A simple method in preparing peripheral blood smear rich in mononuclear leucocytes with abundant cytoplasm for micronucleus test

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Abstrak

Penelitian ini bertujuan mengembangkan cara sederhana untuk membuat sediaan darah tepi yang banyak mengandung lekosit mononuklir bersitoplasma banyak. Sediaan demikian penting untuk melakukan uji mikronukleus yang sederhana dan murah. Sampel darah didapat dari PMI, Jakarta Pusat. Telah dicoba berbagai cara pembuatan sediaan, menggunakan berbagai kombinasi perlakuan hipotonik dan fiksasi. Sembilan macam cara skala kecil yang memberikan hasil konsisten dan baik, diulang pada 10 orang. Analisis dilakukan pada sel mononuklir, dalam hal rasio sel utuh, diameter sel dan lebar sitoplasma. Analisis statistik (balanced Anova) dilakukan dengan program Minitab untuk Windows. Cara pembuatan sediaan yang terbaik adalah dengan perlakuan hipotonik plasma:aquabides 4:1, 3:1, dan 2:1, cara apusan dua garis memanjang dan fiksasi sediaan menggunakan metanol:asam asetat glasial 9:1.

Abstract

This study aimed to develop a simple method to make peripheral blood smear rich in mononuclear leucocytes with abundant cytoplasm. Such blood smear is essential for a simple and inexpensive micronucleus test. Blood samples were obtained from the Indonesian red cross, Central Jakarta. Various methods using combinations of hypotonic treatment and fixation were performed. Nine small scale methods showing good and consistent results were repeated on 10 individuals. Analysis on the ratio of intact cells, cell diameter and cytoplasm width were done on mononuclear cells. Statistical analysis (balanced Anova) were done using a Minitab program for Windows. The best method consists of hypotonic treatment using plasm:aquabidest 4:1, 3:1, and 2:1, smearing method resulting in 2 slender smears, and slide fixation using methanol:glacial acetic acid 9:1.

Keywords: *Hypotonic treatment, fixative, cell diameter, cytoplasm width*

Mass screening of the effect of mutagen exposure in human population is uncommon. The reason for this is the lack of an economical and simple method. Mutagen exposure in human can be detected by chromosomal aberration analysis, sister chromatid exchange analysis or micronucleus assay.[1] The first and second method are laborious and expensive, due to the need to culture the peripheral lymphocytes.

Micronucleus assay is usualy done on peripheral lymphocytes[2-4] or polychromatophilic erythrocytes.[5] The first need lymphocyte culture in the presence of cytochalasin B which is expensive and not readily available in Indonesia; the second is a simple method, but not popular due to the need of a bone marrow puncture, which is an invasive and painful procedure.

In peripheral blood, the most abundant cell is erythrocytes, and micronucleus assay on erythrocytes should be very simple. However, micronucleus containing erythrocytes will be destroyed in the spleen, therefore erythrocytes cannot be used in micronucleus assay except in splenectomized individuals.[6] Polymorphonuclear leucocytes are not suitable candidates, due to their segmented nuclei. Mononuclear leucocytes (lymphocytes and monocytes) can be used, provided that they have abundant cytoplasm in which the micronucleus shoud be searched. Monocytes have abundant cytoplasm, but monocyte count in peripheral blood is low; therefore using monocytes alone will be difficult to fullfil the minimum cell requirement needed (800 cells,[3] 1000 cels[1,7]). In conclusion, mononuclear leucocytes will be suitable candidates.

The aim of this study is to develop a simple method to make peripheral blood smear rich in mononuclear leucocytes with abundant cytoplasm.

METHODS

Blood was obtained from the Indonesian Red Cross (Palang Merah Indonesia, PMI), central Jakarta, Indonesia.

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Chemicals: NaH2PO4 (BDH), K2HPO4 (BDH), NaCl (Merck), KCl (Merck), methanol (Wako), glacial acetic acid (Merck), heparin (Leo), Giemsa solution (Merck) and aquabidest (Ikapharmindo Puramas) were purchased from CV Bumi Indah, or PT Elokarsa Utama. LOC (Amway) was purchased from PT Amindoway Jaya.

Large scale experiments

The main procedure was blood centrifugation to obtain the plasm, centrifugation of the plasm to get the cells, hypotonic treatment, followed by cell fixation and smearing of unfixed cell suspension on slides, or smearing on slides and slide fixation. Finally the slides were stained using 5% Giemsa solution in Sorensen buffer pH 6.7, and analyzed.

