Reduction in Platelet Aggregation (in vitro) by Diallyl Sulphide in Female Participants with Type 2 Diabetes Mellitus

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ABSTRACT

Diallyl sulphide an active principle of garlic has been shown to have many medicinal properties. Thus present study is undertaken to see the effect of diallyl sulphide on platelet aggregation in women participants with type 2 diabetes mellitus. Women participants (18) with type 2 diabetes mellitus with >2 years duration formed the study group. The body mass index of the study group was 26±3.4 kg m–2 and age was 47±8.1 years. Platelet aggregation induced by adenosine diphosphate at 10 and 20 μM concentration was measured in platelet rich plasma. The effect of diallyl sulphide (2.2 μg) on platelet aggregation was seen after 1 min of incubation with platelet rich plasma. The women participants were divided into two groups (fast responders for adenosine diphosphate induced aggregation >5.0% slow responders aggregation <5.0%) based on maximal aggregation percentage. The participants had high abdominal circumference (102±9.4) and were hypertensive. Glucose (162.5±47.18) and glycosylated hemoglobin levels (11.7±2.82) suggest lack of metabolic control. The triglycerides levels (185±58.53) were also high. The platelet aggregation with adenosine diphosphate showed variation (10 μM adenosine diphosphate 2.0-81.0% and 20 μM adenosine diphosphate 9.0-93.0%) in fast responders group. The reduction in platelet aggregation with diallyl sulphide also varied with the participants. The relative reduction of adenosine diphosphate induced aggregation with aspirin ranged from 1.0-65.0%. The fibrinogen levels were in normal range. There was reduction in adenosine induced platelet aggregation by diallyl sulphide. The relative reduction in platelet aggregation by diallyl sulphide as compared to aspirin was higher in four participants in fast responder participants. The data unequivocally demonstrated the platelet aggregation inhibition properties of diallyl sulphide.

Key words: Diallyl sulphide, platelet aggregation, type 2 diabetes mellitus, adenosine diphosphate, aspirin

INTRODUCTION

Diabetes mellitus (DM) is a clinical condition related to glucose metabolism affecting both sex and all age groups. The prevalence as well as morbidity and mortality due to diabetes is on the rise. The type 1 diabetes mellitus (DM 1) is marked by early onset whereas type 2 diabetes (DM 2) which is more prevalent has higher age onset. Several ethnic groups are particularly susceptible to DM 2: Hispanics, Native Americans and Asians especially who migrated to western world.
Epidemiological and pathological data documents diabetes as an independent risk factor for Cardio Vascular Diseases (CVD) in both men and women (Avogaro et al., 2007). Cardiovascular diseases are the cause of death in approximately 65% in participants with DM. Myocardial ischemia due to coronary atherosclerosis commonly occurs without symptoms in patients with diabetes (Buse et al., 2007). As a result, multivessel atherosclerosis often is present before any symptoms of CVD's are manifested. Thus delayed recognition of various forms of Coronary Heart Diseases (CHD) undoubtedly worsens the prognosis for survival for many participants with DM.

Abnormalities are observed in platelets in patients with atherosclerosis. Platelet aggregation has been shown as risk factor in the process of atherosclerosis (Hemingway et al., 2008). Platelet activation is characterized by shape change, induction of fibrinogen receptor expression, release of granular content of platelets etc., leading to aggregation and plaque formation. Fibrinogen, a glycoprotein is involved in the process of homeostasis and other functions. The glycoprotein metabolism is altered in participants with DM. The synthesis of fibrinogen and mucoprotein depends on glycemic control in the participants. Fibrinogen is now being considered as an independent risk factor for CVD's especially carotid atherosclerosis (Paramo et al., 2004).

The process of atherosclerosis may be hastened in some participants with prolonged hyperglycemia. This condition may lead to stroke and infarction in the blood vessels. Thus hyperglycemia may affect the process of platelet activation an initial step in the process of platelet aggregation (Matthew et al., 2009).

