

## Original Paper

# Effect of Physical Stress on Platelet Aggregation in Patients with Ischemic Heart Disease

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## ABSTRACT

*Effect of physical stress on platelet aggregation was evaluated in thirty patients with ischemic heart disease (IHD) - After collecting fasting blood samples, they were subjected to treadmill test up to stage III, and blood samples were again collected. All the blood samples were subjected for the study of platelet aggregation using ADP and collagen as aggregating agents. The study demonstrated that ADP induced platelet aggregation is variably affected by physical stress while collagen induced platelet aggregation was significantly ( $p < 0.05$ ) and consistently increased after physical exercise, in patients with IHD.*

**KEYWORDS:** Stress, ADP, Collagen, Aggreganometer, IHD

## INTRODUCTION

Experimental and clinical data suggest that platelets may contribute to the adverse clinical events associated with atherosclerotic coronary disease<sup>1</sup>. Platelet aggregation in coronary vessels occurring during stress has been implicated as a mechanism of imbalance between myocardial blood supply and demand<sup>2</sup>. The evidence of platelet involvement in myocardial ischemia is derived from several animal and human studies<sup>3,4</sup>.

Platelet upon activation generate a potent pro-aggregant and a vasoconstrictor prostaglandin, thromboxane-A<sub>2</sub>, which has been shown to be increased in patients with coronary artery disease during spontaneous or pacing induced myocardial ischemia<sup>5,6</sup>. Several studies relating

platelet aggregation function to exercise have been reported<sup>7-9</sup>. Moderate and strenuous exercise is known to enhance the platelet aggregation in patients with coronary artery disease<sup>10-12</sup>. Not only has this, but the effect of low-grade exercise also demonstrated enhanced platelet aggregability in-patient with obstructive coronary artery disease. In a recent study, shear induced platelet aggregability before and after mild exercise in 27 patients with documented coronary artery disease was assessed. *ex vivo* platelet aggregability was assessed in flowing whole blood as the time to occlude collagen and ADP coated ring. Patient with coronary artery disease showed a significant increase in aggregation at peak compared with base line, whereas no significant change occurred in controls. This effect is independent of myocardial ischemia and has been

observed despite aspirin consumption<sup>13</sup>. The failure of aspirin to attenuate the platelet response to exercise has also been reported by others<sup>11,14,15</sup>.

It is interesting to note that even low grade exercise also enhances platelet aggregation which is probably dependent on shear stress. The mechanism by which shear stress induces platelet aggregation is not clear however it involves the increase in intracytoplasmic ionized calcium, von Willibrand Factor and functional platelet receptor complex Gp Ib/IX/V and Gp III b/II a. vWF and Gp 1 b a interaction causes associated increase in intracytoplasmic (Ca<sup>2+</sup>) and platelet aggregation. Both of these are potentiated by vWF binding to activated platelet GPIII b/II a complex in presence of released ADP. Fibrinogen, Gp 1 b a and extra cellular Ca<sup>2+</sup> are absolutely required for these above reactions. If the effect of released ADP is blocked, shear induced platelet aggregation is inhibited without affecting shear induced increase in calcium. It is also observed that neither calcium nor aggregation response to shear stress is inhibited by blocking platelet cyclooxygenase with acetyl salicylic acid<sup>16,17</sup>.

The present study has been envisaged to evaluate the effect of physical stress on platelet aggregation in patients of coronary artery disease.

## MATERIAL AND METHODS

### Patients' Selection:

The study included 30 male patients of ischemic heart disease (IHD) between the ages of 40 to 60 years. Patients of ischemic heart disease were either of old healed myocardial infarction (> 6 months) or stable angina pectoris with or without positive Treadmill Test (TMT). The criteria for diagnosis of IHD were same as that of WHO<sup>18</sup>.

### Exclusion Criteria:

The following patients were not included in this study:

1. Patients with valvular heart disease;
2. Bleeding tendency;
3. Uncontrolled diabetes;
4. Patients with peripheral arterial disease;
5. Those patients who were consuming tobacco in any form or taking treatments with corticosteroid, anticoagulant and antiplatelet drugs.

### Protocol:

After informed consent of the selected study subjects, initial fasting blood samples were collected. All selected individuals were subjected to stress testing on a computerized 12 lead TMT machine for less than or equal to stage three of Bruce protocol. Blood samples were again collected at the end of the test. All the blood samples were subjected for measurement of platelet aggregation.

