In vitro effects of fermented papaya (*Carica papaya*, L.) on platelets obtained from patients with type 2 diabetes


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**Abstract**
Background and aim: Oxidative stress is associated with insulin resistance pathogenesis, insulin secretion deficiency, and complication onset. Fermented papaya preparation (FPP), a dietary supplement obtained by fermentation of the papaya fruit, may be used as an antioxidant in the prevention of diabetic complications.

Methods and results: Platelets from 30 patients with type 2 diabetes mellitus (DM 2) and 15 healthy subjects were analyzed to evaluate the in vitro effects of FPP incubation. Na\(^+\)/K\(^+\)-adenosine triphosphatase (ATPase) activity, membrane fluidity, total antioxidant capacity (TAC), superoxide dismutase (SOD) activity, and conjugated diene levels were determined. In vitro FPP incubation improved platelet function, by enhancing Na\(^+\)/K\(^+\)-ATPase activity and membrane fluidity, and ameliorated the antioxidant system functionality, through an increase in TAC and SOD activity and a parallel decrease in conjugated diene levels in patients with DM 2.

Conclusion: Our data suggest that the incubation with FPP may have a protective effect on platelets from patients with DM 2, by preventing the progression of oxidative damage associated with diabetes and its complications.

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**Introduction**

Type 2 diabetes mellitus (DM 2) is caused by a combination of insulin resistance and a relative deficit of insulin production (American Diabetes Association – ADA 2008). The diabetic onset is preceded by chronic hyperglycemia, which increases the mitochondrial production of oxygen and nitrogen reactive species. In physiological conditions, the production of these molecules is balanced by the cellular antioxidant pools; when this balance is disrupted, a condition of oxidative stress is established [1].

Moreover, the pancreatic β cells have a reduced antioxidant activity, so they are more exposed to oxidative damages [2].

Many studies have demonstrated that oxidative stress is associated with the pathogenesis of insulin resistance, insulin secretion progressive deficiency, and chronic complication onset, and may cause these effects either directly or indirectly, acting as a second messenger able to stimulate stress-inducible cellular pathways [3].

The strong socioeconomic impact of DM 2 justifies the interests of scientific research towards new molecules capable of influencing the various pathogenic mechanisms of the disease, considering that lifestyle intervention is essential.

To date, numerous studies have been directed to the use of antioxidants in the prevention of diabetic complications,
and several researchers have demonstrated the effectiveness of supplementation with vitamin E and α-lipoic acid [4–6].

More recently, there has been an interest in fermented papaya preparation (FPP), a dietary supplement obtained by a natural fermentation process of the papaya fruit. The fermented papaya is obtained from ripe but still green fruit, and it is prepared in order to have the greatest amount of enzymes and all the active ingredients of the plant, among which vitamins C and E and carotenoids are the most important for their antioxidant properties.

In the literature, there are many studies on the effectiveness of FPP supplementation in different pathologies, including diabetes mellitus; in particular, it has been demonstrated that this preparation is able to reduce both basal and postprandial glycemia; that it can improve the lipid profile and the wound healing; and that it ameliorates the redox balance of erythrocytes, platelets, and polymorphonuclear cells by reducing reactive oxygen species (ROS) levels and increasing the concentration of reduced glutathione [7–10]. Moreover, it has been shown that FPP supplementation can reduce the redox damage and increase the expression of genes encoding the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase in a healthy elderly population [11,12].

Based on the evidence reported so far, the aim of this study was to evaluate the in vitro effects of fermented papaya incubation on platelets obtained from human subjects with DM 2 and healthy controls, in order to provide its use as a daily adjuvant therapy. In particular, before (T₀) and after (T₁) such incubation, the following parameters were tested: Na⁺/K⁺-adenosine triphosphatase (ATPase) activity, membrane fluidity by means of anisotropy of the fluorescent probes 1-6-phenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), SOD activity, total antioxidant capacity (TAC), and conjugated diene levels.

**Methods**

**Study population**

The study was performed on 30 patients, 16 women and 14 men (mean age: 57.03 ± 6.65 years), consecutively admitted to the Department of Endocrinology and Metabolic Diseases of Ospedali Riuniti in Ancona between February and June 2013. The average duration of disease (i.e., the time between the diagnosis and the day on which the blood sample was taken) was 11 ± 5 years. The mean value of HbA₁c was 59 ± 2.1 mmol/mol (a.v.: 20–42 mmol/mol, according to the International Federation of Clinical Chemistry and Laboratory Medicine — IFCC).