Diet plays an important role in the management of any disease. Excess calorie intake, high fat diet with restricted physical activity is some of the causes of obesity. Obesity is a well known risk factor for CVD's, DM and may other diseases. The adverse effects of obesity may hinder the management of DM as well as CVD's. The quality and quantity of fat with excess calories may lead to central obesity, which is known as an independent risk factor for CVD's and DM. Hypertriglyceridemia, hypercholesterolemia and low levels of HDL-cholesterol are some of the biochemical parameters that may result in imbalance in diet, genetic factors, environmental factors and changes in life style may contribute to the secondary complication of DM.

All nutrients play an important role in diabetes management. The major goals of therapy are to achieve metabolic sugar control and to prevent or delay the macro and micro vascular complications of diabetes. The non nutrient components in the diet can also be used as therapeutic agents in the management of chronic diseases including DM and carotid atherosclerosis (Shai et al., 2010). These non nutrient components of foods are also known as phytoneutrients. Garlic and its active ingredients namely ajoene, allicin and diallyl sulphide (DAS) are known for their antibacterial, hypolipidemic and antioxidant properties (Folasa and Krishnaswamry, 1997; Reinhart et al., 2009).

Fifty three relevant clinical trials were reviewed for efficacy of plants. This study showed significant decrease in total cholesterol and LDL cholesterol after treatment with garlic powder (Allicor), black tea, green tea etc., (Hasani-Ranjbar et al., 2010).

Garlic has over 300 sulphur containing compounds with varying biological properties (Nagini, 2008). Garlic preparation (Garlicin i.v. 60 mg day⁻¹) showed marked improvement in electrocardiogram and lowering of glucose level in participants with unstable angina pectoris. (Li et al., 2000). There are abnormalities in platelets in participants with DM (Vinik et al., 2001).

The prevalence of atherosclerosis is less in females as compared to males. However, the HDL-cholesterol levels are lower in females with DM 2. Female participants with DM were found to have more lipid abnormalities when compared to male participants with DM (Siegel et al., 1996).
In a study, the female participants with dislipidemia and DM 2 showed an increase in spontaneous platelet aggregation when compared to male participants (Menys et al., 1995). These are some of the factors for CVDS and developing secondary complication in fair sex with DM. The risk factors in females with DM are higher when compared to normal females and males with DM (Avogaro et al., 2007). Thus a study was under taken to assess the effect of DAS an active ingredient present in garlic on platelet aggregation induced by adenosine diphosphate (ADP) in female participants with diabetes mellitus.

MATERIALS AND METHODS
The study group: The study was carried out at National Institute of Nutrition, Hyderabad, India from August 2009 to June 2010. Eighteen female participants with DM2 who attended out patient clinics of Osmania general Hospital, Hyderabad, India, formed the study group. The participants in the study were recruited in the study as per the guidelines suggested by Al-Shahi and Warlow (2000).

Inclusion criteria: Adult Women participants aged between 30-60 years formed the study group. The participants were taken on the confirmation of DM 2 for more than 2 years and also on their willingness to participate in the study. Participants if on medication should be on drugs for controlling blood sugar and blood pressure only.

Exclusion criteria: Women with any other major ailments and participants with intake of garlic and garlic preparations and on drugs like anti-inflammatory, anti coagulants and other drugs affecting platelet aggregation and women who were using drugs prescribed by alternate medical professionals and adult women participants using health and nutritional supplements were excluded from the study.

The clinical examination like blood pressure was measured by calibrated sphygmomanometer in the sitting posture by competent personnel. Trained personnel measured anthropometric parameters, family history and other details.

Blood collection: A venous blood sample of 15 mL after 12 h of overnight fasting was drawn from cubital fossa and collected in different anti-coagulant tubes (1.0 mL in Fluoride tube for glucose, 3.0 mL in EDTA tube for lipid parameters 1.0 mL in serum clot activator for creatinine and 10.0 mL in 3.8% sodium citrate for platelet aggregation and fibrinogen estimations). The blood for lipid and glucose was transported on ice from hospital to the investigating unit.

