ORIGINAL ARTICLE

A simple slide test to assess erythrocyte aggregation in acute ST-elevated myocardial infarction and acute ischemic stroke: Its prognostic significance

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ABSTRACT

A simple slide test and image analysis were used to reveal the presence of an acute-phase response and to determine its intensity in subjects of acute myocardial infarction and acute ischemic stroke. Erythrocytes tend to aggregate during an inflammatory process. Evaluation of erythrocyte adhesiveness/aggregation is currently available to the clinicians indirectly by erythrocyte sedimentation rate (ESR), but ESR correlates poorly with erythrocyte aggregation, hence a simple slide technique using citrated blood was used to evaluate erythrocyte aggregation microscopically and also by using image analysis. **Aims:** (1) To study erythrocyte aggregation/adhesiveness by a simple slide test in subjects with acute ST-elevated myocardial infarction (STEMI), acute ischemic stroke and healthy controls. (2) To study the prognostic significance of ESR and erythrocyte aggregation/adhesiveness test (EAAT) in predicting the outcome after 1 week in subjects of acute myocardial infarction and acute ischemic stroke. **Patients and Methods:** Three groups of subjects were included in the study; 30 patients of acute STEMI, 30 patients of acute ischemic stroke, and 30 subjects with age- and gender-matched healthy controls. Citrated blood was subjected to simple slide test and ESR estimation by Westergren's method. Stained smears were examined under 400× and graded into four grades. Images were taken from nine fields; three each from head, body, and tail of the smear. The degree of erythrocyte aggregation was quantified using a variable called erythrocyte percentage (EP), by using the software MATLAB Version 7.5. A simple program was used to count the number of black and white pixels in the image by selecting a threshold level. **Results:** The mean ESR of the subjects with acute myocardial infarction (29 + 17.34) was significantly higher ($P = 0.001$) than the mean ESR of the control group (15.5 + 12.37). The mean EP of the subjects with acute myocardial infarction (69.91 + 13.25) was significantly lower ($P < 0.001$) than the mean EP of the control group (85.16 + 8.41). The mean ESR of the subjects with acute stroke (40.46 + 33.75) was significantly higher ($P = 0.0005$) than that of the controls (15.5 + 12.37). The mean EP of the stroke patients (70.59 + 11.30) was significantly lower ($P < 0.001$) than the mean EP of the controls (85.16 + 8.41). In subjects with acute myocardial infarction there was a significant negative correlation (r = –0.623) between ESR and EP. In acute stroke patients there was a significant negative correlation (r = –0.69) between ESR and EP. On performing standard error of proportions, P value was < 0.05. **Conclusion:** EAAT is a simple bedside test for erythrocyte aggregation, which indirectly reveals the presence and proportion of inflammation. This test has the potential to assess the prognosis of acute myocardial infarction and acute stroke. It can also be used as a screening test for high-risk individuals, so that necessary interventions could be adopted. However, further studies need to be conducted to establish standard protocols.

KEY WORDS: Acute myocardial infarction, acute ischemic stroke, erythrocyte aggregation/adhesiveness test, erythrocyte percentage

Access this article online
Website: www.ijpmonline.org
DOI: 10.4103/0377-4929.77327
Quick Response Code:

INTRODUCTION

Erythrocyte acts as a scavenger toward reactive O_2 and nitrogen species under normal physiologic conditions. If the amount of reactive O_2 and nitrogen species produced is beyond the antioxidant capacity of the erythrocyte, it becomes a source of reactive species and consequently loses its structural features. This results in increased aggregation and adhesiveness to the endothelium and other blood cells, contributing to vascular damage.[1] Erythrocyte adhesiveness/aggregation has been proposed as a useful marker to detect the inflammatory state in unstable angina[2,3] and stroke.[4] Increased synthesis of adhesive proteins[5,6] and hyperlipidemia[7] are some of the reasons for increased erythrocyte aggregation in subjects with ischemic vascular disease.