### Exercise Testing<sup>19</sup>:

Measurements of cardiovascular function during rest are poor predictors of circulatory performance. Exercise is currently the most convenient way of stimulating the myocardium to demand maximal blood flow so that even a moderate impairment of coronary blood flow capacity becomes detectable.

### Modes:

#### 1) Dynamic lower extremity testing:

1. Treadmill: It is most widely used method for exercise testing.
2. Bicycle ergometer: It is used when a patient is unable to do treadmill exercise, i.e., during radionuclide ventriculography and for dynamic testing during cardiac catheterisation.
3. Master's step test: Seldom used

#### 2) Dynamic upper extremity testing:

1. Arm ergo meter: sometimes used in patients with PVD, orthopaedic abnormality and other limitation to lower extremity effort.

### Exercise Test Protocols:

#### 1) Heart rate limited or sub maximal exercise

#### 2) Symptom limited testing

#### 3) Treadmill protocols: There are several protocols available, the choice of which depends upon expected effort tolerance of the patient:

**a) Bruce protocol:** This is preferred for evaluating patients with little or no symptomatic limitation. This has a relatively higher initial workload with greater subsequent work increments. The subjects start out at 1.7 mph on a 10% inclined & progress to their maximal capacity at 3 minutes intervals.

**b) Naughton protocol:** This has low initial workload and small work increment with subsequent stages, and is used after myocardial infarction or coronary artery bypass graft surgery.

The workload achieved in all protocols is expressed in terms of MET (metabolic equivalent). This term is used to describe the energy cost of physical activity. One MET is approximately equal to an oxygen consumption of 3.5 ml/ Kg/min.

### Preparation of Patient:

A detailed clinical examination and evaluation resting 12 lead ECG is done before treadmill test is ordered. The patient should be fasting for at least 2 hours before the test; whether any medication being taken should be discontinued depends on purpose of testing. Leads monitored are 12 lead ECG. The patient is continuously monitored as he goes through various stages of exercise. Reassurance & encouragement of the patient during test enable one to obtain a truly symptom limited

exercise test.

In the post exercise period, the sitting position is mostly frequently employed. Cardiac auscultation is carried out to detect out any gallop sound, mitral regurgitation or pulmonary rales. Observation after exercise is continued for 6 minutes or longer till all exercise-induced abnormalities have disappeared.

### Exercise Test Response

Symptomatic end points:

In symptom limited exercise test, the following symptoms are used as end points:

- 1) Angina pectoris
- 2) Dyspnea and Fatigue
- 3) Leg fatigue, Claudication, Joint pain

### ECG Patterns and their Significance

#### Normal Exercise Electrocardiogram

When the heart rate increases with exercise, a number of predictable changes occur in the ECG tracing. PR interval shortens, P wave becomes taller & atrial repolarisation rate (Ta wave) becomes prominent causing depression of the PQ segment. This results in J point depression, which is usually of short duration (0.04) sec. The normal ST segment with exercise is up sloping and slightly convex in form and returns to base line within 0.04 to 0.06 sec after J point.

#### ST segment changes:

- 1) Up sloping segment: These are abnormal when the degree of depression at 0.08 sec from the J point is 1.5 mm or more below base line.
- 2) Horizontal segment: When ST segment depression is horizontal or down sloping, 1.0 mm depression at 0.08 sec from the J point is considered a positive response and correlates well with the actual presence of CAD.
- 3) Rounded segment depression: A rounded configuration of ST segment depression represents a positive response - patients with this pattern are at higher risk of future coronary events
- 4) ST segment depression late in recovery period: In few patients depression is absent during and immediately after exercise but occurs three to eight minutes into the recovery period. This is almost associated with normal coronaries.
- 5) ST Elevation: When ST segment elevation occurs with exercise it usually indicates a ventricular kinetic or dyskinetic segment or presence of high-grade lesion in the proximal left anterior descending coronary artery.

#### T wave changes:

- 1) Tall T wave in lateral precordial leads after exercise are normal and are due to increased stroke volume.

- 2) T wave inversion during exercise is a non-specific finding and not considered in the evaluation of ischaemia. On the other hand, the evolution of a downsloping T wave after exercise is often associated with ischaemia. Normalisation of T wave with exercise such as a flat or inverted T wave at rest becoming upright with exercise has been considered as a sign of ischaemia.

