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Research Article

SIGNIFICANCE OF ERYTHROCYTE AGGREGATION TEST IN ACUTE MYOCARDIAL INFARCTION

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ABSTRACT
Erythrocyte acts as a scavenger. When reactive O_2 species are produced beyond its antioxidant capacity, it loses its structural integrity resulting in aggregation. Thus, aggregation has been a useful marker to detect inflammatory state. This is assessed by slide test in this study, which was currently available indirectly through ESR. To test for erythrocyte aggregation by a slide test in myocardial infarction (MI) cases and controls and to correlate ESR values with that of erythrocyte aggregation on slide test. Our study included 40 cases of acute MI and 40 matched healthy controls. Blood samples were collected. ESR by Westergren's method and slide test were performed. Slides were prepared and analysed under 40X magnification and grades A to D were assigned, based on degree of erythrocyte aggregation. Statistical Analysis carried out by Fisher exact "t" test and Independent "t" test. The mean age of the subjects was 55±9years. Aggregation was significantly severe in MI cases, where 19(47.5%) showed grade C and 18(45%) showed grade D. Controls showed predominantly grade A and B, constituting 37.5% and 50% respectively. ESR was significantly higher in MI patients. 37.5% cases and 77.5% controls with Grade A, B and D aggregation had ESR <20 and 32.5% cases with Grade D aggregation had ESR >20 mm/hr.
Erythrocyte aggregation test is a simple, cost-effective, indirectly reveals the presence and proportion of inflammation. It can be used as screening test in high-risk individuals for MI.

Keywords: *Acute MI, Slide Test, Erythrocyte Aggregation, ESR*

INTRODUCTION
Erythrocyte acts as a scavenger under normal physiologic conditions (Lakshmi *et al.,* 2011). When amount of reactive O_2 and nitrogen species are produced beyond erythrocyte antioxidant capacity, it becomes the source of reactive species and loses its structural integrity, leading to increased aggregation. Thus, aggregation has been a useful marker to detect inflammatory state (Lakshmi *et al.,* 2011). Diseases like ischemic heart disease and sepsis are associated with inflammatory response, resulting in production of acute phase proteins involved in induction and maintenance of increased erythrocyte aggregability (Avitzour *et al.,* 2003; Ami *et al.,* 2001; Rotstein *et al.,* 2002).

RBC aggregation is dependent on plasma and cellular factors. Normal blood flow sufficiently causes dispersion of RBC aggregates and maintains tissue perfusion. Aggregation is dependent on the cohesive forces within the aggregates (Rotstein *et al.,* 2002). Pathological RBC aggregation is characterized by strong intercellular links, associated with microcirculatory disorders, triggering the flow (Ami *et al.,* 2001; Weng *et al.,* 1998).

Erythrocyte aggregation is currently evaluated indirectly through ESR. Our study focuses on whether erythrocyte aggregation test can be used as a rapid inexpensive biomarker.

Aim
1. To test for erythrocyte aggregation by a slide test in cases with myocardial infarction and healthy controls.
2. To correlate ESR values with that of erythrocyte aggregation on slide test.

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MATERIALS AND METHODS

Inclusion Criteria

Two groups of subjects were included in the study:-

1. Cases of acute myocardial infarction admitted to our hospital, within 6 hrs of onset of chest pain. Patients who met atleast two out of three WHO criteria (Pedoe *et al.*, 1994) were included in our study.
   - Ischemic type chest discomfort.
   - ECG changes (ST- elevation in two contiguous leads or new left bundle branch block; LBBB)
   - Elevation of serum levels of cardiac markers (CK-MB or Cardiac troponin)
2. Subjects of age and gender matched healthy controls, which were non-diabetic and non-hypertensive with no recent history of fever.

Informed consent was taken. History was noted. Routine general and systemic examination was done.

Sample Collection

EDTA anticoagulated venous blood sample was collected immediately after admission, before starting treatment. ESR by Westergren's method and Slide test - Erythrocyte aggregation/adhesiveness test (EAAT) were performed.

Preparation of Slide (Lakshmi *et al.*, 2011)

A single large drop of EDTA anticoagulated blood was placed on a slide using a pipette, with inclination of 45 degrees. The drop size and the angle at which the slides were placed, was maintained constant for all slides. The slide was left in that position for 10 seconds, during which the blood was allowed to run down by gravity, forming a fine film. The slides were dried at room temperature, in completely horizontal position. The dried slides were stained with Leishman stain. The slides were subjectively assessed by two pathologists, under 40X (400X) magnification and grades were assigned to the slides based on the degree of erythrocyte aggregation.

