

Controversies on the osmotic fragility test

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Osmotic fragility tests are based on the measure of red blood cell lysis as a function of osmotic stress. When erythrocytes are placed in hypotonic solutions, they begin to take on water osmotically. This results in swelling of the cell until the critical volume is reached, afterward the membrane at first leaks and then bursts releasing hemoglobin. The susceptibility to osmotic lysis is primarily determined by the surface area to volume ratio of erythrocytes and increased osmotic fragility typically occurs in hereditary spherocytosis. Because of their reduced surface membrane area respect to the cell volume, spherocytes cannot expand as much as normal discoid erythrocytes and result osmotically more fragile. Increased osmotic fragility is also seen in acquired causes of spherocytosis such as autoimmune hemolytic anemia. On the contrary, increased resistance to hemolysis is characteristic of thalassemia, in both the homozygous and heterozygous forms, in iron deficiency and in any other condition in which an increase of the surface area to volume ratio of the red cells is present.

The osmotic fragility tests are mainly useful in the diagnosis of hereditary spherocytosis but they are also used as a screening test for beta-thalassemia in under-resourced countries where automated blood-cell counters are available in only a limited number of major centres.

Red cell osmotic fragility can be evaluated by means of different methods. The traditional osmotic fragility test (OFT), originally described by Parpart et al. (1), requires the preparation of a series (n=14-16) of hypotonic solutions with NaCl content ranging from 0.1 % to 0.9 %, to which a small amount of fresh blood is added. After centrifugation and absorbance reading at 540 nm, the percent hemolysis is calculated for each solution and plotted against NaCl concentrations. The resulting osmotic fragility curve is then compared with that obtained with normal controls. The result of the OFT may be expressed as the concentration of NaCl causing 50 % hemolysis, i.e. the median corpuscular fragility (MCF) that for fresh normal samples is between 4.0 and 4.45 g/L NaCl. No standards as such are available. A sample from known normal individual is usually collected at the same time as the test sample and run in parallel as a control. The OFT is usually performed on freshly drawn blood (within 2 hours from the collection) but 24-hour incubation at 37 °C of the blood sample, has been found to improve the sensitivity of the test since this stress condition results in an increase of osmotic lysis of a greater extent for the abnormal cells respect to the normal ones.

Another method used to measure the osmotic fragility is the acidified glycerol lysis test (AGLT) (2,3). This method offers the advantages respect to the conventional OFT, to have a higher sensitivity, to require less amount of blood and shorter execution time. For these reasons it is particularly useful as a screening procedure for the diagnosis of hereditary spherocytosis in large family studies and population surveys. AGLT is based on the measure of the time taken by the erythrocytes to lyse when exposed to glycerol. After the suspension of the blood sample in a

buffered isotonic solution and the addition of glycerol, the kinetic of hemolysis is recorded as the decrease of absorbance at 620 nm. The results are expressed as GLT50 i.e. the time necessary to have 50 % hemolysis that corresponds to the time taken for the absorbance to fall to half of its original value. Spherocytes, being unable to resist swelling, lyse in a shorter time than erythrocytes. The test can be performed on blood stored for up to 24 hours.

The Pink Test is a further modification of the acidified glycerol test. It is an end-point method based on the measure of the hemolysis of a small volume of blood in a buffered solution contained 135 mmol/L glycerol (4). The Pink Test shows a diagnostic sensitivity similar to AGLT. Moreover it is very easy to perform and the results do not critically depend on the pH of the lysis solution, differently from those obtained with AGLT. This results in a good reproducibility of the test.

Up to now, several modifications of the standard multi-tubes OFT have been proposed. In particular, single-tube methods have been developed with the aim to provide simple, rapid, low cost and reliable tests for thalassemia mass screening, particularly useful in developing countries where the prevalence of thalassemic disorders is high and the resources limited (5,6). The single-tube test, also called NESTROFT (naked eye single tube red cell osmotic fragility test), is based on the use of a single hypotonic solution having a critical saline concentration able to differentiate between normal and thalassemic subjects. The test is interpreted by visualization, negative samples being clear and positive cloudy. The use of different buffered saline solutions containing from 0.32 to 0.40 % NaCl have been proposed and evaluated. The 0.36 % saline solution seems to give the most satisfactory results providing the best sensitivity usually ranged between 95 % and 100 %. The specificity of this solution is not so high and false positive results can be observed in presence of iron deficiency and some common hemoglobin variants such as HbE, HbS and HbC. The use of the 0.34 % or 0.32 % saline solutions gives fewer false-positive results but on the other hand, provides unacceptable sensitivity.

Finally, a pilot survey on the way osmotic fragility test is performed among the ENERCA network was run in 2007. Sixteen laboratories (from Belgium, 1; France, 1; Germany, 3; Italy, 3; Portugal, 2; Spain, 6) participated to the survey. The large majority of them (81.2 % of participants) uses the multi-tubes test described by Papart (1), a minority (18.8 %) the one-point pink test, and some (37.7 %) the acidified glycerol lysis-time test (some laboratories is currently running more than one test). EDTA is the anticoagulant of choice for 61.1 % of the laboratories, while 38.9 % of them prefer heparin. EDTA and heparin seem to be used equally for the various lysis procedures. Concerning the replication of the test after 24 h of incubation at 37 °C, this procedure is followed by 70.6 % of laboratories, all of them agreeing that this further step increase the sensitivity of the test. Concerning the reference intervals, a wide variation was remarked even for the same laboratory procedures run in different laboratories.

In conclusion, the data obtained by the above-mentioned pilot survey, and the evidences actually available from the scientific literature, prove that there is an urgent need to harmonize the laboratory procedures for the determination of osmotic fragility, and for using such a test in the diagnosis of hemolytic anemias.

References

1. Parpart AK, Lorenz PB, Parpart ER, Gregg JR, Chase AM. The osmotic resistance (fragilità) of human red cells. *J Clin Invest* 1947;26:636-40.

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2. Gottfried EL, Robertson NA. Glycerol lysis time as a screening test for erythrocyte disorders. *J Lab Clin Med* 1974;83:323-33.
 3. Zanella A, Izzo C, Rebulli P, Zanuso F, Perroni L, Sirchia G. Acidified Glycerol lysis test: a screening test for spherocytosis. *Br J Haematol* 1980;45:481-6.
 4. Vettore L, Zanella A, Molaro GL, De Matteis MC, Pavesi M, Mariani M. A new test for the laboratory diagnosis of spherocytosis. *Acta Haematologica* 1984;72:258-63.
 5. Kattamis C, Efremov G, Pootrakul S. Effectiveness of one tube osmotic fragility screening in detecting beta-thalassemia trait. *J Med Genet* 1981;18:266-70.
 6. Chow J, Phelan L, Bain BJ. Evaluation of single-tube osmotic fragility as a screening test for thalassemia. *Am J Hematol* 2005;79:198-201.