#### U wave:

Occurrence of inverted or diphasic U waves with exercise is often associated with CAD and in particular with high grade proximal left anterior descending artery stenosis.

#### R wave amplitude:

The R wave amplitude in lateral precordial leads normally decreases with exercise. In patients with severe CAD, R wave amplitude increases with exercise.

#### Predictive Implications

Two of the important factors in the analysis of patients undergoing stress test are:

1. **Pre-test disease prevalence:** Patients with typical anginal pain have 95 % chance of having the disease; in those with atypical chest pain it is 60% and in patients with nonanginal pain it is 10%. It is in the group of a typical chest pain that treadmill test is of considerable value in masking CAD.
2. **Sensitivity & specificity of the test:** Sensitivity provides an index of the capability of the test to detect an abnormality. Specificity indicates the ability of the test to recognize a normal subject.

#### Prognostic Significance

Stress test can be a useful tool in patients with CAD to predict the prognosis of the disease and assess the result of developing future cardiac event.

#### Platelet Aggregation<sup>20</sup>

Most important function of platelets is their role in hemostasis i.e. adhesion to damaged tissue surface and cohesion to one another. This cohesion phenomenon is known as aggregation and may be initiated by a variety of substances including collagen, adenosine 5-diphosphate (ADP), epinephrine, serotonin and ristocetin. Aggregation is one of the numerous *in vitro* test performed as a measure of platelet function. The described procedure is a turbidimetric method of measuring the effect of collagen, ADP and epinephrine on platelets.

#### I. Reagents

- 1) 3.8 per cent citric acid (Trisodium salt dehydrate): prepared by dissolving 3.8 gm citric acid in 100 ml of deionised water.
- 2) Tris buffer: Tris (hydroxyl methyl), methylamine, 1.21 gm (0.01 M), disodium ethylene diamine tetra acetic acid 0.372 gm (0.001 M), sodium chloride 8.76 gm (0.15 M), dissolved in distilled water adjusted to pH 7.5 with hydrochloric acid and made upto one litre with distilled water.

- 3) ADP reagent --- Adenosine 5-diphosphate lyophilized with buffer salts (supplied by sigma diagnostics). ADP solution was prepared by reconstituting ADP reagent with 1.0 ml deionized water to yield solution containing ADP  $2 \times 10^{-4}$  mol/lit. It was swirled to mix and allowed to stand at room temperature (18-26°C) for 15 minutes before use. It should be kept at room temperature only for duration of testing. The reconstituted reagent is stable for one month if stored in refrigerator (2° to 6°C).
- 4) Epinephrine reagent : Epinephrine bitartrate lyophilized with buffer salts. Epinephrine solution was prepared by reconstituting epinephrine reagent with 1.0ml deionized water to yield solution containing epinephrine  $1 \times 10^{-4}$  mol / lit. It was allowed to stand at room temperature for 15 minutes before use. The reconstituted reagent is stable for one month, if stored in refrigerator (2° to 6°C).
- 5) Collagen reagent: Collagen (calf skin) acid soluble, approximately 2 mg, lyophilized with buffer salts. Collagen solution was prepared by reconstituting vial of collagen with 1.0 ml deionised water. The vial was allowed to stand undisturbed for at least 15 minutes at room temperature before use. Warming to 37°C may be necessary for complete dissolution. It was swirled to mix prior to each assay. It should not be vortexed. The solution should be kept at room temperature only for the duration of testing. It is usually stable for at least 2 weeks refrigerated (2-8°C). Stability may be extended by freezing.

## II. INSTRUMENTS & MATERIALS

### 1. Instruments (Fig. 1)

1. Platelet aggreganometer (Elvi 840)
2. Chart recorder (Omniscribe recorder dual pen type <176 USA)

### 2. Materials

1. Cuvette 250µl
2. Teflon coated magnetic stirring bars (micro agitators)
3. Pipettes with disposable plastic tips 50µl and 250µl
4. Centrifuge machine
5. Plastic tubes with caps
6. Plastic transfer pipettes

## III. SPECIMEN COLLECTION

Blood was collected by avoiding stasis and contamination with tissue fluids into plastic tubes containing 0.1 ml buffer and 3.8 per cent sodium citrate in a ratio of blood to anticoagulant in a ratio of 9:1.