Evaluation of erythrocyte aggregation is currently available to the clinicians only indirectly through erythrocyte

sedimentation rate (ESR). However, ESR correlates poorly with erythrocyte aggregation because of the confounding effects of hematocrit, plasma albumin levels, and hemodilution with anticoagulants.[8] In many studies erythrocyte aggregation was measured by using erythrocyte aggregometer, which needed expensive equipment with software for image analysis. A study conducted by Rainer *et al.* demonstrated increased erythrocyte aggregation in 16 patients of coronary artery disease using an erythrocyte aggregometer.[9] Another study by Tanahashi *et al.* found markedly higher erythrocyte aggregation in 80 subjects of cerebrovascular disease using a whole blood aggregometer.[3,10]

A simple slide technique using citrated blood with image analysis (INFLAMET) was designed in Israel to quantify erythrocyte aggregation in the peripheral venous blood.[11] This test is also called erythrocyte aggregation/adhesiveness test (EAAT), but needed expensive equipment. In the present study, patients with ischemic heart disease and brain disease were analyzed, assuming both the conditions to have similar etiologies in terms of atherosclerosis (smoldering inflammation), as well as similar risk factors.

In the present study we analyzed the slide subjectively by assigning grades to the degree of aggregation under the microscope and also measured the area of aggregated erythrocytes as erythrocyte percentage (EP) by using simple software.

AIMS AND OBJECTIVES

1. To test for erythrocyte aggregation/adhesiveness by a simple slide test in subjects with acute ST-elevated myocardial infarction (STEMI), acute ischemic stroke and healthy controls.
2. To study the prognostic value of ESR and erythrocyte aggregation/adhesiveness test (EAAT) in predicting the 1-week outcome of the subjects of acute STEMI and acute ischemic stroke.

PATIENTS AND METHODS

Three groups of subjects were included in the study.

1. Thirty subjects of acute STEMI admitted to the Department of Cardiology, within 6 h of onset of chest pain, subjects with substernal chest pain persisting for more than 30 min and with one of the following: 1 mm ST segment elevation in at least two contiguous electrocardiogram leads.[12]
2. Thirty subjects of acute ischemic stroke admitted to the acute medical care unit, Department of Medicine, within 24 h of onset of disease. According to the WHO definition, stroke is defined as rapidly developing clinical signs of focal or global disturbance of cerebral function, lasting more than 24 h or leading to death with no apparent cause other than of vascular origin.[13] In subjects with an acute transient ischemic attack or acute stroke, hemorrhage was ruled out by means of computed tomography.
3. Thirty subjects of age- and gender-matched healthy controls, who were nondiabetic and nonhypertensive with no recent history of fever.

Informed consent was taken from the subjects. History was taken and routine general and systemic examination was done. A detailed history of risk factors of the diseases was also recorded.

Excluded from the present study were individuals who had a recent (less than 8 weeks) history of myocardial infarction, sepsis, bacterial infection, malignant disease, pregnancy, severe renal or hepatic failure, thrombocytopenia, or deep vein thrombosis, as well as those who were on steroids, aspirin, clopidogrel, and other nonsteroidal anti-inflammatory drugs (within the previous 2 weeks). Blood samples were collected immediately after admission, before starting any intravenous infusion or giving any drug from antecubital vein without producing venous stasis, with 20-gauge needles in plastic disposable syringes. Subsequently, the subjects were treated according to the standard treatment protocol of our hospital. The blood samples were immediately taken to the laboratory and ESR by Westergren's method and slide test (EAAT) were performed.

Preparation of Slide

A single large drop of citrated blood (one volume of citrate to three volumes of blood) was placed using a syringe on a slide, which was positioned at 45°. The slide was left in that position for 10 s during which the blood was allowed to run down by gravity leaving a fine film. An adsorbing paper was used to wipe the blood from the lowest part of the slide. The slides were then dried at room temperature while in a completely horizontal position.[11] The drop size was maintained constant for all the slides and also the angle at which the slides were placed. The slides were then stained with 4–5 drops of Leishman stain and left for 2 min. Then twice the amount of distilled water was poured gently. After 8 min the slides were washed under tap water, dried, and examined.

Analysis of the Slides

First the slides were subjectively assessed without the diagnosis. All areas of the slides were studied at 40× (400×) magnification and grades were assigned to the slides based on the degree of erythrocyte aggregation.

