Aggregation of shape-altered erythrocytes: An *in vitro* study

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Normal erythrocytes (discocytes) obtained from the blood samples of healthy volunteers are subjected to shape transformation into stomatocytes and echinocytes by treatment with various concentrations of Triton × 100 and sodium salicylate, respectively. Aggregation index of these samples is measured by Myrenne aggregometer. As deformability of these cells contributes to their aggregation process, this parameter is also measured by Myrenne filtrometer. The results show that the morphological changes in these cells are dose-dependent which affect the aggregation and deformability of these cells. The deformability of echinocytes does not show significant increase with increasing concentration, whereas, a significant decrease for stomatocytes is observed. Despite the varying pattern of deformability, the aggregation of these shape-altered cells is reduced.

THE shape of a normal erythrocyte is a biconcave disc (discocyte) which represents an equilibrium state between the two distinct shapes, i.e. echinocyte and stomatocyte. Red cells can easily undergo shape transformations *in vitro* under the influence of certain agents and these changes are reversible by removal of causative agents by the addition of antagonists[1–3].

The mechanism by which mature red blood cells change their shape under physiological and pathological conditions has been the subject of considerable interest[4]. The dominating interpretation of shape changes is explained by differential increase in surface area of the two leaflets of erythrocyte membrane[5]. Sheetz and Singer[6] have proposed that the membrane whose proteins and lipids are distributed asymmetrically in the two halves of the bilayer can act as a bilayer couple, and can respond differentially to a perturbation. Anionic drugs mainly interact with the lipids in the exterior half of the bilayer leading to expansion of that layer relative to the cytoplasmic half, and thereby induce crenation (exvagination), while permeable cationic drugs do the opposite and cause the cells to form cups (invagination).

Several groups have reported that drug-induced shape changes of erythrocytes are accompanied by alterations in their flow properties[1,7–9]. These alterations in the erythrocytes could be through direct modification of the cell geometry (surface to volume ratio) or through associated alteration of the membrane skeleton. Thus an inter-relationship exists between the capacity of the cell for the shape change and the deformability of its membrane[2,10,11].

Erythrocytes in clinical conditions are associated with altered morphology leading to abnormal rheological behaviour, as observed in several hematological disorders[10,12–16]. The inability of the erythrocyte to shape alteration contributes to its early removal from the circulation[2].

Aggregation of erythrocytes, which depends on their shape and deformability, is an important mechanism associated with cardiovascular blood flow. The aggregates are observed in the axial regions of large vessels[17] and under low flow conditions in smaller vessels[18]. Although the effect of shape alteration on blood viscosity has been demonstrated[1,19], its effect on aggregation of erythrocytes which contributes significantly under low shear rate conditions is not well elaborated. Hence the aim of the present study is to analyse the effects of the shape alterations induced by sodium salicylate and Triton × 100 on aggregation of erythrocytes under *in vitro* conditions. As deformability of erythrocytes makes an important contribution in this process, the change in this parameter, under shape altered conditions is also determined.

Fresh blood samples ($N = 10$) were drawn by venepuncture from normal young adult volunteers in tubes containing heparin as an anticoagulant. After centrifugation at 3000 *g* for 10 min, the plasma was separated and the buffy coat and uppermost erythrocyte layer were discarded. The cells were then washed in phosphate buffered saline solution with glucose (1 g/l) at pH 7.4. Blood samples with normal hematocrit, plasma viscosity, deformability and aggregation behaviour were considered for these studies. For discocyte–echinocytic transformation, the normal cells were treated with sodium salicylate. From the stock solution of 1 g/l the end concentrations of 1 mg/l and 2 mg/l were obtained. Triton × 100 was used for discocyte–stomatocytic transformation. From the stock solution of 0.8 g/l the desired end concentrations of 0.1 mg/l and 0.2 mg/l were obtained. To avoid any dilution effect, a part of the plasma was replaced by the respective stock solutions.

The morphological analysis was performed using a phase contrast microscope. For this, a small sample of blood cell suspension at 1% hematocrit was placed on a microscope slide and covered with a cover slip. This was placed on a microscope stage and viewed at magnification 100×. For proper refractive index matching, a drop of oil was also placed over the cover slip. The shapes of the cells before and after their exposure to drugs were photographed.