The Grading Criteria (Lakshmi *et al.*, 2011)

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

Grade B: Erythrocyte aggregates are seen in some areas of slide with small clear spaces (mild aggregation) (Figure 2)

Grade C: Variable sizes of aggregates over all the areas of the 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)

The whole slide was assessed for about 50 fields, and the predominant grade was considered.

Exclusion Criteria

Subjects with recent history of MI (less than 8 weeks), sepsis, bacterial infection, malignant disease, pregnancy, severe renal or hepatic failure, thrombocytopenia or deep vein thrombosis.

Statistical Analysis

Fisher exact "t" test was used to compare the independent proportions in two groups, that is grading of aggregation in myocardial infarction cases and controls. Independent "t" test was used to correlate ESR values with the grades of erythrocyte aggregation obtained by slide test. p value of < 0.05 was considered significant.

Ethical clearance was obtained by the Institutional Ethics Committee.

RESULTS AND DISCUSSION

Results

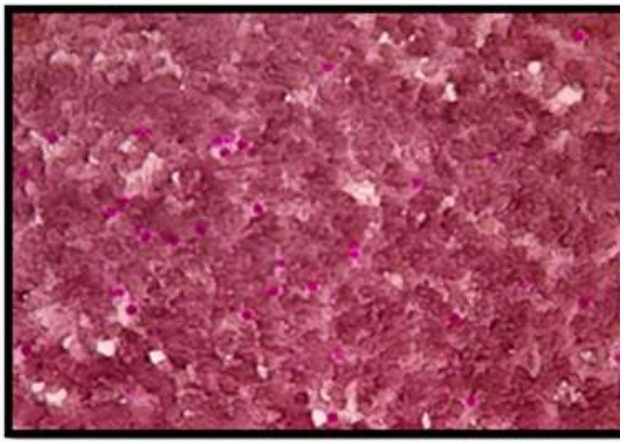

Figure 1: Photomicrograph showing erythrocytes distributed uniformly throughout, with no clear spaces (Normal aggregation- Grade A) (400X, Leishman stain)

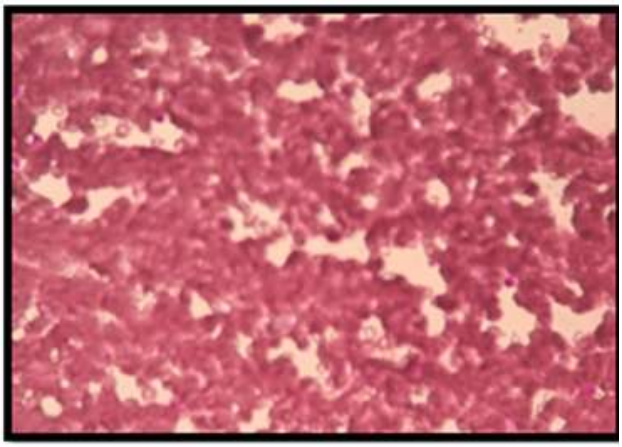

Figure 2: Photomicrograph showing erythrocyte aggregation in some areas with small clear spaces (Mild aggregation- Grade B) (400X, Leishman stain)

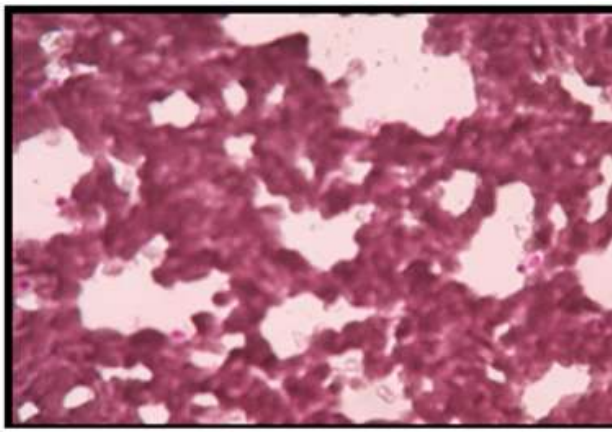

Figure 3: Photomicrograph showing erythrocyte aggregates of varying sizes with small clear spaces (Moderate aggregation- Grade C) (400X, Leishman stain)