Platelet aggregation assay and fibrinogen estimations were carried out within 3 h after sample collection. Diagnostic kits (Bio Systems, S.A. Costa Brava, 30 Barcelona Spain) were used for plasma glucose, triglycerides (TG), Total Cholesterol (TC) and HDL-cholesterol and serum creatinine The Very Low Density Lipoprotein+Low Density Lipoprotein (VLDL+LDL) values were computed from TG, TC and HDL-cholesterol by using Friedewald equation (Friedewald et al., 1972; Warnick et al., 1990).

1 mmol L\(^{-1}\) adenosine diphosphate solution: The 4.272 mg of adenosine diphosphate sodium salt (Sigma Chemical Co. A 2754) was weighed and dissolved in buffered (pH 6.8) saline was used in appropriate volumes to platelet rich plasma (PRP) to induce platelet aggregation.

DAS solution: Ten microliter of DAS (Concentration 0.89 g mL\(^{-1}\)) was dissolved in 10.0 mL of dimethyl sulphoxide.
**Acetyl salicylic acid:** Commercial preparation of acetyl salicylic acid was purchased and dissolved in 3.8% sodium citrate, appropriate volume was added to PRP to get 200 µg mL⁻¹ acetyl salicylic acid.

All the glassware used for platelet aggregation studies were coated with 'Sigmacote' for free flow of the blood and its components. PRP was prepared from citrated blood by centrifugation at 400 g for 10 min at room temperature. The platelet aggregation was studied using turbidometric procedure (Born and Cross, 1963). Platelets in PRP were counted using brilliant cresyl blue stain and the counts were adjusted with Platelet Poor Plasma (PPP). In platelet aggregation studies, measurement of aggregation before treatment is considered as control value (Bordia, 1978). ADP induced platelet aggregation was measured at 10 and 20 µM conc. in 0.5 mL of PRP against PPP on Chronolog Dual Aggregometer. The aggregation was measured for 3 min and expressed as maximum aggregation percentage and relative reduction was calculated as follows:

\[
\%\text{Aggregation with ADP} = \frac{\%\text{Aggregation with ADP and DA5} - \%\text{Aggregation with ADP}}{\%\text{Aggregation with ADP}} \times 100
\]

To 0.5 mL of PRP 2.2 µg of Diallyl sulphide was added and incubated for 1 min, for the reaction after which aggregation was induced by ADP at different levels. Acetyl salicylic acid (200 µg) i.e., aspirin was incubated in PRP for 5 min to study the inhibition of ADP induced platelet aggregation as positive control in the study. There was no spontaneous aggregation induced by these two compounds during the incubation period. The time of incubation was standardized for both the compounds. The platelet aggregation by ADP is receptor (G1-coupled ADP receptor) mediated hence the participants can be classified into two groups. The groups being fast responder group (ADP induced aggregation ≥5%) and slow responder group (ADP induced aggregation ≤5%) of diabetic participants (Fontana et al., 2003). Fibrinogen was measured in the PPP fraction by spectrophotometric method (Ratinoff and Menzie, 1951).

**Statistical analysis:** Statistical Package for Social Science (SPSS) windows version 14.5 was used for statistical analysis. The clinical chemistry parameters were expressed as Mean±SD. The t-test was used for equality of means for the two groups and Levene’s test was used for equal variances.

**RESULTS**

The age of the female participants with diabetes mellitus ranged from 35 to 60 years, suggesting that all the participants are adults. The mean body mass index of the study group is

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Fast responders (6)</th>
<th>Slow responders (12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>50±17.7</td>
<td>45±8.2</td>
</tr>
<tr>
<td>Body mass index (kg m⁻²)</td>
<td>27±4.1</td>
<td>25±3.9</td>
</tr>
<tr>
<td>Abdominal girth (cm)</td>
<td>106±9.3</td>
<td>98±8.9 (11)</td>
</tr>
<tr>
<td>Blood pressure (mm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>143±15.1</td>
<td>143±16.4 (11)</td>
</tr>
<tr>
<td>Diastolic</td>
<td>91±11.2</td>
<td>89±13.5 (11)</td>
</tr>
<tr>
<td>Pulse rate</td>
<td>73±10.3</td>
<td>72±9.6</td>
</tr>
</tbody>
</table>