All patients have taken oral hypoglycemic agents (insulin sensitizers) and bedtime insulin glargine. The daily average amount of glargine was 25 ± 5 UI.

The control group consisted of 15 healthy subjects, matched for age and sex to patients with DM 2.

At the time of recruitment, only three patients have had microalbuminuria, while the others have had neither signs of chronic micro- and macroangiopathic complications nor hypoglycemic episodes. Moreover, nobody has had cardiovascular and cerebrovascular diseases, chronic renal failure, chronic liver disease, cancer, or a secondary form of diabetes, and none has taken immunosuppressive agents. Everybody took antihypertensive drugs; 20 subjects showed control on their blood pressure, taking only one drug (ACE inhibitors or angiotensin receptor blockers (ARBs)), and seven patients by using a combination of two drugs (ACE inhibitors and thiazide diuretics, and ARBs and thiazide diuretics); finally, three subjects did not have adequate control on their blood pressure despite treatment. All patients and control subjects were nonsmokers and nonalcoholics.

The study was performed in accordance with the principles contained in the Declaration of Helsinki as revised in 2001, and it was approved by the Bioethical Committee of Università Politecnica delle Marche. Written informed consent was provided by all subjects enrolled in the study.

**Sample collection**

Peripheral venous blood was drawn after overnight fasting, and immediately mixed with anticoagulant citrate dextrose (ACD) (36 ml citric acid, 5 mM KCl, 90 mM NaCl, 5 mM glucose, 10 mM ethylenediaminetetraacetic acid (EDTA), pH 6.8).

**Platelet isolation and FPP incubation**

Platelets were isolated by differential centrifugation in anti-aggregation buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 5 mM glucose, pH 7.4) [13]. Each platelet sample was divided into two aliquots, one of which remained as it was, while the other was incubated with FPP to a final concentration of 50 μg/ml for 3 h at 37 °C. FFP, a product of yeast fermentation of Carica papaya, L. was supplied by Osato Research Institute, Gifu, Japan. FPP was dissolved in sterilized and filtered (<0.22 μm) phosphate buffer saline (PBS). Its final concentration was chosen based on previous studies by Aruoma et al. [14] Platelets were thus analyzed at recruitment (T₀) and after in vitro FPP incubation (T₁).

**Na⁺/K⁺-ATPase activity**

Na⁺/K⁺-ATPase activity was determined in platelets according to the Kitao and Hattori method, and expressed as μmol Pi/mg prot/h [15,16]. The protein concentration was determined with the Bradford BioRad protein assay [17].

**Membrane fluidity**

Platelet plasma membrane fluidity was studied by determining the fluorescence anisotropy (reciprocal of fluidity) of probes TMA-DPH, which is incorporated at the lipid–water interface of the membrane bilayer, and DPH, which is a totally hydrophobic fluorescent probe, as previously described [18,19].
Steady-state fluorescence anisotropy \( r \) of TMA-DPH and DPH was calculated using the following equation:

\[
r = \left( I_v G - I_h \right) / \left( I_v + 2I_h \right)
\]

where \( G \) is the instrumental factor that corrects the \( r \) value for an unequal detection of vertically \( (I_v) \) and horizontally \( (I_h) \) polarized light. A decrease in the \( r \) value indicates a higher mobility of probe, that is, an increased membrane fluidity.

**SOD activity**

The SOD activity was evaluated using the colorimetric-based Superoxide Dismutase Activity Assay (Assay Designs, Stressgen, Ann Arbor, MI, USA), following the manufacturer’s instructions. Superoxide anion, generated from the conversion of xanthine and oxygen to uric acid and hydrogen peroxide by xanthine oxidase, converts WST-1 (water-soluble tetrazolium salts) to WST-1 formazan, a colored purple product that absorbs light at 450 nm. SOD reduces the superoxide ion concentration and thereby lowers the rate of WST-1 formazan formation. Therefore, SOD activity is determined by the percent inhibition of the rate of WST-1 formazan production and expressed in units/\( \mu \)L.

