The Effect of Ellagic Acid on Coagulation in Vivo

By Antonio Girolami, Domenico Agostino and Eugene E. Cliffton

THROMBOSIS and embolism remain a most important factor in morbidity and mortality, particularly of hospitalized patients, despite all forms of treatment. A tendency to increased thrombosis has been reported in patients with cancer, myocardial infarction, atherosclerosis, pregnancy and puerperium, in postoperative or posttraumatic states, and in patients receiving ACTH and cortisone.

Tests for the tendency to thrombosis have been reported but apparently have not been of much value, since none are in general use. Investigations of the hypercoagulable state and increased tendency to thrombosis have involved studies of the effect of serum lipids and soaps, thrombin and thromboplastic substances, toxins and some chemicals, including celite and tannic acid derivatives. All substances which increase thrombosis also tend to cause hypercoagulability with secondary alterations in fibrinogen and other coagulation factors.

Ellagic acid has recently been reported to have an effect in vivo. It was reported to cause a shortening of the glass and silicone clotting time, a decrease in serum prothrombin activity (with increased prothrombin consumption), a shortening of partial thromboplastin time, and an acceleration of the thromboplastin generation test. It has been suggested that ellagic acid induces hypercoagulability in vivo by activation of the Hageman factor.

The present investigation was undertaken to evaluate the effect of ellagic acid in the dog and in other species of animals.

Materials and Methods

Ellagic acid (4.4', 5.5', 6.6'-hexahydroxydiphenic acid 2.6:2.6' dilactone) as supplied by K & K Lab., Jamaica, N. Y., is a dark green, odorless powder.

The ellagic acid was dissolved in 0.025 M sodium barbital buffer in 0.125 M sodium chloride at pH 7.5. It was prepared in a solution with a concentration of 10^-4 M. Ten ml./Kg. of body weight were infused directly into an exposed femoral vein in 5 minutes in each animal. The buffer alone was injected in control animals.

Animals

Dogs: 12 mongrel males or nonpregnant females weighing from 12.5 to 17 Kg.

Rats: 96 females (Carworth Farms) weighing 200-220 Gm. were used.
Cats: 12 males or nonpregnant females weighing 2.5 to 5 Kg. each (Long Trail Farms).
Rabbits: 13 females weighing 2.5 to 4 Kg. (Rockland Farms).

Anesthesia
Sodium pentobarbital (Philadelphia Lab., Inc., Phila., Pa.) 65 mg./ml.
Dogs, cats and rabbits: Each animal received 24 mg./Kg. by intravenous injection.
Rats: Each animal received 15 mg. by intraperitoneal injection.

Procedure
Dogs: After anesthesia was established, both femoral veins were exposed surgically. One vein was used for the injection of the ellagic acid or buffer, and the other was used for withdrawal of blood for tests. Nine animals were injected with ellagic acid, and three animals (controls) were injected with buffer. All animals were followed for a minimum of 4 hours and a maximum of 24 hours.
Cats and rabbits: After anesthesia was established, a femoral vein was exposed for injection of the ellagic acid or buffer. Nine cats and ten rabbits were injected with ellagic acid while three animals of each species received only buffer. A laparotomy was performed, and blood samples were drawn from the vena cava. All animals were followed for a minimum of 2 hours and a maximum of 4 hours.
Rats: After anesthetization, a femoral vein was exposed for injection of the ellagic acid or buffer. Seventy-seven rats were given 2 ml. of the ellagic acid solution in 5 minutes, and 17 rats received buffer. The 77 ellagic acid-treated rats were divided into 5 groups of 15 to 17 animals. Animals in each group were sacrificed at 5, 10, 20, 30 and 60 minutes after injection. Blood samples were drawn from the vena cava of 17 animals at 5 minutes, 16 at 10 minutes, 15 at 20 minutes, 15 at 30 minutes, and 16 at 60 minutes.

In dogs, several coagulation tests were carried out. In rats, cats and rabbits only the glass and silicone clotting times were studied.