## IV. PREPARATION OF PLATELET RICH PLASMA (PRP)

1. The anticoagulant sample was centrifuged at 400 RPM for 10 minutes.
2. PRP was removed carefully using a plastic transfer pipette.
3. PRP was expelled into a plastic tube covered and kept at room temperature for duration of the test.

## V. PREPARATION OF PLATELET POOR PLASMA (PPP)

1. It was prepared by re-centrifuging the PRP at 6000 RPM for 10 minutes.
2. Supernatant was transferred to a labelled PPP tube, covered and kept at room temperature for the duration of the test.

The platelet count of PRP was adjusted to the range of 4 to 5 lac / cu mm when necessary by addition of autologous PPP to PRP samples. The caution should be taken to assay platelet aggregation within 30 minutes of collection of test samples.

## VI. AGGREGATING AGENTS

**ADP** - ADP induced aggregation may occur in one or two phases and it may be followed by rapid disaggregation, which may be seen in normal man without any hemorrhagic disease.

**Epinephrine** - Epinephrine induced platelet aggregation may occur in one or two phases and is largely irreversible and epinephrine induced platelet aggregation sometimes absent in patients who appear to be totally normal and may be related to temporary saturation of catecholamine binding sites on platelets.

**Collagen** - Collagen induced platelet aggregation may occur in an irreversible single-phase curve or a reversible single-phase curve depending on the collagen concentration in the PRP.

## VII. PROCEDURE

After preparing PRP and PPP the aggregation was recorded as follows:

1. Cuvette with PRP was introduced into the aggregometer.
2. The electromagnetic agitation was started by means of stirred control after having introduced a small stirring bar into the sample.
3. Agitation speed was maintained at 1000 RPM.
4. Baseline of the recorder was adjusted by means of the zero control.
5. The cuvette with PRP was removed and cuvette with PPP was inserted.



# PLATELET AGGREGATION

## ELVI - 840 Aggregometer and Omniscribe Recorder



### Aggregating Agents (SIGMA)

**ADP** - 50 $\mu$ l. of  $2 \times 10^{-4}$  mol/L

**EPI** - 50 $\mu$ l. of  $1 \times 10^{-4}$  mol/L

**COLL** - 50 $\mu$ l. of 2mg/ml

**Figure 1:** Platelet aggregometer (Elvi 840) with chart recorder (Omniscribe recorder dual pen type 176 USA)

6. By means of gain control the maximum excursion of the pen to the recorder was adjusted.

7. Cuvette with PPP was removed and the cuvette with PRP was reinserted and it was readjusted if necessary by means of the zero control.

8. The sliding of the recorder paper was started.

9. The aggregating agents (ADP, and collagen) were added to the PRP by means of micropipette (50 $\mu$ l)

The aggregation was recorded for a minimum of 5 minutes and results were expressed as percentage aggregation.

$$\begin{aligned} \text{Percentage Aggregation} &= \frac{90 - \text{CR}}{90 - 10} \times 100 \\ &= \frac{90 - \text{CR}}{80} \times 100 \end{aligned}$$

CR is chart reading in terms of number of segments.

### VIII. EXPECTED VALUES

Platelet aggregation studies were performed on 20 healthy adults using the three aggregation reagents. The results were as follows -

ADP = 80-100

Epinephrine = 67-97 % (subjects with secondary aggregation phase)

Collagen = 26-59 % (subjects without secondary aggregation phase)

Collagen = 80-100%

However due to differences in instrumentation and technique, each laboratory should establish its own normal ranges for each reagent.

## IX. PERFORMANCE CHARACTERISTICS

Duplicate aggregation determinations on platelet rich plasma from normal individuals yielded an average difference of  $\pm 3\%$  aggregation for collagen, ADP and epinephrine aggregations determinations.

### STATISTICAL ANALYSIS<sup>21</sup>

Results were statistically analysed with Student's t-test and a 'p' value of less than 0.05 was considered as significant difference in analysis.

### OBSERVATIONS AND RESULTS

The following observations were made on 30 male patients of known ischemic heart disease. The mean age of subjects was 57 years, they were subjected to treadmill stress (TMT) test. On an average, they exercised to stage III  $\pm$  I of Bruce protocol. 17 patients had positive test and 13 had negative stress test.

Collagen induced platelet aggregation was increased from mean value of 49.75 to 54.90%. This had led to increase in post exercise aggregation to the extent of 10 percent and that is statistically significant ( $p < 0.05$ ). However, ADP induced platelet aggregation was increased to the extent of 5 percent but it was not significant ( $p$  NS) statistically [Table 1].