**Total antioxidant capacity**

TAC was measured in platelets with the Total Antioxidant Capacity Assay Kit (Biovision Inc., Milpitas, CA, USA). Cu\(^{2+}\) ions reduced by the antioxidants present in the sample are chelated with a colorimetric probe giving a broad absorbance peak at around 570 nm, proportional to the TAC.

The results were expressed as nmol/\( \mu \)L or mM Trolox equivalents.

**Determination of conjugated dienes**

Conjugated dienes were determined by monitoring the increase in absorbance at 234 nm, in a single-point assay, as previously described [20].

**Statistical analysis**

Statistical analysis was performed using SAS statistical package (Statistical Analysis System Institute, Cary, NC, USA). All experiments were carried out in triplicate, and data were expressed as means ± standard deviation (SD). Student’s t-test was used to analyze the differences between groups. Correlation studies were performed by linear regression analysis, using Pearson’s correlation coefficient \( \rho \). The significance level was set at \( p < 0.05 \).

**Results**

In the present study, we analyzed the platelets isolated from 30 patients with DM 2 and 15 healthy controls, age- and sex-matched. With respect to the clinical parameters, there were statistically significant differences between the two groups for fasting glucose, body mass index (BMI), plasma creatinine, malondialdehyde, and total antioxidant status (Table 1).

As regards Na\(^+\)/K\(^+\)-ATPase activity, incubation with FPP provoked a significant increase in both patients with DM 2 and controls at \( T_1 \) compared to \( T_0 \) (Fig. 1, \( p < 0.05 \)). Furthermore, at \( T_1 \), the enzyme activity was still significantly lower in patients compared to controls \( (p < 0.05) \), while at \( T_0 \) there was no significant difference between the two groups.

The fluorescence anisotropy of TMA-DPH showed a statistically significant decrease in both patients and controls at \( T_1 \) compared to \( T_0 \) (Fig. 2, upper panel, \( p < 0.05 \)), thus indicating an increase in outer membrane layer fluidity after FPP incubation. In addition, at both \( T_1 \) and \( T_0 \),

| Table 1 Clinical parameters of patients with DM 2 and controls. Values are expressed as mean ± SD. |
|---------------------------------|------------------|------------------|
| Number                          | Patients with DM 2 | Controls         |
| Age (years)                     | 57.05 ± 6.65      | 57.50 ± 6.48     |
| Sex (F/M)                       | 16/14             | 7/8              |
| Disease duration (years)        | 11 ± 5            |                  |
| Fasting glucose (mg/dl)         | 146 ± 8.50        | 84 ± 3.80        |
| HbA1C (mmol/mol)                | 59 ± 2.10         |                  |
| Body mass index (BMI-kg/m²)     | 29.16 ± 4.43      | 24.00 ± 3.20     |
| Plasma creatinine (mg/dl)       | 1.13 ± 0.12       | 0.91 ± 0.08      |
| Total cholesterol (mg/dl)       | 181 ± 15          | 184 ± 17         |
| LDL cholesterol (mg/dl)         | 104 ± 9           | 99 ± 8           |
| HDL cholesterol (mg/dl)         | 46 ± 4            | 49 ± 3           |
| Triglycerides (mg/dl)           | 154 ± 13          | 129 ± 11         |
| Malondialdehyde (µM)            | 3.58 ± 0.61       | 1.95 ± 0.53      |
| Total antioxidant status (mM)   | 0.58 ± 0.11       | 1.72 ± 0.83      |

\( ^a p < 0.05. \)

Figure 1 Na\(^+\)/K\(^+\)-ATPase activity in platelets of patients (diabetes) and controls, before \( (T_0) \) and after \( (T_1) \) FPP incubation. Values are expressed as mean ± SD. \(^* p < 0.05. \)
The anisotropy of TMA-DPH was significantly higher in patients with DM 2 compared to controls \((p < 0.05)\), showing a reduced fluidity of diabetic platelet membranes.

Similarly, the anisotropy of DPH showed a statistically significant decrease, in DM 2 subjects and controls at \(T_1\) compared to \(T_0\) (Fig. 2, lower panel, \(p < 0.05\)), indicating an increased membrane fluidity in the inner part of the platelet membranes after incubation with FPP. Moreover, at both \(T_1\) and \(T_0\), the DPH anisotropy was significantly lower in patients compared to controls \((p < 0.05)\), thus demonstrating a higher fluidity of this part of diabetic platelet membranes.