The Grading Criteria

Grade A: Erythrocytes are discrete with uniform distribution throughout, clear areas are not seen (normal aggregations) [Figure 1].

Grade B: Erythrocyte aggregates are seen in some areas of the slide with small clear space (mild aggregation) [Figure 2].

Grade C: Variable sizes of aggregates over all the areas of slide with small clear spaces (moderate aggregation) [Figure 3].

Grade D: Large thick aggregates with rounded/clear borders and large clear spaces (severe aggregation) [Figure 4].

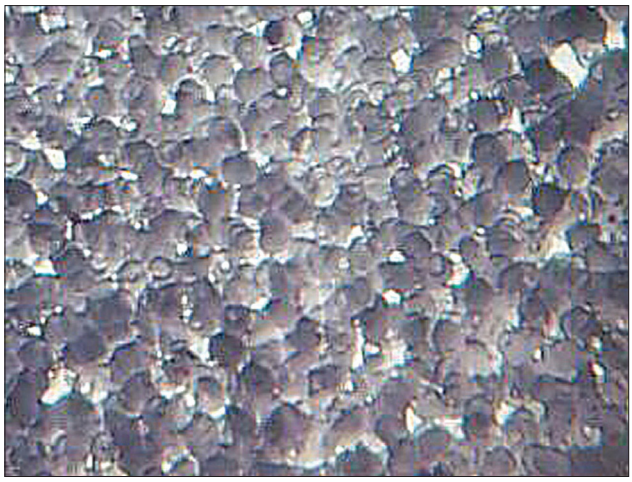

Figure 1: Photomicrograph showing erythrocytes that are arranged discretely with uniform distribution and no clear spaces. [Normal aggregation (Grade A) (Leishman stain, ×400)]

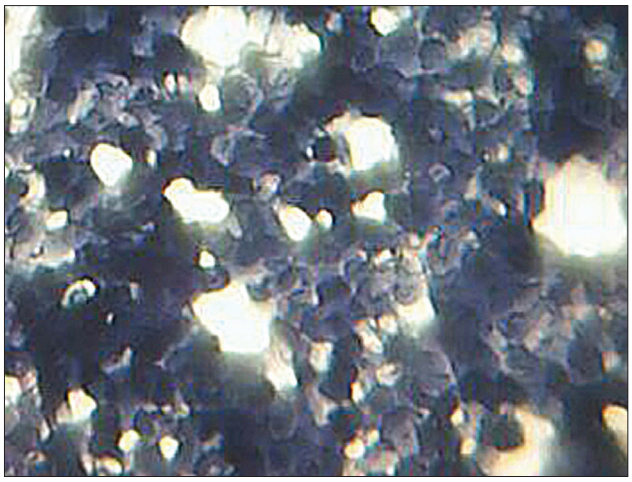

Figure 2: Photomicrograph showing aggregation of erythrocytes in some areas with small clear spaces. [Mild aggregation (Grade B) (Leishman stain, ×400)]

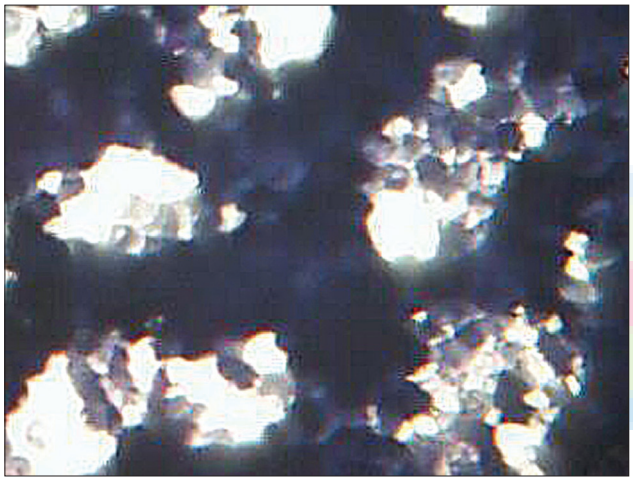

Figure 3: Photomicrograph showing variable sizes of erythrocyte aggregates with small clear spaces. [Moderate aggregation (Grade C) (Leishman stain, ×400)]