## DISCUSSION

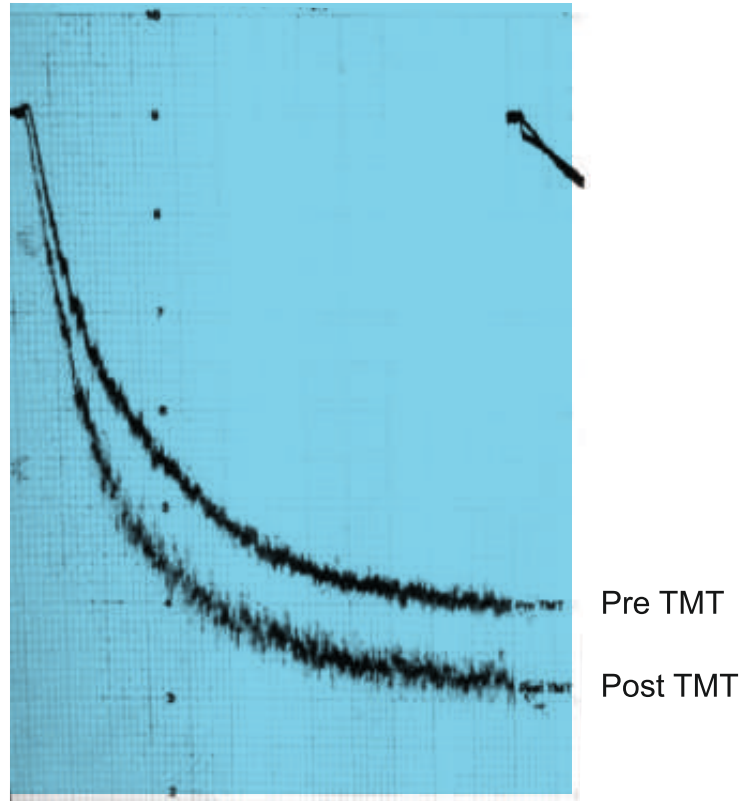
The present study was conducted on 30 male patients of known ischemic heart disease between the ages of 40 - 60 years. They were subjected to treadmill exercise to the stage III. Blood samples were collected before and after exercise test for platelet aggregation.

The mean age of the patient selected was 57 years, 17 patients had positive ischemic response to exercise while 13 had negative test. Exercise had significantly ( $P < 0.05$ ) increased collagen induced platelet aggregation to the extent of 10% while ADP induced platelet aggregation was not significantly altered (Table 1).

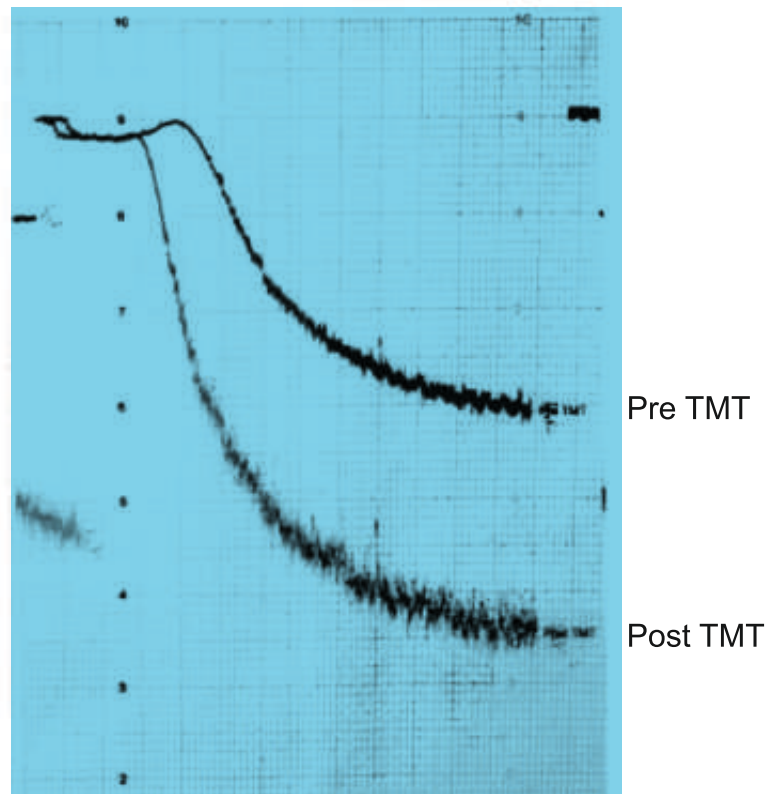
The present study therefore suggests that treadmill exercise test which is basically a physical stress test does not significantly increase ADP induced platelet aggregation in patients with documented IHD. Collagen induced platelet aggregation on the other hand has been observed to increase significantly ( $< 0.05$ ) after exercise [Table 1] [Fig. 2, 3, 4 and 5].