With respect to the SOD activity, a statistically significant increase at \(T_1\) compared with \(T_0\) in both patients and controls was observed (Fig. 3, upper panel, \(p < 0.05\)).

Figure 2 Anisotropy of the fluorescent probes TMA-DPH (upper panel) and DPH (lower panel) in platelets of patients (diabetes) and controls, before \((T_0)\) and after \((T_1)\) FPP incubation. Values are expressed as mean ± SD. \(*p < 0.05\).

Figure 3 Superoxide dismutase (SOD) activity (upper panel), total antioxidant capacity (TAC) (middle panel), and conjugated diene levels (lower panel) in platelets of patients (diabetes) and controls, before \((T_0)\) and after \((T_1)\) FPP incubation. Values are expressed as mean ± SD. \(*p < 0.05\).
Furthermore, this enzymatic activity was significantly higher in patients compared to controls, both at T₀ and T₁ (p < 0.05).

Parallel, TAC showed a statistically significant increase in both DM 2 subjects and controls, following incubation with FPP (Fig. 3, middle panel, p < 0.05). In addition, a significantly higher TAC was observed in patients compared to controls at T₀ as well as at T₁ (p < 0.05).

Finally, platelet peroxidation, measured by means of conjugated diene detection, showed a statistically significant decrease at T₁ compared to T₀ in both patients and controls (Fig. 3, lower panel, p < 0.05). Furthermore, significantly higher levels were found in patients compared to controls at T₀ as well as at T₁ (p < 0.05).

With respect to the correlation studies, a significant negative correlation between Na⁺/K⁺-ATPase activity and both TMA-DPH (T₀ ρ = −0.955, T₁ ρ = −0.943, p < 0.05) and DPH anisotropy (T₀ ρ = −0.943, T₁ ρ = −0.950, p < 0.05) (i.e., a positive correlation with platelet membrane fluidity) was demonstrated. Moreover, Na⁺/K⁺-ATPase activity showed a significant positive correlation with SOD activity (T₀ ρ = 0.953, T₁ ρ = 0.888, p < 0.05), as well as with TAC (T₀ ρ = 0.901, T₁ ρ = 0.897, p < 0.05).

SOD activity and TAC were also negatively correlated with both TMA-DPH (correlation with SOD: T₀ ρ = −0.958, T₁ ρ = −0.954; with TAC: T₀ ρ = −0.859, T₁ ρ = −0.926, p < 0.05) and DPH anisotropy (correlation with SOD: T₀ ρ = −0.898, T₁ ρ = −0.886; with TAC: T₀ ρ = −0.918, T₁ ρ = −0.926, p < 0.05) (i.e., they were positively related with sperm cell membrane fluidity).

Finally, significant positive correlations were found between conjugated diene levels and both TMA-DPH (T₀ ρ = 0.910, T₁ ρ = 0.968, p < 0.05) and DPH (T₀ ρ = 0.888, T₁ ρ = 0.884, p < 0.05) anisotropy (reciprocal of membrane fluidity), while conjugated diene content was negatively related with Na⁺/K⁺-ATPase (T₀ ρ = −0.954, T₁ ρ = −0.881, p < 0.05) and SOD (T₀ ρ = −0.958, T₁ ρ = −0.949, p < 0.05) activity, as well as with TAC (T₀ ρ = −0.879, T₁ ρ = −0.932, p < 0.05).

Discussion

Diabetes is characterized by a slow and progressive evolution, whose outcome is poor in the absence of suitable therapies, because of the high risk of vascular complications inducing an increased mortality risk. The appropriate therapeutic interventions, as well as educating the patient to observe a proper diet, have significantly improved the prognosis; however, in many cases, the current diabetic treatment does not allow achieving and maintaining a glycometabolic compensation to avoid complication onset.

Studies about diabetes onset, progression, and secondary complications are increasingly focused on the role of oxidative stress, although the specific mechanisms have not yet been clarified [21]. In diabetes, characterized by chronic hyperglycemia, the glucose autoxidation leads to an excessive production of ROS; moreover, because of the reduced antioxidant activity of pancreatic β cells, the oxidative stress may contribute to insulin resistance and deficient insulin secretion onset, as well as to appearance of complications [22, 22].