In large scale experiments, each kind of experiment used 10 ml blood, and some were repeated using 5 ml blood. We used various combinations of hypotonic solution and fixative. We used also various amount of hypotonic solution and fixative.

Centrifugation (to obtain the plasm, to get the cells, and centrifugation after hypotonic treatment and after cell suspension fixation) were done using various speed and over various time, i.e. 1900 g (10 min), 700 g (10 or 8 min), or 500 g (10, 8, or 5 min). Most experiments used the same speed and time in the whole procedure, but some experiments used a higher speed to obtain the plasm and /or to get the cells, compared to the speed used after hypotonic treatment or cell suspension fixation.

In each experiment, the hypotonic solution used was NaCl 0.9%:KCl 0.56% (10:1, 9:1, 8:1, 7:1, 6:1, 5:1, 4:1, 3:1, 2:1, 1:1, 1:1.75, or 1:2), or plasm:aquabidest (100:8, 100:9, 9:1, 8:1, 7:1, 6:1, 5:1, 4:1, 3:1, 2:1, 1:1, 1:1.5, 1:1.75, or 1:2). The amount of hypotonic solution used was 5 ml, 3 ml, or 1ml, and the hypotonic treatment was done instantaneously, or in 3 or 10 minutes.

In each experiment, the fixative used was a modified Carnoy solution (methanol: glacial acetic acid, 3:1 or 2:1), or 45% glacial acetic acid, which was used as cell suspension fixative; or methanol or 1% formaldehyde/50% alcohol as smeared cell fixative. The amount of fixative used was 5 ml, 3ml, 1 ml or a very small amount.

The analysis of slides was focused on mononuclear cells and the parameters observed were the amount of mononuclear cells/10x40 field, the frequency of lysis (screening of 100 cells), and the nucleus/cell diameter ratio of mononuclear cells (screening of 50 cells). The measurements of the diameter of the cells and their nuclei were done on the direction where the cytoplasms were maximal. Analysis of slides were done after each experiment, and relatively good results were repeated 4-5 times.

Small scale experiments

The main procedure was natural separation to obtain the plasm, hypotonic treatment, and slide preparation (smearing on slides and slide fixation). Finally the slides were stained using 5% Giemsa solution in Sorensen buffer pH 6.7, and analyzed.

The hypotonic solution used was aquabidest, 1 µg/ml or 0.1 µg/ml LOC/aquabidest; the plasm:hypotonic solution ratio was 2:1, 3:1, or 4:1 (the volume of the hypotonic solution was always 50 µl). The hypotonic treatment time was 3 min, 1 min, or very short (instantaneously dried using a hair dryer) The fixative used was heat, 80% methanol, 100% methanol or modified Carnoy solution (methanol:glacial acetic acid= 4:1, 6:1, or 9:1).

Slide preparation was done using 15 µl, 10 µl or 5 µl of hypotonic treated plasm. Smears on slides were made using the routine spreading method in preparing blood smears (by putting the material on the end of a slide, and pulling the material behind a polished object glass held at an angle of 45^0), or a modification of it (by putting 2 drops of material, and pulling the drops one by one using a long narrow piece of plastic, resulting in 2 narrow smears on one slide). Slide fixation should be done very fast, by applying fixative on standing slides and immediately air dried.

In small scale experiments, each kind of experiment used only 100 µl, 150 µl or 200 µl of plasm. In these experiments, we used various combination of hypotonic solution and fixative. The experiments that showed good results were listed in Table 1, and were repeated 10 times, using 10 different individuals.

The analysis of slides was focused on mononuclear cells and the parameters observed were the frequency of lysis (screening of 200-400 cells), and the measurement of the diameter of mononuclear cells and their nuclei (screening of 20 cells). Screening was only done on swelled cells (diameter of the cell or the nucleus in case of lysis >9 µ). The measurements of the diameter of the cells and their nuclei were done on

the direction where the cytoplasms were maximal. The measurement was only done on intact cells having round, oval, or slightly indented nuclei.

Table 1. Small scale experiments which gave good and consistent results

Experiment	Hypotonic treatment (plasm:aquabidest)	Fixative (methanol: glacial acetic acid)
I	4:1	4:1
II	4:1	6:1
III	4:1	9:1
IV	3:1	4:1
V	3:1	6:1
VI	3:1	9:1
VII	2:1	4:1
VIII	2:1	6:1
IX	2:1	9:1

The results were analyzed statistically using Minitab program for Windows. The ratio of intact cells was analyzed in 10 individuals using balanced Anova (2 way crossed design), with hypotonic treatment and fixation as fixed factors. The diameter of the cell and cytoplasm width (=diameter of the cell - diameter of the nucleus) were both analyzed in 6 individuals using balanced Anova (3 way crossed design) with hypotonic treatment and fixation as fixed factors and individual as random factor.