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The aggregation of erythrocyte was measured by a Myrenne Aggregometer (MA2, Myrenne GmbH, Germany). Basically this instrument consists of a transparent cone-plate viscometer with photometer, measuring the extent of aggregation immediately after their dispersion by high shear stress. A small amount of blood sample was placed between the cone and plate and sheared at 600 s^{-1} to introduce hydrodynamic dispersion and then brought to a sudden stop. The intensity of light emitted by the LED after transmission through the sample was measured by a photodiode. The aggregation process is accompanied by an initial decrease and a subsequent increase in light transmission. The rate constant of aggregate formation was determined by a procedure that normalizes the area under the transmission intensity curve by integration of the photodiode output signal during the first five seconds after shearing was stopped. This parameter is taken as the aggregation index and is calculated automatically by the instrument. Further details of this instrument and the measuring procedure are given elsewhere[20,21].

The deformability of erythrocytes was measured by a microcomputer-based filtrometer, model MF4 (Myrenne GmbH, Germany). This is a fully automatic instrument equipped with four specially designed U-shaped glass sample holders which record optometrically the changes in flow rate under decaying pressure through a Nuclepore membrane of pore diameter 5 μm. Prior to measurement, the erythrocyte suspension was filtered through a column of Imugard Cotton Wool (IG 500) (Terumo, Tokyo, Japan). By this process, small sub-populations of leucocytes are effectively removed[22]. The flow of cells are recorded for 10 min and the corresponding filtration curves are generated. The deformability index of the red cells is calculated from the initial flow rate of the filtration curve. The functional details of this instrument are discussed elsewhere[23,24]. All the experiments were performed within two hours of sample collection as per the recommendations of the International Committee on Standardization in Hematology[25]. Statistical analysis of the samples was carried out by Student's *t*-test.

The photomicrograph of normal erythrocytes is shown in Figure 1. The transformation of discocytes to echinocytes at a concentration of 2 mg/l of sodium salicylate is shown in Figure 2. The transformation to stomatocytes at concentration of 0.2 mg/l of Triton × 100 is shown in Figure 3. Due to variation in shape, the stomatocytes appear distinctly different from the normal and echinocytic cells.

Figure 4 shows the variation of aggregation index of normal erythrocytes and echinocytes as obtained by treating normal cells at various concentrations of sodium salicylate. The decrease in aggregation index is significant at 1 mg/l and highly significant at 2 mg/l compared to that of normal erythrocytes. Similarly, the effect of Triton × 100 on aggregation index of erythrocytes is shown in Figure 5. A similar significant to highly significant decrease with the increase of its concentration is observed.

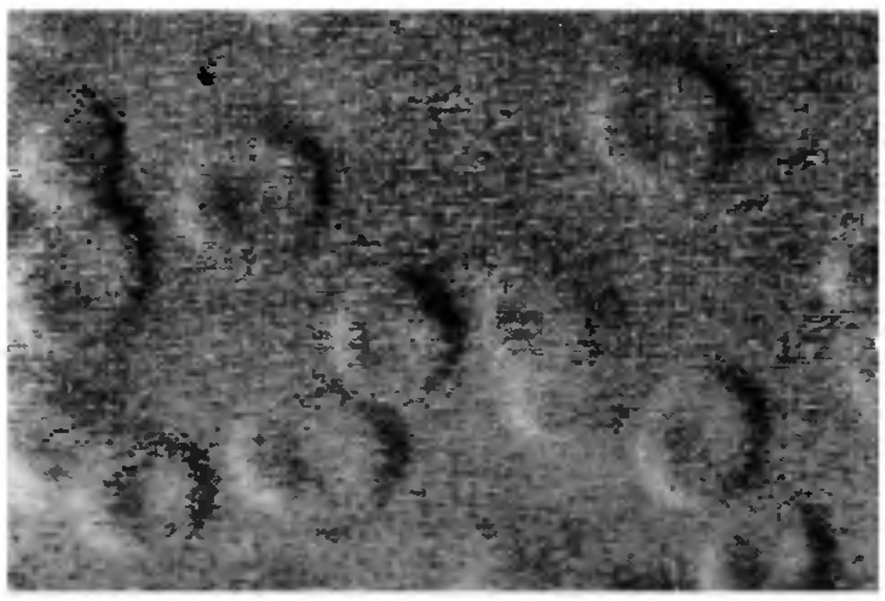

Figure 1. Photomicrograph of normal erythrocyte as obtained by phase contrast microscope at 100 × magnification.