Chronic hyperglycemia also affects platelet functionality, by activating protein kinase C and by reducing the membrane fluidity, which is essential for the hemostatic process [3]. Furthermore, as insulin inhibits platelet activation, its deficiency, absolute or relative, appears to be an additional pathogenic factor, responsible for the increased platelet adhesiveness and aggregation typical of diabetes, and promoting the formation of atherosclerotic plaques [14].

The use of dietary antioxidants and nutriceuticals is one of the strategies aimed at reducing the oxidative stress for the prevention of diabetes and its chronic complications [23].

FPP is an excellent nutriceutical, functional food, or a supplement, which acts in both nutritional and physiological terms. Its multiple beneficial effects are due to the high amount of antioxidants, the high content of simple carbohydrates that provide energy charge, and the presence of proteolytic enzymes, including papain that helps the digestion, and it is the wealthiest of minerals [24]. FPP is also able to modulate the inflammation and to improve the immune function, thus supporting the idea that it may have potential benefits in the management of chronic diseases mediated by a pro-inflammatory state. Nevertheless, in the literature, there are some studies that do not confirm the antioxidant capacity of the papaya [25], but this could depend on species differences or on different methods used for the papaya crop.

Our data concerning Na⁺/K⁺-ATPase activity showed a significant increase after incubation with FPP, in both diabetics and controls, probably due to an ion transport rebalancing dependent on the intrinsic properties of papaya.

Several studies have shown a decreased platelet Na⁺/K⁺-ATPase activity in diabetes, which probably depends on a high nonenzymatic glycation of NH₂ residues and/or on an increased ROS production, which alters the enzyme structure [26, 27].

Na⁺/K⁺-ATPase has a key role in regulating cellular homeostasis, and it is also a marker of membrane functionality, as its activity depends on the physicochemical properties of the microenvironment where it is embedded [28].

With respect to platelet membrane fluidity, our study showed a significant increase after FPP incubation in patients with DM 2 and controls. Specifically, in patients with DM 2, a decreased fluidity in the outer layer was found with respect to the controls, both before and after incubation; this could be explained by a higher exposition of the external surface to the increased extent of glycosylation and the altered plasma lipoprotein profile, characteristic of diabetes [26]. Instead, with regard to the inner part of the membrane, an enhanced fluidity was observed before and after incubation with FPP, in patients with DM 2 compared to controls; this may probably be due to a rearrangement of the plasma membrane to counteract the decreased fluidity of the external layer.
Our correlation studies demonstrated a significant negative correlation between the Na⁺/K⁺-ATPase and both TMA-DPH and DPH anisotropies, and thus a positive correlation with platelet membrane fluidity. Therefore, the in vitro incubation with FPP seems to improve the platelet function, thus delaying the complication onset.

Moreover, our results showed that incubation with FPP induces a significant decrease in oxidative stress in platelets from patients with DM 2 compared to controls; specifically, an increase in SOD activity and TAC as well as a decrease in conjugated diene levels, which are the early products of lipid peroxidation, were observed, in agreement with previous studies [8,9,12,29].

These data could be explained as defense mechanisms that are established in the diabetic patient to counteract the peroxidation. Indeed, our results may also suggest that control platelets take advantage of FPP incubation, by opposing oxidative stress.

Our correlation studies confirmed these results, showing significant positive correlations between Na⁺/K⁺-ATPase activity and both SOD activity and TAC, as well as between TMA-DPH and DPH anisotropy and conjugated dienes levels. On the contrary, SOD activity and TAC were negatively correlated with TMA-DPH and DPH anisotropy, as well as with conjugated diene content, which is also negatively related with Na⁺/K⁺-ATPase activity.

In conclusion, it could be hypothesized that FPP is able to contrast the oxidative damage associated with diabetes and its complications; on the one hand by improving platelet function through an increase in membrane fluidity and Na⁺/K⁺-ATPase activity, and on the other by enhancing the antioxidant system functionality and thus decreasing the conjugated diene levels. This results in a reduction of platelet hyperactivity and hyperaggregability, and suggests a new approach for the prevention of diabetes complications associated with platelet dysfunction.

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