Blood samples in all animal species were drawn through disposable needles No. 19 (dogs and rats) or No. 20 (cats and rabbits) into siliconized syringes. Slow or foamy samples were discarded. The glass and silicone clotting times were determined by keeping tubes with 1 cc. of blood in a 37 C. water bath, checking them every 30-45 seconds. The time at which the tubes could be inverted without flow of blood was considered as the clotting time. The prothrombin time was determined by the method of Quick.33 Factors II, V and VII were determined according to the methods of Owren, modified by Lewis et al.34 The prothrombin consumption test was carried out on serum from blood oxalated after 15 and 60 minutes at 37 C.32,34 The blood fibrinogen was determined according to the method of Cullen and Van Slyke, modified by Quick.33 The fibrinolytic activity was studied by the euglobulin method.35 Proteolytic activity was studied by the method of Downie and Clifton.36 Partial thromboplastin time was measured by the method of Langdell et al.,37 utilizing a kaolin-activated partial thromboplastin as suggested by Proctor and Rapaport.38 The thromboplastin generation test was performed according to the method of Hicks and Pitney.39 Sedimentation rate was read in a standard Westergren apparatus.40

RESULTS
The effects of ellagic acid on the several tests performed are summarized in table 1. In all animals tested the silicone clotting time was significantly shortened (fig. 1–5). The glass clotting time was shortened in about 80 per cent of the animals. The thromboplastin generation time was accelerated in the dog in about 60 per cent of the animals (fig. 6). There was no constant change in the other coagulation factors studied (table 1, fig. 7). The effect of ellagic acid on the clotting times lasted longer in dogs than in rats, rabbits
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Table 1.—The Influence of Ellagic Acid on Clotting Tests and Other Hematologic Parameters

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass clotting time</td>
<td>Slightly shortened in about 80 per cent of animals</td>
</tr>
<tr>
<td>Silicone clotting time</td>
<td>Strikingly shortened in all cases.</td>
</tr>
<tr>
<td>Thromboplastin generation test</td>
<td>Accelerated in about 60 per cent of dogs.</td>
</tr>
<tr>
<td>Partial thromboplastin time</td>
<td>No change</td>
</tr>
<tr>
<td>Prothrombin consumption test</td>
<td>No change</td>
</tr>
<tr>
<td>Prothrombin time (one-stage)</td>
<td>No change</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>No change</td>
</tr>
<tr>
<td>Fibrinolytic activity</td>
<td>No change</td>
</tr>
<tr>
<td>Proteolytic activity</td>
<td>No change</td>
</tr>
<tr>
<td>Factor II</td>
<td>No change</td>
</tr>
<tr>
<td>Factor V</td>
<td>No change</td>
</tr>
<tr>
<td>Factor VII</td>
<td>No change</td>
</tr>
<tr>
<td>Sed. rate</td>
<td>No change</td>
</tr>
</tbody>
</table>

or cats. In dogs a shortening of the silicone clotting time was sometimes detectable 4 hours after the administration of ellagic acid (fig. 1). The changes were statistically significant (p < 0.01) up to 3 hours after the ellagic acid injection. In cats and rabbits, the average value of the silicone clotting time was shortened significantly (p < 0.01) at 5, 10, 20, 30 and 60 minutes after the ellagic acid administration.

The effects were of shortest duration in rats. In these animals the silicone clotting time was back to normal range (p > 0.6) within 20 minutes, and the glass clotting time was back to the control levels (p > 0.6) within 10 minutes after the administration of ellagic acid.

Sex of the animals had no effect on the response. In control animals no changes in clotting times or other clotting tests were ever observed. There was no apparent toxic effect in any of the animals studied. No thrombosis was detected in any of the animals studied, but no attempt was made to induce thrombosis by stasis or trauma to vessels.

One striking observation was the decrease in oozing from the fresh wounds made to expose the femoral veins. There was also a significant decrease in bleeding from the venipuncture wounds in the femoral veins of the treated animals as compared with controls. This decrease in bleeding was observed in all 4 species studied, dogs, cats, rabbits and rats.

DISCUSSION

Our results appear to be substantially in agreement with the data reported by Botti and Ratnoff. However, we did not detect a shortening of the partial thromboplastin time or an increase in the prothrombin consumption. Acceleration of the thromboplastin generation time was observed only in about 60 per cent of animals. The absence of increased prothrombin consumption was noted in samples of blood oxalated both after 15 and 60 minutes at 37°C. Botti and Ratnoff found a definite decrease in serum prothrombin ac-
Fig. 1.—Shortening of glass and silicone clotting times in dogs treated with ellagic acid.

Fig. 2.—Glass clotting time in controls and ellagic acid-treated rats. Each point represents one animal, the bars illustrate the mean in each group of rats.

tivity, indicating an increased prothrombin consumption in the samples oxalated after 15 minutes at 37 C. These discrepancies may be due to the different dose of ellagic acid used or to the different experimental conditions. Our failure to show any shortening of P.T.T. may be due to the fact that we used a kaolin-activated partial thromboplastin in performing the test.