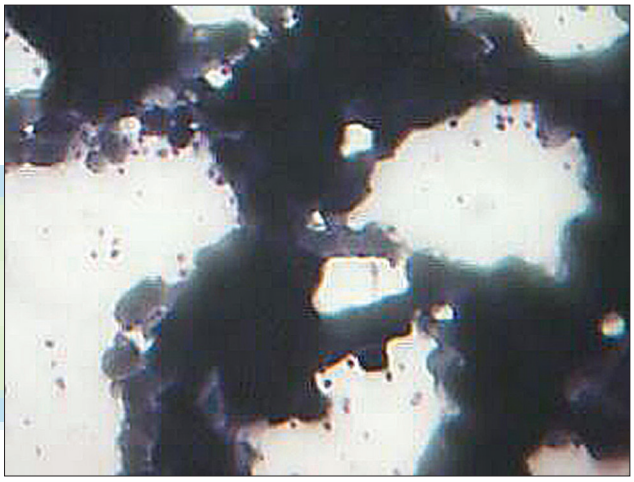

Figure 4: Photomicrograph showing large thick aggregates and large clear spaces. [Severe aggregation (Grade D) (Leishman stain, ×400]

After assigning grades to the slide, it was then divided into three equal parts: (head, body, and tail). Three digital images were taken from each part randomly. So, a total of nine images from each slide were obtained. The black and white images were captured by a single pathologist in order to decrease the possible subject-to-subject variation.

In each image, the degree of aggregation was quantified using a variable called EP, which is defined as the percentage of image area occupied by erythrocytes. To measure this EP, we used software MATLAB Version 7.5, where a simple program in the command window was given to count the number of black and white pixels in the image by selecting a threshold level. In the workspace we get the number of black pixels and the number of white pixels for each image. The total number of pixels for any image is 76,800, that is, 320 × 240 pixel image. Then the average number of black pixels calculated for every 9 images was taken from each slide.

EP for subject = average number of black pixels × 100

This EP value was taken as the erythrocyte aggregation of the subject. EP is inversely proportional to erythrocyte aggregation. The higher the valve of EP, lesser is the erythrocyte aggregation.

To assess the prognostic significance, the subjects were followed-up for 1 week. The outcome of subjects at 1 week follow-up was recorded in the following categories.

Acute myocardial infarction outcome includes complete recovery, postmyocardial infarction, angina, re-myocardial infarction, left ventricular failure, cardiogenic shock, arrhythmias (ventricular tachycardia, atrial fibrillation, ventricular fibrillation), and death from cardiac causes and death from noncardiac consequences.

Ischemic stroke: stroke outcome was graded as follows:

Grade 1: subjects with neurologic symptoms but no signs and no impairment of activity

Grade 2: subjects with symptoms and signs and no impairment in activity

Grade 3: subjects with impairment in one limb or impairment of speech

Grade 4: subjects with impairment in two limbs and of speech

Grade 5: deaths from ischemic stroke.

Data analysis

Data were analyzed for two separate or independent groups, namely, myocardial infarction with controls and stroke with controls. In order to compare two independent proportions in two groups, χ^2 test was used, that is, grading of aggregation in subjects and controls. To compare the mean values in two independent groups (ie, EP in subjects and control); unpaired "*t*" test, two-tailed, unequal variance were used. To find out the association between ESR and EP values in the same group, Pearson Correlation coefficient was used and "*t*" test for correlation was used to find out the significance of association. To test the prognostic significance of erythrocyte aggregation/ adhesiveness in stroke, standard error of proportions was used.

RESULTS

Among 30 subjects of acute myocardial infarction, 21 were males and 9 were females of age ranging from 26 to 80 years. Male to female ratio was 2.3:1. The mean age of the subjects with acute myocardial infarction was 51.87 + 13.11 years. Among 30 subjects of acute ischemic stroke, 19 were males and 11 were females of age ranging from 20 to 80 years. Male to female ratio was 1.7:1 .The mean age of acute ischemic stroke subjects was 57.72 + 15.16 years. Among 30 control group subjects, there were 17 males and 13 females of age ranging from 22 to 70 yrs. Male to female ratio was 1.3:1. The mean age of the controls was 40.966 + 12.73 years.