All values are Mean±SD, values in parenthesis indicates number of participants
Table 2: Clinical chemistry of study groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Fast responders (6)</th>
<th>Slow responders (12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg dL⁻¹)</td>
<td>139.1±47.18</td>
<td>174.3±50.14</td>
</tr>
<tr>
<td>HbA1C (%)</td>
<td>12.8±1.22</td>
<td>10.6±3.39 (9)</td>
</tr>
<tr>
<td>Triglycerides (mg dL⁻¹)</td>
<td>226.3±46.66</td>
<td>165.2±54.33</td>
</tr>
<tr>
<td>Cholesterol (mg dL⁻¹)</td>
<td>228.8±47.34</td>
<td>214.0±66.33</td>
</tr>
<tr>
<td>HDL-Cholesterol (mg dL⁻¹)</td>
<td>38.7±4.30</td>
<td>37.2±9.64</td>
</tr>
<tr>
<td>VLDL+LDL (mg dL⁻¹)</td>
<td>144.8±53.39</td>
<td>143.2±73.60</td>
</tr>
<tr>
<td>Creatinine (mg dL⁻¹)</td>
<td>1.4±0.51</td>
<td>1.6±0.33 (11)</td>
</tr>
</tbody>
</table>

All values are Means±SD. Values in parenthesis indicate number of participants. *p<0.01 compared to slow responders

Table 3: Platelet aggregation (%) in type 2 diabetic participants

<table>
<thead>
<tr>
<th>Participant No.</th>
<th>ADP (10 μM)</th>
<th>ADP (10 μM) + DAS</th>
<th>ADP (20 μM)</th>
<th>ADP (20 μM) + DAS</th>
<th>ADP (20 μM) + Aspirin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>NE</td>
<td>9</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>81</td>
<td>68</td>
<td>88</td>
<td>73</td>
<td>76</td>
</tr>
<tr>
<td>3</td>
<td>84</td>
<td>76</td>
<td>93</td>
<td>72</td>
<td>92</td>
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<tr>
<td>4</td>
<td>12</td>
<td>7</td>
<td>26</td>
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<td>26</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>NE</td>
<td>18</td>
<td>8</td>
<td>7</td>
</tr>
</tbody>
</table>

NE: Not estimated as platelet aggregation in ADP (10 μM) is less than 5%

Fig. 1: Relative reduction (%) in platelet aggregation by DAS and aspirin

25.3±3.4 kg m⁻². Five of the eighteen participants had body mass index <24.0 kg m⁻². The abdominal circumference (102±49.4 cm) is also on the higher side in all the participants, the normal cut off suggested is 80 cm in Asian women. There are no significant differences in body mass index, age, abdominal circumference and blood pressure between the fast responders and slow responders (Table 1).

The glycosylated hemoglobin in all the participants is 12.8% suggesting that their long term blood sugar levels are not in the normal (<7.0%) range (Table 2). The lipid parameters, total cholesterol and HDL-cholesterol were normal in both the groups. While the triglyceride levels were significantly higher in participants whose platelet responded to ADP induced aggregation.

The ADP induced platelet aggregation was studied at 10 and 20 μM conc. of ADP in PRP. (Table 3). The percentage of aggregation at 10 μM ranged between 7.0-76.0%, with a relative reduction of 9.0-42.0% with DAS. The aggregation percentage with 20 μM ADP in six participants
was higher when compared to 10 μM ADP. In one subject DAS completely inhibited the process of platelet aggregation. The Relative Reduction (RR) ranged from 0-73.0% with DAS. Aspirin (200 μg mL\(^{-1}\)) was used as positive control to test the inhibition of platelet aggregation. The relative reduction with aspirin ranged between 1.0-85%. In four participants the relative reduction with DAS was more than aspirin while in one subject the RR was less than aspirin. However in one subject there was no difference in RR between two compounds (Fig. 1). The Fibrinogen levels in these participants (responders) ranged between 207-377 mg dL\(^{-1}\). These fibrinogen levels were in the normal range (150-400) of these hemostatic parameters.