RESULTS

Large scale experiments

Large scale experiments gave relatively good results. However, repetition of those experiments gave very inconsistent results. The amount of mononuclear cells/10x40 field (<1 - >10 cells/field), and the frequency of lysis (29 -99 %) showed a very broad range. The experiments which gave good results in terms of the amount of mononuclear cells/10x40 field and the frequency of lysis, gave sufficient amount of intact cells to be evaluated for the nucleus/cell diameter ratio. The results of these experiments showed that the range of the ratio was 0.62 - 0.73.

Fixation using methanol or 45% acetic acid showed noisy background. Furthermore, fixation using 45 % acetic acid generated too many acytoplasmic cells. Fixation using modified Carnoy solution gave the best result (methanol: acetic acid = 3:1), while other modification (methanol acetic:acid = 2:1) resulted in the increase of acytoplasmic cells. Acytoplasmic cells were also seen on control slides (without hypotonic treatment) fixed with modified Carnoy solution.

The hypotonic treatment that gave relatively good result in terms of the frequency of lysis, and nucleus/cell diameter ratio was the treatment using plasm/aquabidest or NaCl 0.9%:KCl 0.56% instantanously. These experiments were listed in Table 2.

Table 2. Large scale experiments which gave relatively good results

Experiment	Hypotonic solution	Fixative (MeOH:HAc)	Procedure
I	N/K= 10:1 (5ml)	3:1 (5ml)	B (10ml)-C-C-H-C-F-C
II	N/K= 7:1 (5ml)	3:1 (3ml)	B (10ml)-C-C-H-C-F-C
III	N/K= 6:1 (5ml)	3:1 (3ml)	B (10ml)-C-C-H-C-F-C
IV	N/K= 3:1 (5ml)	3:1 (3ml)-2X	B (10ml)-C-C-H(t=0)-F-C-F-C
V	N/K= 2:1 (5ml)	3:1 (3ml)-2X	B (10ml)-C-C-H(t=0)-F-C-F-C
VI	N/K= 2:1 (3ml)	3:1 (3ml)-2X	B (5ml)-C-C-H(t=0)-F-C-F-C
VII	P/A= 4:1 (5ml)	3:1 (0.5ml)	B (10ml)-C-H-C-F-C
VIII	P/A= 3:1 (4ml)	3:1 (0.5ml)	B (10ml)-C-H-C-F-C
IX	P/A= 4:1 (0.5ml)	3:1 (3ml)	B (5ml)-C-C-H-C-F-C
X	-	3:1 (3ml)	B (5ml)-C-C-F-C

N/K= NaCl:KCl, P/A= Plasm:aquabidest, MeOH= methanol, HAc= glacial acetic acid, C= centrifugation, H= hypotonic treatment, t= time (in minutes), F= fixation, B= blood

Note: Hypotonic treatment instantaneously using plasm:aquabidest caused a large amount of pellet after fixation. However, cells obtained were very scarce.

Small scale experiments

In small scale experiments, fixation using heat caused cell damage and blurred slides, and fixation using methanol (80 or 100%) gave noisy background. Fixation using modified Carnoy solution gave good and relatively consistent results, but prolonged fixation in this solution showed increase in the frequency of lysis. The experiments which gave good and consistent result were listed in Table 1. Most of the swelled cells were located at the periphery of the smears, therefore the modified method (2 narrow smears) gave more swelled cells/slide, compared to the routine spreading method.
A small proportion of the cells screened showed 1-3 micronuclei (Figure 1).