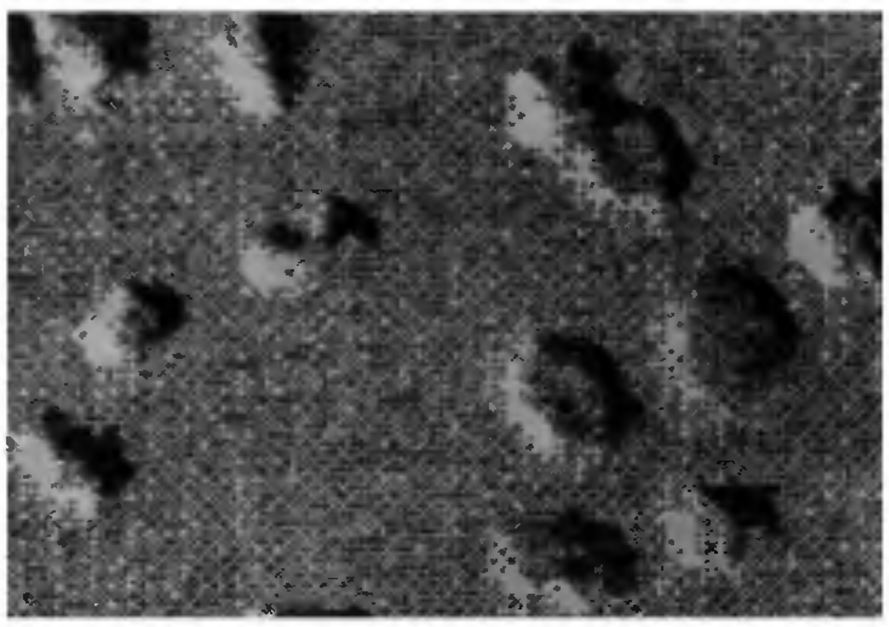

Figure 2. Photomicrograph of echinocytes at 100 × magnification as obtained by treating normal erythrocytes with sodium salicylate at concentration 2 mg/l.

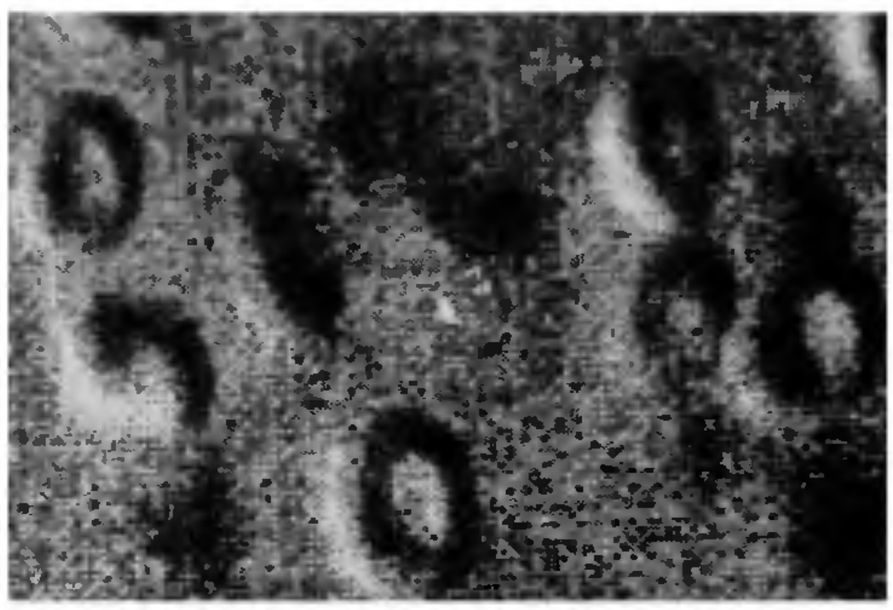

Figure 3. Photomicrograph of stomatocytes at 100 × magnification as obtained by treating normal erythrocytes with Triton × 100 at concentration of 0.2 mg/l.