DISCUSSION

Diabetes is a clinical condition with altered carbohydrate metabolism. The incidence of this abnormality is on rise, may be due to altered life style leading to changes in the physiological activity in the cells. Optimal maintenance of the glucose levels (random blood sugar <150 mg dL\(^{-1}\)) in the body could be the main strategy which would also prevent the secondary complications attributed in participants with diabetes mellitus. The Disability Adjusted Loss Years (DALYS) is on rise with increase in incidence and the associated clinical conditions (Waugh et al., 2007). Interventions are needed for better quality of life in the population.

The age of the participants suggests that diabetes mellitus could also be due to altered metabolism by life style changes vis-à-vis expression of genes. The body mass index of the participants indicates that, the caloric intake is higher than the energy expenditure. In a study from the same region, an association between hypertension and the BMI especially in women was observed by Vijaylakshmi et al. (1999). The abdominal circumference has been used as a surrogate indicator of obesity in a specified group of the population (Vir and Love, 1980). Present results suggest that the participants with diabetic mellitus are obese based on BMI and abdominal circumference. The blood pressure measurements both systolic and diastolic are elevated in the study group. These parameters and elevated glucose levels are at risk for the development of secondary complications including cardiovascular diseases.

The triglyceride levels in these women are higher than the normal levels (<150 mg dL\(^{-1}\)), which is supported by the BMI data. The higher levels of the parameters could be attributed to the dietary habits in the region. These participants may belong to the group termed as ‘common soil’, wherein the levels of triglyceride and cholesterol are high in families for generations (Stern, 1995). Hence in take of garlic with its anti-lipidemic properties could be suggested for prevention of secondary complications.

Fibrinogen, a glycoprotein is involved in the homeostasis and other functions. The glycoprotein metabolism is altered in diabetic participants. The synthesis of fibrinogen and mucoproteins depends on glycemic control. Fibrinogen is now being considered as an independent risk factor for clinical cardiovascular disease especially for carotid atherosclerosis (Paramo et al., 2004). The fibrinogen levels of 331 mg dL\(^{-1}\) or above have odds ratio of 7.45 in an angiographically defined CAD participants from three hospitals from the same region (Gheyie et al., 1999). The results of fibrinogen in the present study indicate that two participants have values >331 mg dL\(^{-1}\) suggesting the risk of coronary artery disease.

Garlic displays strong antioxidant properties and activates Nitric Oxide Synthase (NOS), leading to an increase in platelet-derived NO. It can also interact directly with the GPIIb/IIIa receptors, thus reducing the ability of platelets to bind to fibrinogen. In vitro studies stated that garlic inhibits platelet aggregation by suppressing mobilization of intraplatelet Ca\(^{2+}\) and by
increasing levels of cAMP and cGMP. It is concluded that garlic may have a role in preventing cardiovascular disease (Rahman, 2007).

The phytonutrients present in plant foods are used in traditional medical practice to treat many ailments including diabetes. The herbs, spices and condiments are also well known for their health promoting effects. The phytonutrients and other compounds present in them are supposed to confer these beneficial effects (e.g., Digoxin) and are being exploited presently in the development of new chemical compounds for use as nutraceuticals and for food fortification.