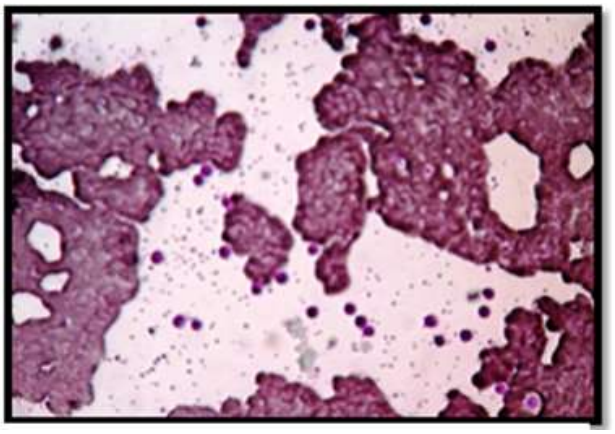

Figure 4: Photomicrograph showing large thick aggregates with rounded/clear borders and large clear spaces (Severe aggregation- Grade D) (400X, Leishman stain)

Among 40 cases of acute myocardial infarction and 40 controls, 35 were males and 5 were females. Male to female ratio was 7:1. The age ranged from 38 to 75 years, with the mean age of 55±9 years.

Aggregation was significantly more severe in cases with myocardial infarction, where 18 of 40 (45%) cases showed grade D and 19 of 40 (47.5%) cases showed grade C aggregation. Among the controls, 15 of 40 (37.5%) subjects showed grade A and 20 of 40 (50%) subjects showed grade B aggregation and only 5 of 40 (12.5%) subjects showed grade C aggregation. Grade C and grade D were predominantly seen in MI patients and was statistically significant with $p < 0.001$(Table 1).

The mean ESR of patients with myocardial infarction was 22.7±20.86. The mean ESR of control group was 11.55±8.26. ESR was significantly higher in MI patients, with p-value =0.001. ESR was more than

20 in 13(32.5%) cases with grade D aggregation. Only 5(12.5%) cases with grade D aggregation had ESR < 20. Among controls with grade A, 13(32.5%) subjects had ESR < 20 and 2(5%) subjects had ESR 20 to 40 mm/hr. Fifteen (37.5%) cases and 31(77.5%) controls with Grade A, B and C aggregation had ESR < 20. Independent "t" test showed significant correlation of ESR with erythrocyte aggregation grades, with p = 0.001 (Table 2).

Table 1: Degree of erythrocyte aggregation in cases with acute myocardial infarction and controls

Grade	Controls (n=40)	Myocardial infarction (n=40)
A	15 (37.5%)	0
B	20 (50%)	3 (7.5%)
C	5 (12.5%)	19 (47.5%)
D	0	18 (45%)

**Significant with p-value < 0.001*

Table 2: Correlation of ESR with erythrocyte aggregation in cases and controls

ESR	< 20		20-40		41-60		> 60		Total
Grade	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	
A	0	13 (32.5%)	0	2	0	0	0	0	15
B	2 (5%)	15 (37.5%)	1 (2.5%)	5	0	0	0	0	23
C	13 (32.5%)	3 (7.5%)	6 (15%)	2(5%)	0	0	0	0	24
D	5 (12.5%)	0	7 (17.5%)	0	4 (10%)	0	2 (5%)	0	18

**Significant with p-value = 0.001*

Discussion

Human diseases associated with inflammatory response shows acute phase reaction, which is characterised by increased leucocyte count, accelerated ESR, hyperfibrinogenimia, hypergammaglobulinemia, increased synthesis of C- reactive protein (CRP) and other acute phase proteins. These proteins induce and maintain the erythrocyte aggregation, predominantly fibrinogen (Avitzour *et al.,* 2003). The intensity of acute phase response is evaluated, either by measuring the concentrations of these individual acute phase proteins or indirectly by Westergren erythrocyte sedimentation rate. This can be quantified directly by degree of aggregation on slide, which is the final effect of these proteins (Avitzour *et al.,* 2003).

In acute MI, aggregation is predominantly influenced by plasma factors (Ami *et al.,* 2001). In normally perfused tissue, dispersion of erythrocyte aggregates is maintained by blood flow. Thus, in low flow states such as microcirculatory disorders there may be increased erythrocyte aggregation (Ami *et al.,* 2001). Enhanced erythrocyte aggregability in occlusive cardiovascular disease suggest the local events in cerebral ischemia, which could be due to changes in RBC aggregability of systemic circulating blood. Blood factors which can be correlated with erythrocyte aggregability in stroke patients are globin

concentration, fibrinogen concentration, A : G ratio and Mean corpuscular volume (MCV) (Tanahashi *et al.*, 1989).