The hypercoagulable state produced by ellagic acid appears to be a safe,
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Fig. 3.—Silicone clotting time in controls and ellagic acid-treated rats. Points and bars have the same meaning as in figure 2.

Fig. 4.—Shortening of glass and silicone clotting times in ellagic acid-treated cats.

prolonged, reversible condition characterized mainly by a striking shortening of the silicone clotting time. The most obvious effect of ellagic acid administration is the disappearance of the difference normally existing between glass and silicone clotting times. This phenomenon has been observed in vitro and occasionally in vivo with other substances such as celite and some tannic acid derivatives.30,32
Fig. 5.—Shortening of glass and silicone clotting times in rabbits given ellagic acid.

The data available suggest that the mechanism of action of this substance is through activation of the Hageman factor. The acceleration of the thromboplastin generation test observed in five of our dogs (fig. 6) seems to support this contention. The hypercoagulable state induced by the administration of ellagic acid lasted much longer than the actual time of administration of the substance in all four animal species studied, indicating that the activated HF-PTA system persists in the circulation. Despite this prolonged action, no thrombosis appeared.

The fact that no thrombotic phenomena were detected in our experiment does not rule out the possibility that ellagic acid may cause thrombosis in vivo. The phenomenon may require the concomitant action of other factors such as stasis, or an endothelial lesion. It is to be remembered, however, that activated procoagulants are removed rapidly from the circulation by the reticuloendothelial system.

As already observed by Botti and Ratnoff, ellagic acid did not cause any increase in fibrinolytic activity (fig. 7). This appears to be in conflict with data indicating that stimulation of Hageman factor in vitro and in vivo cause activation of the fibrinolytic system. It is possible that the reported fibrinolytic activity produced with activation of Hageman factor was due to some condition other than the Hageman factor activation itself.

Ellagic acid had a similar effect in the four species studied. The variation in the duration of effects observed in this study may indicate a different behavior of the activated HF-PTA system in the different animal species.
Fig. 6.—Thromboplastin generation test in one dog which is representative of the changes seen in 60 per cent of the ellagic acid-treated animals. Acceleration of thromboplastin generation is evident in the first 5 samples which refer to the first 60 minutes after the beginning of the ellagic acid infusion.

Further studies with this substance are warranted. The persistent hypercoagulable state without apparent undesirable side effects, such as increased thrombosis, makes this a very interesting agent for study of the effect of alterations in the coagulation mechanism on bleeding abnormalities and thrombosis.

The decrease in bleeding from open wounds and needle puncture sites in exposed vessels observed with ellagic acid is of particular interest. If this effect can be confirmed in more serious bleeding conditions and no harmful side effects are found with other experimental methods, we may find this to be a valuable agent in the control of abnormal oozing or bleeding.

SUMMARY

Ellagic acid shortens the silicone and glass clotting times of the blood of dogs, cats, rabbits and rats. The silicone clotting time is reduced so as to
Fig. 7.—Demonstrates the effect of ellagic acid on fibrinogen, fibrinolytic activity, factor II, V, and VII, prothrombin consumption (on blood oxalated after 15 minutes at 37°C), and partial thromboplastin and prothrombin times. No significant change occurred in any dog studied.

make it almost equal to the normal glass clotting time for 5 to 30 minutes after the injection of the agent. There is occasional acceleration of thromboplastin generation in dogs. No other clotting factors were altered significantly in our experiments.

There were no obvious toxic effects of ellagic acid, and it appears to decrease bleeding in normal animals.

SUMMARIO IN INTERLINGUA

Acido ellagic reduce le tempores coagulatori del sanguine de canes, cattos, conilios, e rattos tanto in silica como etiam in vitro. Le tempore coagulatori in silica es reduceite de maniera a render lo quasi equal al normal tempore coagulatori in vitro durante periodos de inter 5 e 30 minutas post le injection del agente. Il occurre occasionalmente un acceleration del generation thromboplastinic in canes. In nostre experimentos, nulle altere factores coagulatori se monstrava alterate de maniera significative.
Nulle obvie effectos toxico del acido ellagic esseva notate. Illo pare reducer le sanguination in animales normal.

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ANTONIO GIROLAMI, DOMENICO AGOSTINO and EUGENE E. CLIFFTON

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