Aggregation was significantly more severe in subjects with myocardial infarction (where 15 of the 30 subjects belonged to grades C and D), compared with controls (where only 2 of the 30 subjects belonged to grades C and D). Fifty percent of the subjects with acute myocardial infarction and acute stroke (15 of 30) had grades C and D aggregation (severe aggregation) [Table 1].

The mean ESR of myocardial infarction (29 + 17.34) was significantly higher (P = 0.001) than the mean ESR of control group (15.5 + 12.37). The mean EP of acute myocardial infarction (69.91 + 13.25) was significantly lower (P < 0.001) than the mean EP of control (85.16 + 8.41) [Table 2]. The mean ESR of acute stroke (40.46 + 33.75) was significantly higher (P = 0.0005) when compared with controls (15.5 +12.37). The mean EP of acute stroke patients (70.59 + 11.30) was significantly lower (P < 0.001) than the mean EP of the controls (85.16 + 8.41) [Table 3].

In subjects with acute myocardial infarction, there was a significant negative correlation (r = –0.623) between ESR and EP. In subjects with acute stroke there was a significant negative correlation (r = –0.69) between ESR and EP. Of the 15 subjects of acute stroke with severe aggregation, 6 died and 9 survived. Of the 15 subjects with nil or mild aggregation only 1 died and the cause was not known. Of the 15 patients of myocardial infarction with severe aggregation, 4 died and the rest survived. On performing standard error of proportions, P valve was <0.05.

DISCUSSION

Erythrocytes tend to aggregate in the presence of plasma proteins, especially fibrinogen.[14] The event of erythrocyte aggregation is determined by opposing forces, the repulsive force between the negatively charged cells, the cell-to-cell adhesion induced by plasma protein and the disaggregating shear force generated by blood flow.[15-19] Erythrocyte aggregation is thus dependent both on plasma (extrinsic) factors and on cellular (intrinsic) factors. Normally the blood flow is sufficient for dispersion of erythrocyte aggregates, which is essential for normal tissue perfusion. In low flow states and other pathologic conditions, increased erythrocyte aggregations may contribute in general to circulating disorders and in particular to the microcirculation.[16]

Table 1: Degree of erythrocyte aggregation in subjects with acute myocardial infarction, acute stroke, and controls (P < 0.001)

Grade	*Controls (n=30)*	*Myocardial infarction (n = 30)*	*Stroke (n = 30)*
A	19	02	00
B	09	13	15
C	02	10	09
D	00	05	06

Table 2: Erythrocyte percentage in subjects with acute myocardial infarction, acute stroke, and controls

Erythrocyte percentage	*Controls (n = 30)*	*Myocardial infarction (n = 30)*	*Stroke (n = 30)*
51–60	0	10	6
61–70	2	8	10
71–80	6	2	7
81–90	14	9	7
91–100	8	1	0

Table 3: Mean ESR and EP in subjects with acute myocardial infarction, acute stroke and controls

	Controls (n = 30)	*Myocardial infarction (n = 30)*	*P value*	*Stroke (n = 30)*	*P value*
ESR	15.5 ± 12.37	29 ± 17.34	<0.05	40.46 ± 33.75	0.0005
EP	85.16 ± 8.42	69.91 ± 13.25	<0.001	70.59 ± 11.30	<0.001

ESR, erythrocyte sedimentation rate; EP, erythrocyte percentage.

Erythrocyte aggregation is increased in various conditions associated with inflammatory response, such as ischemic heart disease, acute myocardial infarction, acute ischemic stroke, and bacterial sepsis.[20,21] Evaluation of erythrocyte aggregation is currently available to the clinicians only indirectly through the test of ESR.[8] This test was invented in 1897 by the Polish doctor Edmund Biernacki. It is also called Biernacki reaction. ESR is governed by the balance between prosedimentation factors, mainly fibrinogen and those factors remitting sedimentation, namely, the negative charge of the erythrocyte (Zeta potential). Where an inflammatory process is present, the high proportion of fibrinogen in the blood causes red blood cells to stick to each other. The red cells form stacks called "rouleaux," which settle faster. Rouleaux formation can also occur in association with some lymphoproliferative disorders, in which one or more immunoglobulins are found in high amounts. ESR correlates poorly with erythrocyte aggregation because of the confounding effects of hematocrit, plasma albumin, temperature, and hemodilution by anticoagulants. Furthermore, ESR does not differentiate between the erythrocyte tendency to aggregate because of cellular factors or of plasma factors.[16,20,22,23]