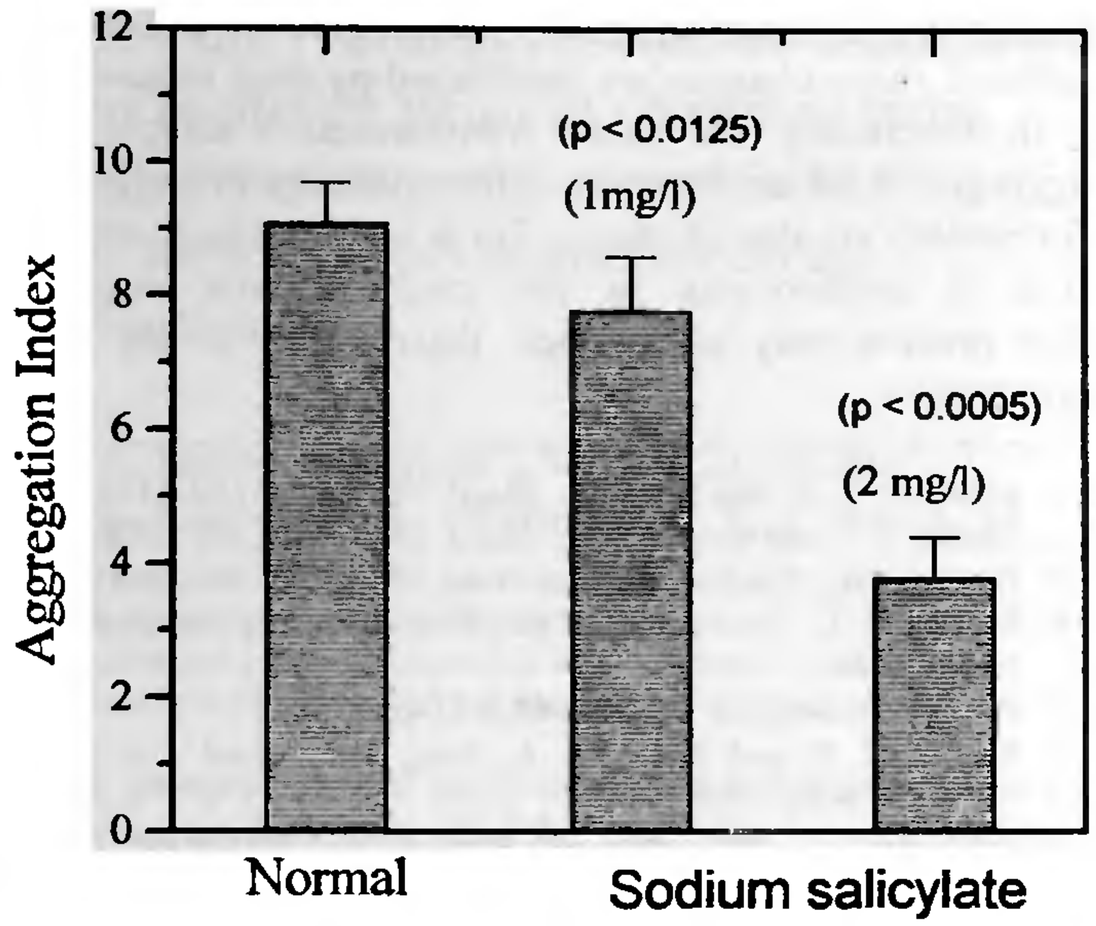

Figure 4. Variation in aggregation index of echinocytes as obtained at various concentrations of sodium salicylate. A significant decrease in comparison with that of normal erythrocytes is observed.

