A NEW METHOD OF OSMOTIC FRAGILITY TEST OF ERYTHROCYTES WITH COIL PLANET CENTRIFUGE

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The conventional method of osmotic fragility test of erythrocytes has inherent disadvantages of poor reproducibility, requirement of a considerable dose of blood sample and a time consuming procedure, which altogether make it difficult for use in some medical investigations and in the routine laboratory tests.

A new method described here gives a high reproducibility with a minute amount (2 to 4 µl) of blood sample and yet requires a relatively simple technic. In this method, red cells are forced to travel through a long linear density gradient between physiological saline solution and distilled water at a constant speed. Thus, cells are exposed to a gradually increasing hypotonic solution down to the point where hemolysis occurs, the hemoglobin distribution recorded in the tube indicating the osmotic fragility of the sample.

The following device is made to move cells through such a gradient prepared in a fine plastic tube. The tube is coiled onto a glass rod which is mounted on the periphery of a cylindrical holder rotating slowly under a high centrifugal acceleration field acting vertically to the axis of rotation. When the centrifugal force is strong enough to prevent the cells from accompanying the rotating tube, they move relative to the tube at a rate equal to the tube rotation. The device has been named "the coil planet centrifuge" and is described in detail in the following chapter.

MATERIALS AND METHOD

Apparatus: The coil planet centrifuge1,2 which was originally devised for particle separation has been found to be a versatile apparatus for scientific use: particle separation, purification and analysis of chemicals, osmotic fragility test of erythrocytes, etc. It involves a slowly rotating coil holder, carrying a coiled tube filled with a sample to be treated, in a high centrifugal acceleration field acting vertically to the axis of rotation. Fig. 1 shows an apparatus specially designed for osmotic fragility test by Sanki Engineering, Ltd., Kyoto, Japan. Its main body (Fig. 1) consists of three parts, each...
NEW METHOD OF OSMOTIC FRAGILITY TEST

FIG. 1. Main body of the coil planet centrifuge used for osmotic fragility test of erythrocytes. Part III: central shaft 1 and central gear 2; Part II: frame composed of discs 3, arms 4, and links 5; Part I: coil holders 6 and interchangeable gears 7. Simultaneous rotation of Parts III and II at a different angular velocity results in a revolution and a rotation of Part I as a planet.

capable of rotation as a unit: coil holder 6 and interchangeable gears 7 with shaft (Part I); frame or a pair of arms 4 and discs 3 bridged with links 5 (Part II); central shaft 1 and gear 2 which interlocks to gear 7 of Part I (Part III). Simultaneous rotation ofParts II and III at a different angular velocity results in a revolution and a rotation of Part I as a planet. The rotation of Part I is determined by the difference in angular velocity between Part II and Part III and by the gear ratio between 2 and 7. The rotation ratio between Parts II and III is fixed at 100:99, while the gear ratio between 2 and 7 is adjustable in four steps, i.e., 1, 2, 3 and 5. The revolution is continuously regulated up to 1600 r.p.m. that is equivalent to about 350 g on the axis of the coil holder. Each coil holder is equipped with six grooves on its periphery to accommodate coiled tubes and is covered with a vinyl cylindrical protector.

Preparation of coiled tube: A polyethylene tube measuring about 0.5 mm in inner diameter is cut into a 3.5 m length. It is coiled uniformly and tightly on a glass rod, measuring 24 cm in length and 6 mm in diameter, making about 200 turns or 20 cm in length. Each end of the coil is fixed to the core with a rubber band, leaving the ends of the tube 20 to 25 cm non-coiled on both sides. The capacity of the tube is calibrated by passing water with a precisely graduated syringe. Then the tube is aspirated and washed with ethanol and water. In order to avoid adherence of erythrocytes to the tube wall, coating of the inner surface of the tube is carried out by passing the tube 0.1% bovine or human albumin solution, 10% formalin solution and distilled water, in
turn, by dipping the tip of the tube several times in the fluids with suction. This is done twice from each side of the tube and then the tube is thoroughly washed with distilled water and dried by passing air through it for a few minutes.

Density gradient preparation: A linear density gradient between physiological saline solution and distilled water is prepared in the above described tube by the aid of a gradient maker. Although any type of the instruments can be used, the gradient must be precisely made so as to fill out a given capacity of the tube calibrated previously. In our laboratory, the device described by Parr (1) or modified Gradientor (Type DGK) made by Hitachi Seisakusho, Tokyo, Japan, was employed with a satisfactory result. The obtained density gradient was examined with Advanced Osmometer by collecting the fractions directly from the outlet of the instrument and also from the coiled tube before and after centrifugation. The results all revealed a gradient close to the theoretical one as illustrated in Fig. 2. The excess of the tube is trimmed on both sides before sample charge.