Erythrocyte aggregation can be determined more accurately by commercially available systems, the Myrene Rheometer and the Laser-assisted optical rotation cell analyzer (LORCA),[24] which employs light transmission through erythrocyte suspension or light scattering to obtain indices of erythrocyte aggregation, expressed mainly, as the average aggregate size at a certain sheer stress. In addition, a cell flow analyzer (CFA) has been developed in the laboratory of Yedgar for monitoring erythrocyte aggregation by direct visualization of the aggregation process under controllable shear stress in a narrow gap flow chamber. The CFA analyzes the aggregate size distribution, namely, the percentage of the erythrocyte population in each aggregate size (expressed as the number of cells per aggregate) as a function of shear stress.[25,26] However, they are expensive and need a well-established laboratory with expensive equipment.

The EAAT is based on the concept that multiple adhesive proteins are involved in the induction or maintenance of increased aggregation. The finding of enhanced aggregation means, the concentration of these proteins is increased and *vice versa*.[27]

A simple slide technique and image analyzer called INFLAMET (Inflammation meter) also called EAAT was designed by the Department of Internal Medicine, Tel Aviv University, Israel.[11] By this test, the state of erythrocyte aggregation can be determined and quantified. They suggested using the degree of erythrocyte aggregation in the peripheral blood as a quantitative measurement for the intensity of acute phase response. Image processing used by them was complex. Three variables were used: EP, AR (aggregation radius), and VR (vacuum radius). It needed sophisticated software, which was designed by them.[11] EP was defined as the proportion of image area occupied by the erythrocytes. In the study conducted by the Department of Gynecology, Tel Aviv University, in 15 patients of pelvic inflammatory disease, EP alone was taken as a variable to measure erythrocyte aggregation and obtained significant difference ($P = 0.001$) between subjects and controls.[28]

As per our availability, we quantified aggregation as EP in images as mentioned in methodology to compare the erythrocyte aggregation between subjects of acute myocardial infarction, acute ischemic stroke, and controls. A strong correlation was also found between erythrocyte aggregation and inflammatory state in unstable angina. Erythrocytes can initiate a potent atherogenic stimulus contributing to the deposition of cholesterol at the atherosclerotic plaque, and the redox changes of erythrocytes have been hypothesized to play a role in the pathogenesis of hypertension and stroke.[12,13]

The present study used a simple slide test, the so-called EAAT to study erythrocyte aggregation in the peripheral blood of subjects with acute ischemic heart and brain disease. The study found a significant difference in the level of erythrocyte adhesiveness/aggregation among subjects of acute myocardial infarction, acute ischemic stroke, and controls. Slides were assessed subjectively by the pathologist who was blinded to the diagnosis and assigned grades A–D as described in Subjects and Methods section.

The study found that 50% (15 of 30) of patients of acute myocardial infarction and acute ischemic stroke had severe aggregation (ie, belonged to C and D grades), whereas only 6.6% (2 of 30) of control group had severe aggregation, so there was significant elevation ($P < 0.001$) of erythrocyte aggregation in subjects of acute myocardial infarction and acute ischemic stroke when compared with controls. The degree of aggregation was quantified by measuring EP (percentage of image area occupied by erythrocytes). Mean EP value in acute myocardial infarction was 69.91 + 13.25 and in subjects of acute stroke it was 70.59 + 11.30, whereas in controls mean EP was 85.16 + 8.417. There was a significant decrease in EP values in acute myocardial infarction and stroke as compared with controls ($P < 0.001$).