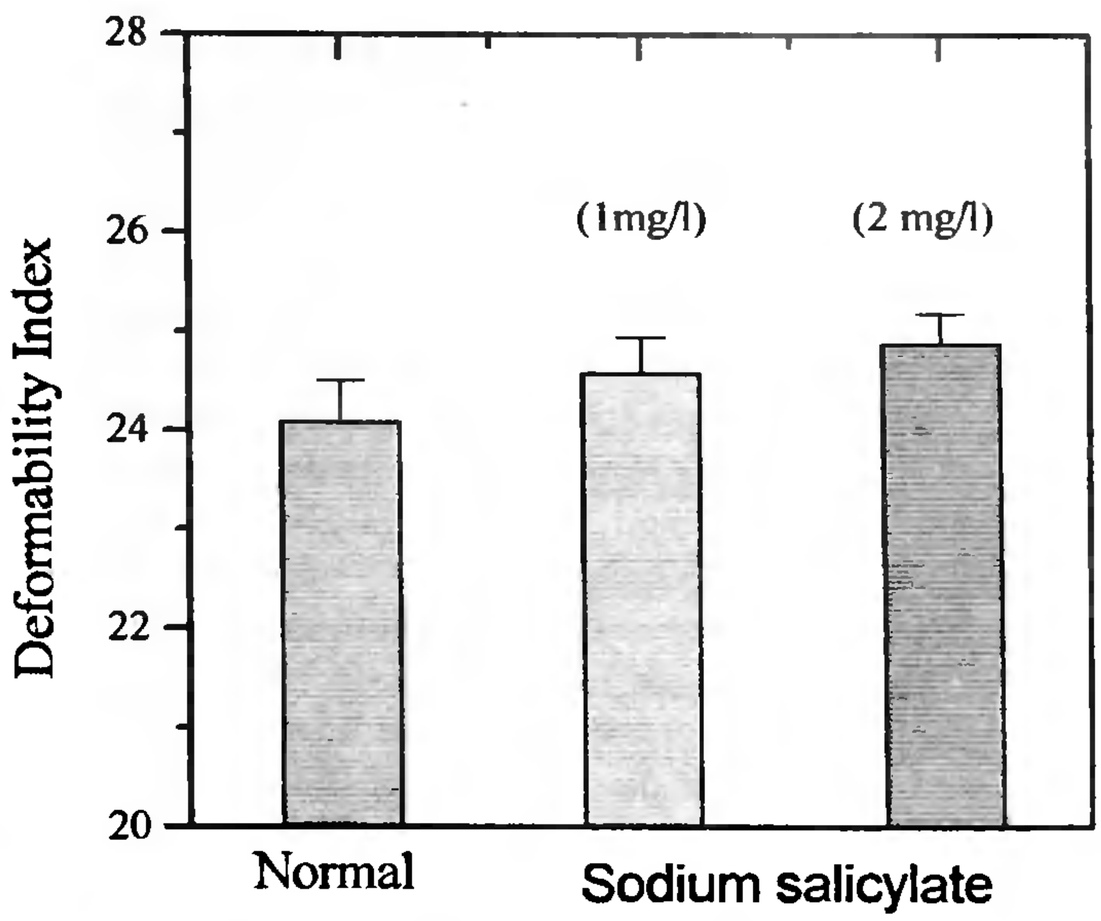

Figure 6. Variation in deformability index of echinocytes obtained after treatment with various concentrations of sodium salicylate and its comparison with that of normal erythrocytes. An increase in deformability index but not significant in comparison with that of normal erythrocytes is observed.