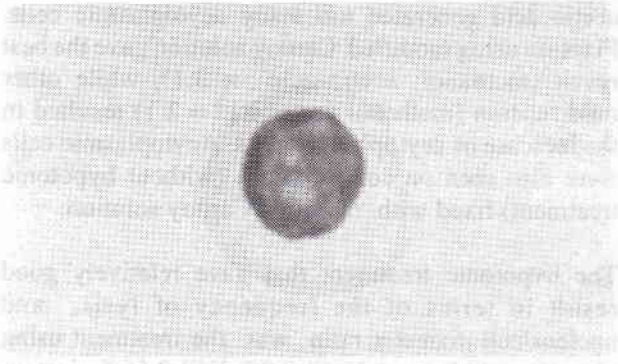

Figure 1. A plasmocyte like cell (photographed using Nikon Optiphot -2, 5x100)

Statistical analysis on the ratio of intact cells showed that there was no difference between hypotonic treatment (P= 0.772), but there were differences between various fixations (P= 0.001, see Table 3). Increase in the strength of hypotonic treatment showed increase in the diameter of the cell and cytoplasm width. Furthermore the mean of the diameter of the cell was maximum on the strongest fixative strength (methanol:glacial acetic acid= 4:1), and the mean of the cytoplasm width was maximum on the lowest fixative strength (methanol:glacial acetic acid= 9:1) (Table 4). However, statistical analysis on the diameter of the cell showed that there was no difference between various hypotonic treatment and between various fixation (P value was 0.019 and 0.655 respectively), and statistical analysis on the cytoplasm width showed that there was no difference between various hypotonic treatment and between various fixation (P value was 0.130 and 0.224 respectively). Though there were differences between individuals both in the diameter of the cell and cytoplasm width (both P= 0.000).

Table 3. The results of various fixatives on the ratio of intact cells in small scale experiments

Fixative	Ratio of intact cells $\bar{X}$ (SD)
Methanol:glacial acetic acid: 4:1	0.6073 (0.1312)
Methanol:glacial acetic acid: 6:1	0.6790 (0.0903)
Methanol:glacial acetic acid: 9:1	0.7153 (0.0956)

$\bar{X}$= mean, SD= standard deviation

Table 4. Cell diameter and cytoplasm width in small scale experiments

Treatment	Cell diameter (μ) $\bar{X}$ (SD)	Cytoplasm width (μ) $\bar{X}$ (SD)
Hypotonic		
P/A= 4:1	11.549 (1.685)	3.7889 (1.3978)
P/A= 3:1	11.637 (1.729)	3.8403 (1.6238)
P/A= 3:1	11.896 (1.794)	4.0097 (1.6316)
Fixation		
MeOH/HAc= 4:1	11.754 (1.760)	3.8542 (1.5808)
MeOH/HAc= 6:1	11.636 (1.708)	3.7958 (1.4916)
MeOH/HAc= 9:1	11.692 (1.758)	3.9889 (1.5925)

P/A= Plasm:aquabidest, MeOH= methanol, HAc= glacial acetic acid, $\bar{X}$= mean, SD= standard deviation

Unexpected result

We got an unexpected, but very interesting result on small scale experiments. Some cells had excentric nuclei with cartwheel appearance, resembling plasm cells (Figure 1).

DISCUSSION

Application of micronucleus test in cytokinesis blocked (CB) lymphocytes showed that the background frequency of micronucleus obtained from 8 normal individuals was 4.4 ± 2.6/500 CB lymphocytes,[3] and the background frequency of micronucleus obtained from 100 non occupationally exposed subjects was 9.5 ± 4/1000 CB lymphocytes.[8] The frequency of micronucleus in exposed individuals should be higher, so the minimum cell requirement might be decreased. A study screened various amount of CB lymphocytes and the lowest amount screened was 482.[2] Therefore, we concluded that to be used in a micronucleus test each specimen should provide at least 500 intact cells having abundant cytoplasm. Therefore the treatment

selected should give a consistent result, in term of the ratio of intact cells and the cytoplasm width. Otherwise, a great variety of hypotonic strength should be applied, and many slides should be made, so that the cost is increased and the work become laborious.

Large scale experiments

To analyze the condition of the cells we used 1000X magnification, and using this magnification, we could not see the cytoplasm of some small lymphocytes on standard blood smears. Therefore, we could not differentiate between cells which have undergone lysis and cells having very scarce cytoplasm that were not swelled. In these experiments, we noted that acytoplasmic cells were also seen on control slides. The control slides were treated using modified Carnoy solution, without hypotonic treatment. In this case, the acytoplasmic cells could represent the cells that have undergone lysis and cells that were not swelled. However, based on the result that fixation using 45% acetic acid or a modified Carnoy solution containing higher amount of acetic acid resulted in the increase of acytoplasmic cells, we concluded that fixatives containing acetic acid increased the frequency of lysis. This result is in accordance with the finding of Matsuoka et al; they found that to preserve the cytoplasm around the nucleus of a Chinese hamster cell line cell, very mild fixative (1% acetic acid in methanol) should be used.[9]