Sample charge: Approximately 0.5 ml of heparinized venous blood is delivered into a small sample bottle. Aeration of the sample is carried out by rotating the bottle kept sideways so as to form a thin membrane of the blood over the inner surface of the bottle for a minute. Then, approximately 3 µl of the blood is drawn with a tip-tapered micropipette and injected into the tube at the saline side of the gradient. When the sample is to be collected from a new born infant, aeration may be omitted and the sample directly drawn into a heparinized micropipette following heel puncture. Before
sealing, the tube is clamped at the end of the sample side and the rest of the tube is hung down until the red cells move down a little. This prevents the cells from heat injury during the sealing. Each end of the tube is sealed by clamping the tube with a teeth-ground needle holder at the point a few millimeters from the end which is then flamed.

Centrifugal treatment: The coiled tube prepared as described is inserted into the groove of the coil holder of the coil planet centrifuge and covered with a vinyl protector. Then the tube is treated under 1500 r.p.m. revolution (ca. 300 g) and about 4 r.p.m. rotation for 50 minutes at room temperature to complete hemolysis.

Recording of the hemolytic pattern: The hemoglobin distribution pattern thus obtained in the coiled tube is stable only for a few weeks even if the tube is stored in a refrigerator. Therefore, a simple device has been made to photograph the pattern directly on a printing paper with the aid of an enlarger. A densitometer and a continuous-flow spectrophotometer both specially designed for recording the hemolytic pattern in the coiled tube are now under construction.

RESULTS

The results of the present method obtained from a human adult sample (4 on the top) and a cord blood sample (4 at the bottom) are shown in Fig. 3. White bands indicate the hemolyzed Hb distribution patterns. In each tube a linear density gradient is formed between the right distilled water side and

![Fig. 3. Osmotic fragility tests of erythrocytes from a healthy adult and a term new born infant. Results were directly photographed on a printing paper with the aid of an enlarger. White bands indicate hemoglobin distribution. A linear NaCl density gradient is formed between the left physiological saline solution and the right distilled water sides so that both minimum and maximum osmotic resistances are easily estimated. The figure shows reproducibility of the present method.](image-url)
the left physiological saline side so that the osmotic resistance is easily estimated. The cord blood sample revealed a broadly spreading pattern of hemolysis compared with that of the adult sample. The peak of the hemoglobin distribution, however, appeared on a similar point in both samples. In each group, the same blood sample was used to demonstrate reproducibility of the present method.

As shown in Table 1 and Fig. 4, minimum and maximum osmotic resistance are 107.6±6.1 m osmol and 72.5±5.0 m osmol for human adult samples and 120.5±5.8 m osmol and 61.9±5.4 m osmol for cord blood samples respectively.

TABLE 1.
Red Cell Osmotic Fragility of Human Adult and Cord Blood Samples Measured by the Present Method.

<table>
<thead>
<tr>
<th></th>
<th>Adult samples (79)'</th>
<th>Cord blood samples (72)'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum resistance</td>
<td>107.6±6.1</td>
<td>120.5±5.8</td>
</tr>
<tr>
<td>Maximum resistance</td>
<td>72.5±5.0</td>
<td>61.9±5.4</td>
</tr>
<tr>
<td>50% hemolysis</td>
<td>90.2±4.5</td>
<td>91.0±5.0</td>
</tr>
<tr>
<td>Hemolytic zone</td>
<td>35.2±3.5</td>
<td>59.3±5.5</td>
</tr>
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' Number of samples examined.

Note: Samples were collected during May, June and July.

FIG. 4. Red cell osmotic fragility of human adult and cord blood samples measured by the present method. Data were obtained during May, June and July.
These samples in each group were collected from over 70 donors during May, June and July.

The hemoglobin distribution pattern thus formed in the coiled tube is fairly stable and can be kept in cold water for a few weeks without any appreciable change.

**MAJOR FACTORS INFLUENCING THE RESULTS OF THE PRESENT METHOD**

The results obtained with the present method are found to be influenced by various factors such as centrifugal force, travelling time of cells, Ph of the media, dose, aeration and storing conditions of the sample, etc., as described below. All experiments were performed at between 20° and 30°C. If not indicated otherwise, approximately 3 μl of aerated heparinized human adult venous blood was charged into a 0.5 mm tube and subjected to approximately 300 g for 50 minutes.

a. Centrifugal force: In this method, revolution and rotation of the tube should be adjusted in such a way that most of the cells move through the gradient at a constant speed of one turn per one rotation of the tube. The experiments revealed that 1600 r. p. m., which is the maximal capacity of the present apparatus producing a centrifugal force of 350 g on the axis of rotation, is necessary for 8 rotation per minute of the coiled tube with 6 mm of helical diameter. When 1600 r. p. m. is applied for a slower rotation, a considerable number of red cells tend to adhere on the inner surface of the tube though the rest are subjected to hemolysis in the usual pattern.