In a study conducted by Fusman *et al.*,[11] Israel, in which they used this slide technique to reveal the state of erythrocyte aggregation in 206 subjects of ischemic vascular diseases, the mean EP for subjects was 75 + 10 and for controls it was 81.5 + 5; they found significant increase ($P < 0.001$) in aggregation.[29] There are multiple causes that can increase the adhesiveness and aggregation of erythrocytes in the peripheral blood of subjects with ischemic vascular diseases, including proteins of acute-phase response,[5] hyperlipidemia,[7] and changes in the composition of erythrocyte membrane.[30] Among plasma factors, fibrinogen is the dominant contributor to erythrocyte adhesiveness/aggregation in the peripheral blood of individuals with atherothrombotic risk factors and healthy ones.[31] Erythrocyte aggregation is also implicated in the pathogenesis of ischemic vascular diseases. An elevated erythrocyte aggregation might have an indirect role in the formation of arterial thrombosis through its effects on the platelets. It is stated that with the increase in erythrocyte aggregation, blood viscosity also increases and local blood

flow decreases. These events cause local acidosis and platelet aggregation leading to endothelial cell damage.[30,31]

There was a significant negative correlation between ESR and EP values ($r = -0.623$ in acute myocardial infarction and $r = -0.69$ in acute stroke). So with increase in ESR, there was a decrease in EP, which corresponds to increased aggregation. The mean ESR in acute myocardial infarction subjects was 29 + 17.34 and the mean ESR in acute stroke subjects was 40.46 + 33.75, whereas the mean ESR in the controls was 15.5 + 12.37. There was a significant increment in the ESR values of acute myocardial infarction and acute stroke compared with that of the controls ($P < 0.001$).

In acute stroke subjects, out of 15 subjects with severe aggregation (ie, belonging to grade C and D), 6 subjects died; and out of 15 subjects with normal or mild aggregation, 1 subject died. There was a significant association between severe aggregation and death ($P < 0.05$). The relative risk calculated was 6.64; that is, subjects with enhanced or severe aggregation on admission had 6.64 times more risk of death when compared with normal or mild aggregation. Clinical worsening at such an early phase of acute stroke is most frequently due to thrombosis. This test can be an indirect marker of thrombus formation. Enhanced aggregation facilitates blood stasis and secondary thrombus progression.[32]

There was no significant association found between high admission ESR (>20 mm is taken as high) and death, but we observed that ESR > 40 mm was significantly associated with death ($P < 0.05$). In a study conducted by Neurology Service, Hospital Clinic i Provincial, Spain, where they evaluated a group of 208 ischemic stroke patients, they found a significant association between higher admission ESR and clinical deterioration during the first 24 h of stroke onset where they took high ESR as >13 mm/h in men and 20 mm/h in women.[29]

In the present study, we observed that 60% of the subjects with acute myocardial infarction were either diabetic or hypertensive. Enhanced aggregation in diabetics is explained by many reasons. The membrane anionic charge in diabetes is decreased, which leads to increased tendency of erythrocyte aggregation.[30] There were 4 deaths in acute myocardial infarction within 2 days with severe or enhanced levels of aggregation. There was no significant association found between ESR and deaths in acute myocardial infarction.

CONCLUSIONS

EAAT is a simple bedside test. The slides can be assessed subjectively and can be graded based on the degree of aggregation. The degree of aggregation can also be quantified by a simple software program as EP. It is definitely a real-time low-cost procedure. In the present study, greater erythrocyte aggregation was seen in subjects of acute myocardial infarction and acute stroke when compared with controls. Erythrocyte adhesiveness/aggregation is a useful biomarker to detect internal inflammation in individuals with atherothrombotic risk factors.

The test may have the potential to assess the risk of acute myocardial infarction or acute ischemic stroke in subjects with unstable angina/transient ischemic attack and also to assess prognosis in subjects with acute myocardial infarction and acute ischemic stroke. As it is a simple test, it can be used as a screening test for high-risk individuals so that necessary lifestyle interventions could be brought up in them.

However, this needs further studies. In the present study, stained images were difficult to read as binary by software, due to the variable intensity of staining. We suggest that unstained slides could be used to calculate erythrocyte aggregation. Ideal quantification of aggregation should also consider thickness of the aggregate.

ACKNOWLEDGMENT

This project work was done in the Department of Pathology as part of ICMR project.

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Source of Support: Nil, **Conflict of Interest:** None declared.