**Table 1:** Effect of Physical stress on platelet aggregation (percent) in Patients with IHD

	ADP ( $2 \times 10^{-4}$ )		Collagen (0.2 $\mu\text{g}/\text{micro lit.}$ )	
	Pre TMT	Post TMT	Pre TMT	Post TMT
Mean	53.63	56.50	49.75	54.90
% Change		5.30		10.35
S.D. $\pm$	10.56	12.84	10.70	12.75
S.E. $\pm$	3.34	4.06	3.38	4.03
P value		NS		$< 0.05$



**Figure 2:** Effect of exercise on ADP induced platelet aggregation



**Figure 3:** Effect of exercise on Collagen induced platelet aggregation

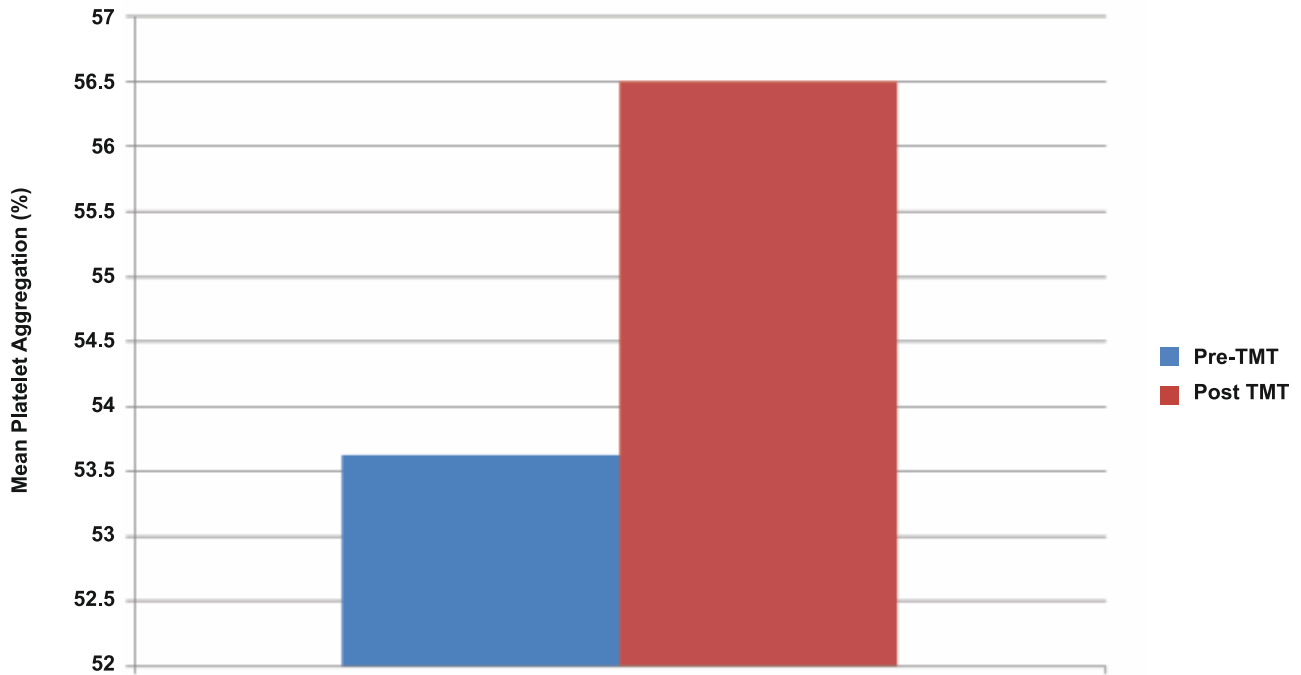


Figure 4: Effect of physical stress on ADP induced platelet aggregation

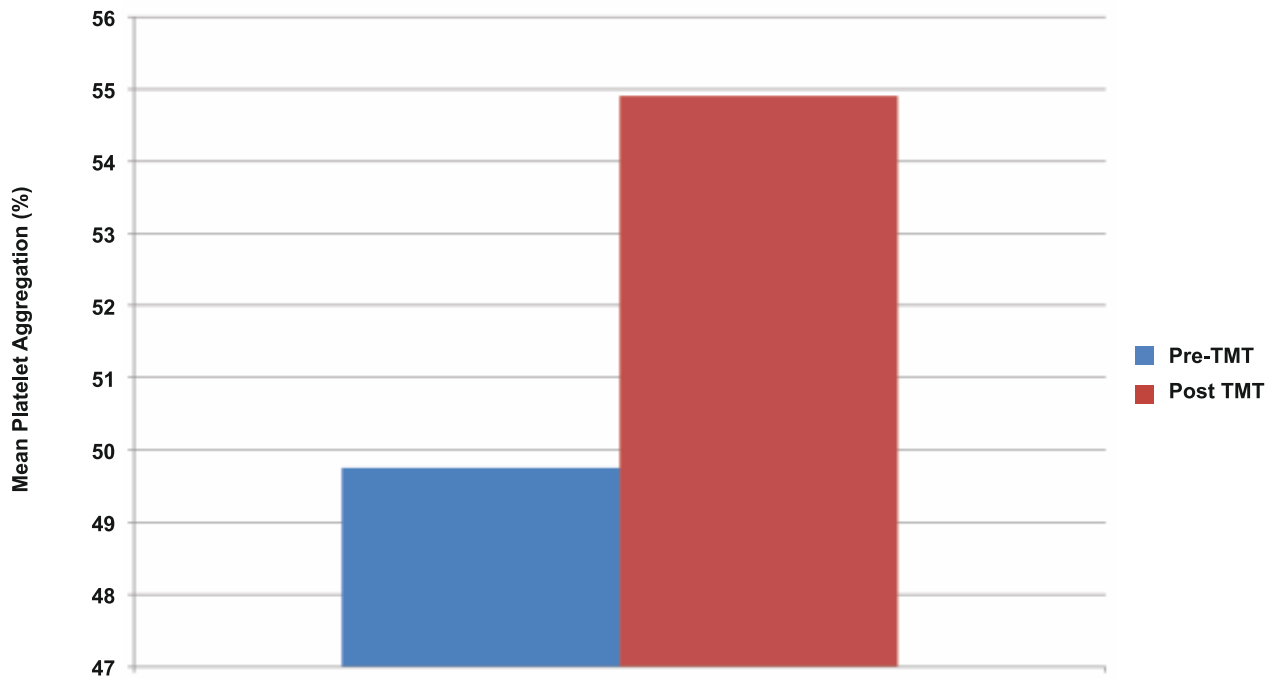


Figure 5: Effect of physical stress on Collagen induced platelet aggregation



There are controversial reports on the effect of exercise on platelet aggregation not only on healthy individuals but also in patients of CAD. Some of the inconsistencies regarding the effect of exercise on platelet aggregation can perhaps be explained by differences in physical conditions, i.e., healthy young individuals who do not engage in regular exercise increase their aggregability during exercise whereas those who are regularly participating in an exercise show the opposite effect<sup>22,23</sup>.

In elderly, the failure of exercise to reduce platelet aggregability can be explained by their generally low level of physical activity. Another factor could be that young people release more platelet inhibitors such as prostacycline and NO (nitrous oxide) from the vessel wall than do the elderly. In mild hypertensive, the response to maximal exercise is an increased *in vivo* platelet activity and a similar reaction has been noted in patients with IHD<sup>24</sup>.

In the present study also, the response to exercise on platelet aggregation is variably observed in patients of IHD with ADP induced aggregation. However, the response of collagen induced aggregation was consistently observed to be increased and was statistically significant. The possible cause of inconsistent observation of exercise induced aggregation may be because of humoral factors which may be stimulated and released by exercise such as catecholamines, vWF, leucocytes, thrombocytes, release of PF-4 which causes platelet leukocyte interaction and alteration in fibrinolytic system. The response to collagen-induced aggregation, which has consistently increased after stress, may be more sensitive indicator of underlying atherosclerosis involving the endothelial functions. This has already been reported that platelet collagen interaction *in vitro* may be comparable to platelet damage to vessel walls interaction *in vivo*<sup>30</sup>.