b. Travelling time of cells: In order to investigate the effect of time required by cells to travel through the gradient upon the hemolytic pattern, various travelling times ranging from 20 to 150 minutes were applied to the same sample. This is easily carried out by applying proper interchangeable gears to adjust the rotation. The results revealed similar hemolytic patterns in all groups.

c. Inner diameter of the tube: Polyethylene tubes with various inner diameters ranging from 1.0 to 0.35 mm are applied with the same sample, the results being shown in Fig. 5. Except for the 0.35 mm tube, the starting points of hemolysis fall on a similar position whereas the ending points show a tendency to shift toward the distilled water side in the thicker tubes. This tailing phenomenon is considered to be an artifact probably due to the disturbance of Hb distribution by centrifugal treatment, while the cause of the early hemolysis in the 0.35 mm tube is unknown and remains for future study.

d. Ph of the media: The Ph of the gradient made between physiological saline solution and distilled water is about 5.8 which is far below the physiological range. Though the buffering action of the cells may overcome this lowered Ph environment, it is interesting to examine the effect of the medial
Fig. 5. Effects of inner diameter of the coiled tube upon hemolytic pattern by the present method.

Fig. 6. Effects of pH of the media upon hemolytic pattern by the present method.
Ph on the hemolytic pattern. Isotonic media with various Ph ranging from 9 to 4 were prepared according to the Michael's veronal buffer recipes, Ph and osmotic pressure of each solution being measured by Ph meter and Advance Osmometer respectively for correction. When the above media were substituted for the physiological saline solution in the gradient, the hemolytic pattern revealed remarkable changes as illustrated in FIG. 6. As is expected, acidic groups showed weakened resistance while the Ph 8.0 group showed a resistance close to that of the control saline group.

e. Sample dose: Various doses of the sample were subjected to hemolysis. The optimal dose was found to be 2 to 4 µl for 0.5 mm polyethylene tube. Overdose resulted in a marked tailing phenomenon while undercharge made it difficult to observe its hemolytic pattern.

f. Aeration of the sample: It is a well established fact that the oxygen content of the blood sample considerably influences its osmotic fragility and therefore, if venous blood is used, oxygenation of the sample is necessary for reproducible results. In the present method, oxygenation of venous blood samples is carried out as described previously by forming a thin layer of the sample over the inner surface of the bottle for one minute.

g. Storing conditions of the sample: Storage of the blood sample, either heparinized or ACD added, at 2° to 5°C for 24 hours, or at 37°C for 5 hours, results in no appreciable change of the osmotic fragility. Incubation of the sample at 37°C for 24 hours, however, produces a remarkable change in the hemolytic pattern. Usually those samples show a spread pattern of hemolysis with an early starting point whereas in some samples a marked tailing is observed indicating the appearance of cells with a stronger resistance as is often seen in the samples incubated in a high glucose (200-400 mg/dl) medium.

DISCUSSION

As shown in TABLE 1, the present method gives figures over 20 m osmol stronger than those with the conventional osmotic fragility test in both minimum and maximum resistances for human adult samples collected during May, June and July. When the samples are collected in winter-time, however, figures are quite different and become similar to those with the conventional method. Since temperature regulation during hemolysis does not significantly affect the results in a range between 18°C and 32°C, we assume that red cells in vivo undergo a seasonal variation which is conveniently disclosed by subjecting the cells to a gradually increasing hypotonic environment for hemolysis. Investigations on this seasonal change of osmotic resistance together with its mechanism are now in progress in our laboratory.

DANON has developed a rapid micro-method for recording red cell osmotic fragility in which a cell equipped with dialyzing membranes and a spectrophoto-
meter are combined so as to record a hemolytic process of red cells under a continuous decrease of salt concentration. Direct comparison between the results obtained by Danon's method and ours, however, is rather difficult because of the difference in manner of the decrease of salt concentration to which cells are exposed: a rapid concave decrease for Danon's method and a slow linear decrease for ours. Although both methods similarly yield remarkably accurate and reproducible data with a minute amount of blood sample, the present method further provides following advantages.

1) Hemolyzed cell contents can easily be fractionated for later investigations such as hemoglobin-type studies, enzymatic studies, etc.

2) Various types of the gradient combined with various time intervals for hemolysis can be applied.

3) Various substances including macromolecules can be chosen for the gradient to examine hemolytic or anti-hemolytic activity.

4) Cells are exposed steadily to new medium so that the effects of the hemolyzed cell contents upon hemolysis can be largely eliminated.

5) Effects of light upon hemolysis are also eliminated.

SUMMARY

A new method of osmotic fragility test with the coil planet centrifuge is presented. The method is highly reproducible and accurate with a minute amount (3 μl) of blood sample and most suitable for medical investigations, routine laboratory tests, and screening tests of blood at blood banks.

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The authors are indebted to Mr. Kan'ichi Nunogaki, President of Sanki Engineering Ltd., for construction of the Coil Planet Centrifuge.

REFERENCES