The results of large scale experiments were very inconsistent; even a same treatment gave different result when it was repeated on different individuals. Based on these results we concluded that mononuclear cell membrane resistancy had great variability between individuals. Therefore, to get enough intact mononuclear cells having abundant cytoplasm, various hypotonic strength should be applied for each individual. As 5-10 ml blood was needed for each hypotonic strength, at least 15 ml of blood was required from each individual (if we applied 3 kinds of hypotonic strength). Considering the amount of blood needed and the inconsistency of the results, we concluded that it was not feasible to elaborate a micronucleus test from these experiments, so it is needless to analyze these results statisticaly. Therefore, we designed small scale experiments requiring small amounts of blood, and the treatments in large scale experiments which gave fairly good results in term of the frequency of lysis (less than 50%) and the nucleus/cell diameter ratio (less than 70 %) were repeated in small scale experiments.

Small scale experiments

Fixatives containing acetic acid increased the frequency of lysis. Therefore in small scale experiments we reduced the amount of acetic acid in the fixative. Furthermore we noticed that prolonged fixation in modified Carnoy solution also increased the frequency of lysis, so we reduced the fixation time. This is in accordance with the recommendation in the standard method of micronucleus analysis in CB lymphocytes, in which the fixation should be minimized.[1] To reduce the fixation time is difficult, when fixation is applied on cell suspension. Therefore we made the slides first, followed by rapid slide fixation. This slide fixation (the spot method) was applied for cytokinesis blocked lymphocytes and showed good results.[4]

The opinions about fixatives containing acetic acid was in accordance with the results of these small scale experiments. These experiments showed that the fixative containing the lowest amount of acetic acid (methanol:glacial acetic acid= 9:1) gave the highest ratio of intact cells (Table 3). Therefore, in elaborating a simple, fast and inexpensive micronucleus test we recommend the use of this fixative.

Statistical analysis on the ratio of intact cells showed that there was no difference between various hypotonic treatment. Theoretically, stronger hypotonic tyreatment should generate more lysis. However, this study showed that mononuclear cell membrane resistancy had great variability between individuals, and this meant that some individuals could not resist even mild hypotonic treatment, while some others could resist strong hypotonic treatment; overall, the variation in cell membrane resistancy could negate the theoretical point of view that stronger hypotonic treatment should generate more lysis.

Statistical analysis on the diameter of the cell and cytoplasm width showed that there were differences between individuals. This meant that the cells and cell nuclei of some individuals could swell more or less than did the cells and cell nuclei of other individuals. Therefore, to ensure the generation of swelled cells, it is important to apply various strengths of hypotonic treatment. Based on the results of these experiments we recommend the use of plasm:aquabidest (4:1, 3:1 and 2:1) as hypotonic solution.

In a micronucleus test, the micronucleus is searched in the cytoplasm.[7] Therefore the cytoplasm width is far more important than the diameter of the cell.

Cytoplasm width was maximum on the mildest fixative used (Table 4). Based on this result we recommend the use of methanol:glacial acetic acid= 9:1 as fixative.

In making smears, at first we use the conventional routine spreading method for making blood smears, and we noticed that most of the swelled cells were located at the periphery of the smears. In order to get more swelled cells, the periphery should be increased, and this was achieved by making 2 slender smears on one slide.

This study showed good results in small scale experiments. However, these results derived from only 10 individuals. Therefore, before using this method for routine micronucleus test, application of this procedure to larger population is needed. Furthermore, a study of micronucleus formation in exposed individuals should be conducted, and the sensitivity and specificity of the test should be measured.

Unexpected result

The plasm cells (plasmocytes) are usually present in lymphoid tissues/organs, but they were absent in normal peripheral blood.[10] Plasmocytes is believed to be generated from B lymphocytes that was stimulated by antigens. Upon stimulation, B lymphocytes differentiate to preplasmocytes and then to plasmocytes. B lymphocytes circulate in the peripheral blood for a short period, leave the circulation to enter the tissues and back to the circulation via the lymph. The swelled cells having excentric nuclei with cartwheel appearance found in this study might be the preplasmocytes, or stimulated B lymphocytes. Another possibility is that B lymphocytes have nuclei with cartwheel appearance upon swelling of their nuclei.

CONCLUSION

To make slides for micronucleus test, three kinds of hypotonic solution (plasm/aquabidest= 4:1, 3:1 and 2:1) should be used, followed by smearing on slides in the form of two slender smears on one slide, and slide fixation using methanol/glacial acetic acid= 9:1.

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