Andreottii & Associates<sup>13</sup> have demonstrated that even low grades of exertion transiently enhance platelet aggregability in patients with CAD and not in subjects without apparent CAD. The increase in aggregability is independent of myocardial ischaemia. These findings suggest that platelet aggregability is enhanced by exercise in the presence of coronary atherosclerosis *per se*, as a result of hemodynamic factors interacting with arterial obstruction or more likely with endothelial dysfunction<sup>26</sup>.

Another study has demonstrated increase platelet aggregability response to shear stress in patients with acute MI<sup>14</sup>. They observed that shear induced platelet aggregation was significantly higher in patients with acute MI than in patients with stable CAD and normal subjects. This variability also explains the observation in present study, which shows that response to stress is variable in patients with stable CAD.

Effect of physical and mental stress on platelet aggregation was also evaluated in 113 patients with stable angina and 50 healthy individuals by Wallen and associates<sup>27</sup>. They observed that platelet functions were more or less same at rest but

physical exercise increased the platelet aggregability in both the groups. Platelet responses to mental stress were highly variable but more pronounced in angina patients than healthy controls.

Platelet activation, aggregation and adhesion is a complex phenomenon responsible for arterial thrombosis. It does involve the coagulation system with fibrin formation. The event starts with response of platelet against tissue or endothelial injury. The platelets come in rescue to produce platelet plug to stop bleeding (primary hemostasis). Platelets, as has been described, have multiple surface receptors. These receptors, when stimulated, produce change in shape of platelets. The major receptors involved are glycoprotein Ib (Gp Ib) receptor, which binds to von Willebrand factor (vWF). Besides these, there are receptors for adenosine diphosphate (ADP), thrombin and thromboxane A<sub>2</sub>.

With the shape change, there is change in the surface of the platelet that leads to expression of a second binding site, the glycoprotein IIb/IIIa (Gp IIb/IIIa) receptor. These Gp IIb/IIIa receptors bind fibrinogen to bridge between adjacent platelets. Furthermore, the surface of the platelet also expresses binding sites for factor V, which is an essential cofactor in the generation of thrombin<sup>28,29</sup>.

The exact underlying mechanism of exercise-induced alteration in platelet aggregability remains unclear. However, there are certain evidences pointing towards the possible mechanism.

It has been observed that intense physical exercise increases plasma level of von Willebrand factor (vWF). Not only this but platelet aggregation is also associated with enhanced expression of adhesion molecules on platelets such as P-selectin (CD 62P) and glycoprotein (Gp) IIb/IIIa<sup>30</sup>.

Exhaustive exercise leads to activation of several 'stress hormones'. Epinephrine and vasopressin are the key regulators of the stress response. The magnitudes of responses are modulated by the relative intensities and duration of physical exercise. Epinephrine and vasopressin trigger the activation of endothelial cells, which may result in the release of ultra large vWF multimers (ULvWFM), which in turn induce platelet activation and thrombus formation under the state of high shear stress<sup>31</sup>.

The biological activity of the platelet activating ULvWFM is regulated by a specific plasma metallo-protease ADAMTS-13 (A Disintegrin and Metallo-protease with Thrombo Spondin-1 Repeats)<sup>32</sup>. Moreover, there is a compelling body of evidence that support the concept of a reciprocal behavior of the proteolytic activity of ADAMTS-13 and plasma vWF<sup>33</sup>.

A similar association could be shown for various (patho-) physiological conditions, including systemic inflammation following the endotoxin challenge and stimulation of endothelial vWF release by desmopressin<sup>34,35</sup>.

## CONCLUSION

The present study clearly demonstrated that physical stress in terms of exercise increases platelet aggregation in patients with IHD. The response to collagen challenge is more specific and predictable, while ADP induced platelet aggregation shows variable response. The exact mechanism of exercise induced platelet aggregability is not clearly understood. However, there is a complex mechanism involving von Willebrand Factor, P-selectin, glycoprotein receptors and a specific plasma metallo-protease ADAMTS-13.

## ACKNOWLEDGEMENT

Authors are highly thankful to the Chief Investigator, Indigenous Drug Research Centre, Department of Medicine, RNT Medical College, Udaipur, Rajasthan for providing facilities to conduct this platelet aggregation study.

**CONFLICT OF INTEREST:** None

**FINANCIAL SUPPORT:** None

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