Garlic and its active ingredients namely ajoene, allicin and diallyl sulphide are well known for their antibacterial, hypolipidemic and antioxidant properties. Garlic has over 300 sulphur containing compounds with varying biological properties. Garlic has been documented to contain antimitogenic and anticarcinogenic properties (Polasa and Krishnaswamy, 1997). Platelet aggregation has been shown as risk factor in the process of atherosclerosis. Platelet aggregation is characterized by shape change, induction of fibrinogen receptor expression and release of granular contents leading to aggregation and plaque formation. Hyperglycemia may affect the process of platelet aggregation. Many agents are known to induce platelet aggregation and in this study ADP was studied at two levels. A study showed that ADP receptor P2Y12 plays a pivotal role in platelet aggregation. There appears to be two phenotypic groups of participants with high and low response to ADP induced platelet aggregation (Fontana et al., 2003). The two haplotypes are designated as H1 and H2 and the H2 is associated with increased platelet aggregation in response to ADP. Micro aggregates were formed in diabetic participants by inducing stress (without any agonist) via P2Y12 receptors (Matsune et al., 2005) suggesting abnormalities in the platelet function. On the platelet membrane, the event that triggers exposure of fibrinogen binding site is not clear. In a recent study investigators have identified five single nucleotide polymorphisms were identified in the receptors for thromboxane A2 in healthy participants (Fontana et al., 2006). Thromboxane molecule is an important molecule in the process of platelet activation a step prior to the process of aggregation.

There are studies to show the abnormalities in platelet function in diabetes mellitus. The abnormalities in platelet function may occur due to clinical conditions and other acquired disorders (George and Shattil, 1991). Tomato juice has been shown to reduce platelet aggregation in type 2 diabetic participants (Lazarus et al., 2004). The results of this study indicated that DAS effectively inhibited the platelet aggregation induced in vitro by ADP in blood samples of some participants with diabetes mellitus. There are three main antiplatelet constituents identified in garlic namely adenosine, allicin and polysulfides. Adenosine and allicin both inhibited platelet aggregation without affecting cyclo-oxygenase and lipo- oxygenase metabolites of arachidonic acid. The polysulfides inhibited platelet aggregation as well as thromboxane synthesis (Banerjee and Maulik, 2002). In blood samples of some diabetic participants platelet aggregation could not be achieved by ADP and may be considered as slow responders group. However, the clinical chemistry was similar in both the groups. The reasons for the slow response by some individuals are beyond the scope of this study.

The current observation is on the anti atherogenic property of DAS. Platelet aggregation is shown to be reduced by some sulphur containing drugs (Clpidogrel). Diallyl sulphide may share the same mechanism of action of inhibiting platelet aggregation with sulphones, clpidogrel and other sulphur containing compounds. The data unequivocally demonstrated the platelet aggregation inhibition properties of DAS and can be compared to that of aspirin a well-known anti-inflammatory agent (Martha et al., 2010). Most of these agents act by inhibition of release of arachidonic acid from membrane by the action of phospholipase A2 and also by inhibition of
cyclo-oxygenase enzyme (Saeed et al., 2004). Some of the anti-inflammatory drugs and analgesics causes irritation of gastric mucus and are therefore given along with antacids or anti H2 receptors. In this context, use of naturally occurring substances in the diet can be alternative with minimal side effects. The inhibitory potential varied between the individuals suggesting there may be genetic variation in the responses.

Use of indigenous products or alternate medicines are becoming vogue as they are less expensive, easily available and have less adverse effects. Regular consumption of spices like garlic by diabetics may confer other beneficial effects than merely platelet aggregation. Garlic is well known anti lipedemic, antioxidant (Nouri et al., 2008; Sukandar et al., 2010) and anti bacterial agent (Jazani et al., 2007). Oxidative stress is also likely to be high in diabetics with altered glucose metabolism. Garlic by virtue of its antioxidant properties will reduce the oxidative stress. Fungal and bacterial infections particularly food infections and urinary tract infections are common due to elevated glucose levels and glucosuria.

Since garlic is an integral part of Indian diet, it is quite easy to advocate its regular consumption through diet. However in depth studies are needed to be undertaken under clinical conditions to demonstrate these effects in order to indicate the observation of this study.

CONCLUSION

Garlic has significant potential for preventing cardiomyopathy. DAS has also been shown to prevent excessive platelet adhesion in humans. This study points out to the long term benefits of DAS, an important constituent of garlic to prevent and possibly slow the progression of atherosclerosis in people at risk.

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