Blood viscosity was upto 10 fold greater in patients suffering from thrombosis and coronary occlusion. The determinants of blood viscosity are hematocrit, intrinsic resistance of the plasma to flow and RBC aggregation. The size of myocardial infarction after coronary arterial ligations is shown to be increased with increased blood viscosity and is more likely to develop complication like cardiogenic shock and heart failure (Tanahashi *et al.*, 1989).

Lakshmi *et al.*, (2011) used simple slide test, the so called EAAT to study erythrocyte aggregation in the peripheral blood of cases with acute ischemic heart and brain disease. They found a significant difference in the level of erythrocyte adhesiveness/aggregation among cases of acute myocardial infarction, acute ischemic stroke, and controls. Slides were assessed subjectively by pathologist; they assigned grades A to D. The degree of aggregation was quantified by measuring erythrocyte percentage (percentage of image area occupied by erythrocytes).

Increased erythrocyte aggregability has been shown to be associated with increased concentration of fibrinogen, lipids, during diabetes, hypertension, smoking, menopause and inflammation (Rotstein *et al.*, 2002). In individuals with ischemic vascular disease, hyperlipidemia and increased fibrinogen levels contributes to erythrocyte aggregation in the peripheral blood (Lakshmi *et al.*, 2011; Weng *et al.*, 1998). Many studies have shown significant correlation between the aggregability and concentration of fibrinogen and gamma globulins and reduced aggregability following reduction in concentration of acute phase reactants, like CRP (Avitzour *et al.*, 2003).

ESR may correlate poorly with RBC aggregation; it may be confounded by gender, age, hematocrit, plasma albumin levels, temperature and hemodilution by anticoagulant. Erythrocyte aggregation assessed by cell flow analyser helps to differentiate that actual aggregation is because of cellular factors or plasma factors by expressing the distribution of RBC population into the aggregate size ranges and the resistance to shear – induced disaggregation. This differentiation is not possible with ESR (Ami *et al.*, 2001). ESR depends on number and size of RBCs and positively charged inflammatory proteins. Thus, ESR is an index of both viscosity and inflammation, reflecting plasma concentration of acute phase response proteins (Natali *et al.*, 2003). Studies have shown that, ESR is an independent predictor of coronary artery disease, even after stratification of other risk factors and confounding factors (Natali *et al.*, 2003; Rana *et al.*, 2005; Timmer *et al.*, 2005). Our study also showed good correlation of ESR with erythrocyte aggregation. ESR was more than 20 mm/hr in cases showing Grade C and D erythrocyte aggregation.

Cell flow properties analyser (CFA) was developed in laboratory by Yedgar for monitoring RBC aggregation by direct visualization of aggregation process under controllable shear stress in a narrow gap (30 µm) flow chamber. CFA provides aggregate size distribution, which is percentage of the RBC population in each aggregate size, as a function of shear stress (Rotstein *et al.*, 2002). Studies have shown that in bacterial infection and unstable angina – both cellular and plasmatic factors contribute to enhanced aggregation by alteration of RBC membrane and antioxidant action in response to oxidative stress (Ami *et al.*, 2001).

The study on acute bacterial infection demonstrated the RBC aggregation reducing the blood flow through the microcirculation in sepsis and septic shock. The formation of "sludge" blood causes tissue hypoxia and acidosis, thus interfering with normal host defences and administered antibiotics to reach sequestered bacteria and facilitates bacterial multiplication (Ami *et al.*, 2001).

Our study on acute myocardial infarction patients showed significantly greater erythrocyte aggregation in cases with acute myocardial infarction when compared with controls. The presence of certain protein doesn't mean that, the protein is involved in the phenomenon of cell aggregation. In erythrocyte aggregation test the final effect of these proteins can be quantified directly by assessing the degree of aggregation (Fusman *et al.*, 2000).

EAAT can also be used to monitor the changes in the erythrocyte aggregation/adhesion following therapeutic interventions (Fusman *et al.*, 2000). Thus, Erythrocyte aggregation test helps in identification

of significant acute phase response, which might have diagnostic and therapeutic implications in small clinics devoid of advanced laboratory facilities (Almog *et al.*, 2005). This may be an useful additional diagnostic criterion or screening tool, rather than a sole marker for diagnosis of heart pain (Eftekhaari *et al.*, 2012).

Conclusion

EAAT is a simple, inexpensive bedside test. The slides can be assessed subjectively and can be graded based on the degree of aggregation. EAAT indirectly reveals the presence and proportion of inflammation. This test is better than ESR by Westergren. Our study showed greater erythrocyte aggregation in cases with acute myocardial infarction when compared with controls. Thus, it can be used as screening test in high-risk individuals for MI.

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