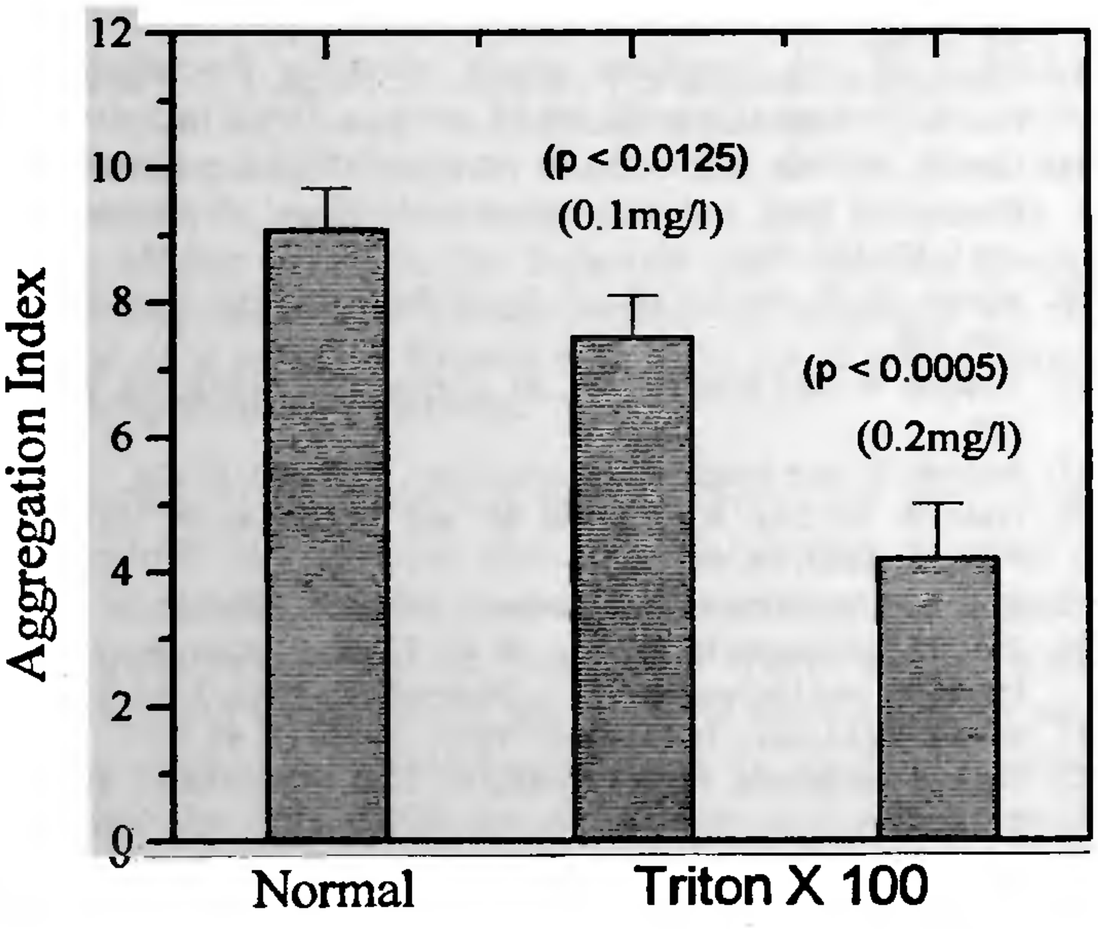

Figure 5. Aggregation index of stomatocytes obtained by treating normal erythrocytes at various concentrations of Triton × 100 and its comparison with that of discocytes. A significant to highly significant decrease in comparison with that of normal erythrocytes is observed.

