen-light green stain, the main nuclei and micronuclei were stained pale magenta (purplish pink) and the cytoplasm green. The main nuclei were clearly

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distinguishable, due to its big size, but the micronuclei appeared as white dots in contrast with the green cytoplasm, their colour (purplish pink) were only distinguishable when the microfocus was used (Fig. 1A).

In this study we used fetal blood of the LMR strain rats, because former results showed that the amount of cells containing micronuclei was high, even when the animals received no treatment.[8] Besides that, fetal blood contains a considerable amount of nucleated cells of the erythrocyte lineage.[9] The micronuclei take the same quality of staining as the main nuclei,[2] thus it is very easy to detect the success of staining, by looking at the nuclei. The Feulgen and Rossenbeck (1924) staining method is a standard technique to demonstrate the deoxyribose in DNA.[7] This method consist of the cleavage of the purine-deoxyribose bond by mild acid hydrolysis to expose a reactive aldehyde group, and the detection of the aldehydes by the use of a Schiff reagent. The reactive aldehyde combined with the leucofuchsin in the Schiff reagent yields the formation of a quinoid compound which gives a red purplish colour.[7,10] In this study, several hydrolization times were tried, as the correct hydrolization time suitable to the fixative used in this study (absolute methanol) was not available. The best result was obtained using hydrolization time of 10 and 15 min. Using hydrolization time of 5 and 25 min. gave a paler nuclei, and less contrast. This finding supports the theory that the hydrolysis is the critical part of the method, with an increasingly stronger reaction as the hydrolization time is increased until the optimum is reached.[7] Stevens and Bancroft (1990) found that 45 min was enough for Schiff's reagent staining,[7] but other investigators suggested shorter or longer time.[6,10,11] Most investigators stained paraffin sections, except Castelain et al[6] who stained blood smear slides, as was done in this study. Therefore, we tried several Schiff's reagent staining times, and we got the same result as Stevens and Bancroft. The recommended staining time for 1% light green solution is 2 min.,[7] but we found that 2 min. gave a very strong result, thus we shorten the staining time. The Feulgen-light green stain does not stain the unevenly distributed RNA in the young erythrocytes, when we use fetal blood. Therefore, no effort is needed in differentiating the micronucleus from RNA aggregates, such as in the modifications of the Romanowsky staining method (Fig 1A and 1B). In addition, in the in vitro micronucleus test, in which the lymphocytes are screened, the Feulgen-light green staining method has an advantage. This staining method only stains the DNA (micronucleus), thus no effort is needed to differentiate the micronuclei from the aggregates of the azurophilic granules stained in the modifications of the Romanowsky staining method.[12] The only disadvantage of Feulgen-light

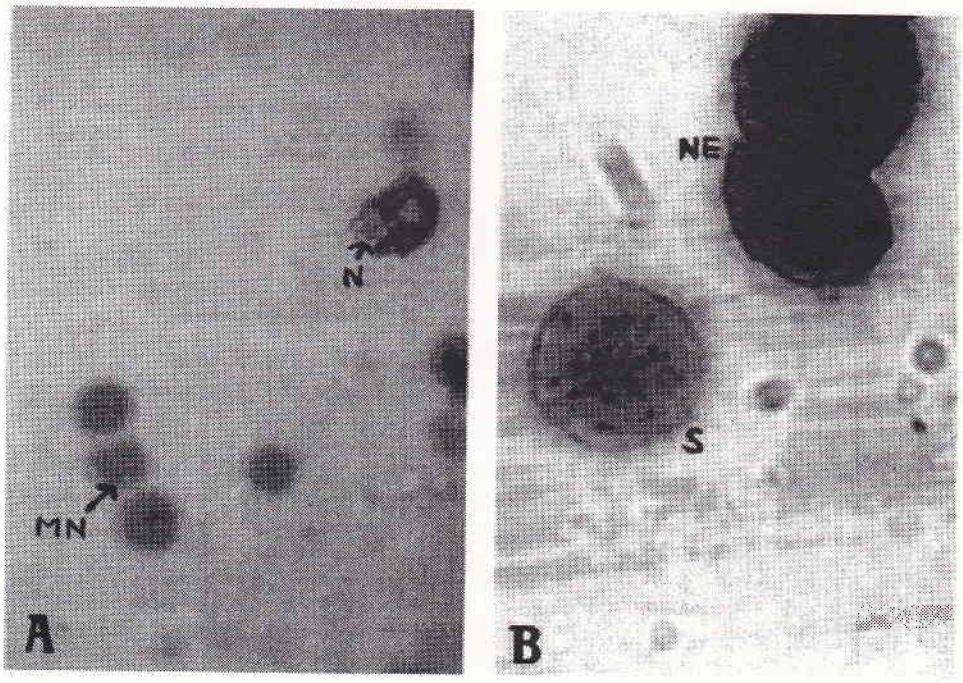

Figure 1. The micronucleus staining. (A) Using the Feulgen-light green staining method, MN = micronucleus (purplish pink), N = nucleus. (magnification X225.) (B) Using Wright's stain, S = stippled erythrocyte, NE = nucleated cell of the erythrocyte lineage. (magnification X1000.)

green staining method is the need to use the microfocus, to differentiate white colour (RNA and other substances) from purplish pink (micronucleus). In this study, the amount of micronuclei was not compared to that found using other staining methods. Therefore, whether there was false positive/negative results was not known, and needs further investigation.

In conclusion, the shortest time needed which gave the best result was obtained at hydrolization time of 10 min., Schiff staining time of 45 min. and light green staining time of 1 second. The results showed pale micronuclei, but whether there was false positive/negative results needs further investigation.

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