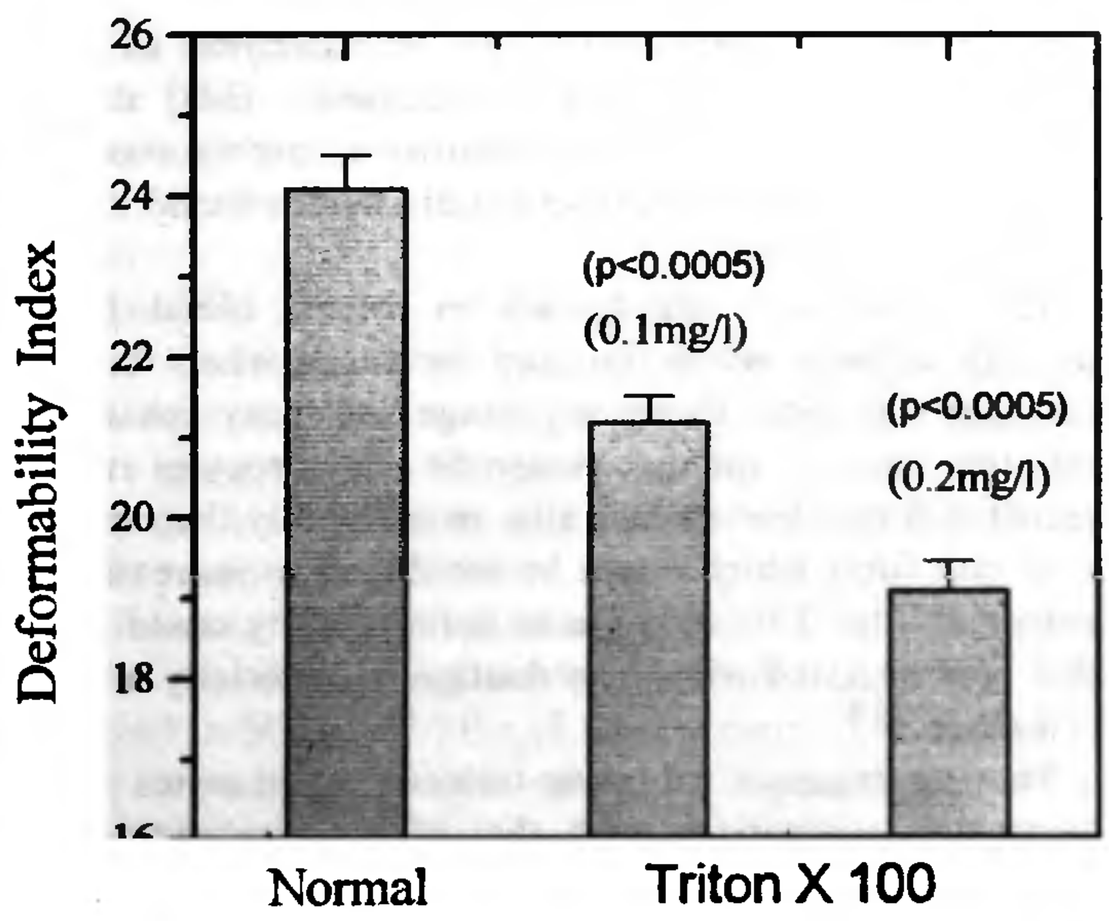

Figure 7. Deformability index of stomatocytes obtained at various concentrations of Triton × 100. A significant decrease in this parameter in comparison with that of normal erythrocytes is observed.

The deformability of cells is affected by the shape alteration procedure. Figure 6 shows the deformability of normal and sodium salicylate-treated cells. No significant change in deformability, compared to discocytes, is observed at various concentrations, indicating that the echinocytic transformation does not significantly affect the passage of these cells through the micropores. In contrast to this, a significant decrease in deformability in stomatocytes, compared to that of discocytes, with increase in concentration of Triton × 100 is observed (Figure 7).

The change in shape of intact erythrocytes in presence of sodium salicylate and Triton × 100 has been demonstated earlier[24]. Our observation confirms the earlier findings that sodium salicylate transforms the discocytes into echinocytes, whereas, Triton × 100 transforms them into stomatocytes. These transformations take place immediately at room temperature and are dose-dependent.

Our observations show that the aggregation and deformability of shape-transformed red blood cells are altered and could be contributing to the changes in the rheological properties of blood[1,8]. Interestingly, stomatocytic as well as echinocytic transformations affect the filtrability of erythrocytes through narrow pores. There is an increase, though not significant, in the deformability of echinocytes when compared to the native discocytes. This is in agreement with earlier reports as analysed by various deformability methods such as high speed centrifugation and ektacytometry[1]. Despite this increase, the aggregation of cells, as observed in the present study, is decreased. As the echinocytes accompany ATP depletion and Ca^{++} influx (by the ionophore A 23187), they consistently stiffen the RBC membrane[9]. We found earlier that the echinocytes produced by the drug RÖKAN (an extract of gingko biloba) also increases erythrocyte deformability as studied by various techniques. Further details of this mechanism for echinocytic cells, which are associated with rounded spicules all over the membrane, are not fully understood. A change in mean curvature of these cells has been reported[24], but reduction in cell size leading to faster movement through pores, needs to be verified. On the other hand, the deformability of stomatocytes as obtained by Triton × 100 is decreased. This dose-dependent reduction in deformability is highly significant at high concentrations and could be attributed to its shape transformation.

The echinocytes are known to impair blood flow through cellular interaction and increased whole blood viscosity despite their advantage of easy passage through narrow splenic sinusoids. Erythrocytes with crenated form clear splenic slits more rapidly than those with cup form which could be attributed to increase in deformability. This increase in deformability could further be associated with the reduction in sphericity of the cell surface[1,6].

The aggregation of drug-treated erythrocytes decreases in comparison with that of the discocytes. In both transformations the rate constant of aggregate formation, a parameter related to aggregation index, is low. At low and high concentrations of sodium salicylate the decrease in aggregation index is comparable to that as induced by Triton × 100. The change in area and sphericity of the outer membrane surface of erythocytes could be contributing to altered aggregation behaviour of the cells. The sialic acid residues of the membrane glycoproteins which contribute to aggregation behaviour of the cell[26] do not appear to be involved since d induced shape changes are unaffected by their remov

In conclusion, the shape transformation affects aggregation of erythrocytes either directly through formability or altered shape. For a similar shape alt tion in erythrocytes in the cardiovascular sys this process may affect their distribution in the crovessels.

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