The purpose of this study was to investigate the effects of ellagic acid on platelet expression via the cyclooxygenase (COX) pathway by examining its effects on platelet activation and comparing them with known COX inhibitors in male Sprague-Dawley rats. Ellagic acid is a major compound found in certain fruits and nuts. It has been attributed as having anti-inflammatory, free radical scavenging, and coagulation properties as well as effects on tumor genesis in multiple forms of cancer.

We assessed the similarities of ellagic acid to known COX-2 specific and nonspecific COX inhibitors by examining their effects on platelet activation via use of P-selectin flow cytometry. Compared with the vehicle group, both the ellagic acid (P = .035) and the ketorolac (P = .038) groups demonstrated a significant decrease in platelet activation (P = .026). Furthermore, compared with all other groups, ellagic acid plus ketorolac group showed a significant decrease in platelet activation (P = .01). Our findings suggest that ellagic acid is likely a nonspecific COX inhibitor. It also suggests that combining ellagic acid with a known nonspecific COX inhibitor such as ketorolac may cause a significant decrease in platelet activity and an increase in blood loss.

Keywords: COX inhibitors, ellagic acid, flow cytometry, platelets, Sprague-Dawley rats.
termine the effects of ellagic acid on the COX pathway by comparing its effects on platelet activation with 2 known COX inhibitors.

• Theoretical Framework. All of the products of the COX pathway are termed eicosanoids. They are a class of signaling molecules that are used throughout the body in a number of different pathways and chemical events. They have effects on vascular and bronchial smooth muscle, platelet function, kidney function, and neurotransmission. The COX pathway is very complex, with a series of enzymes that convert arachidonic acid into various products that include prostaglandins, prostacyclins, and thromboxane. The 2 major isoenzymes involved in this pathway are termed COX-1 and COX-2. The COX-1 enzyme is a ubiquitous enzyme that is involved with normal homeostasis and that helps to regulate numerous body functions, including platelet aggregation, protection of the gastric mucosa, and renal function. The COX-2 enzyme is released during periods of stress, with its effect based on the type of stimulus present. It has been found to be involved in tumor production and shear stress and is a major mediator in both pain and inflammation.

Ellagic acid has been shown to have COX-2 effects, including effects on COX-2 overexpression in carcinogenic tumor cells and anti-inflammatory effects. Ellagic acid has been extensively studied for its anti-inflammatory properties and potential use in cancer, and has been found to cause apoptosis in cancer cells, prevent binding of carcinogens to DNA, and inhibit tumor cell proliferation.

Ellagic acid’s effects on coagulation have also been extensively studied, and a general hypercoagulable state has been attributed to ellagic acids effect on Hageman factor (factor XII). It has been shown that with the administration of ellagic acid, there is a decrease in clotting time (silicone clotting time) and increased prothrombin consumption with an increase in partial thromboplastin time. Further studies on ellagic acid found that after intravenous administration, platelet aggregation increased with swelling of both the lymph nodes and the spleen. This was accompanied by a marked thrombocytopenia in conjunction with a decrease in both plasminogen and fibrinogen plasmin levels that were attributed to an increase in coagulation.

Despite studies indicating the alterations in platelet function as well as the COX-2 effects of ellagic acid, there have been no studies specifically to determine the effects of ellagic acid on platelet expression via the COX pathway. All the information to date has been an extrapolation of information from studies not specifically designed for this intent. With the current study, it is our goal to determine the effects of ellagic acid on platelet activation and to compare them with known COX inhibitors.

We compared the effects on platelet activation of ellagic acid to ketorolac (nonspecific COX inhibitor) and meloxicam (a COX-2 specific inhibitor). If the effects of ellagic acid are similar to that of ketorolac, it is likely a nonspecific COX inhibitor. Conversely, if ellagic acids effects in platelet activation are similar to that of meloxicam, it is likely to be a COX-2 specific inhibitor (Figure 1).

• Instrumentation. The activation of platelets is a result of cell surface receptors, such as the adenosine diphosphate (ADP) and glycoprotein IIb/IIIa, responding to the adhesion of the platelet to a damaged vessel wall. Once activated, the platelet undergoes conformational changes that result in alterations to platelet shape, adhesion, aggregation, and secretion of granule contents. These changes enhance signaling pathways that lead to the activation and aggregation of additional platelets as well as enhanced adherence to other proteins. These processes combine to form thrombin with resulting fibrin deposition that is associated with the maintenance of homeostasis.

During the process of platelet activation, the phospholipid molecules of the platelet membrane transpose themselves from the inner side to the outer side of the platelet. This transposition allows the expression of specific proteins that facilitate the process of coagulation. One such protein is P-selectin that facilitates the adherence of molecules, such as endothelial cells, to the activated platelet. P-selectin is expressed only after platelet degranulation and is therefore specific to activated platelets and not resting platelets. In recent years, P-selectin has been used to identify activated platelets after their expression via flow cytometry. There are many advantages to flow cytometry. These include a more physiologic milieu, no radioactivity, and ability to detect activation-dependent changes in multiple surface receptors.

The process of flow cytometry begins by activating the platelet with an artificial activator, such as ADP, as was used in this experiment. After the platelet is activated and the cell surface antigen is expressed, the platelets are labeled with a fluorescent-conjugated monoclonal antibody. The activated platelets are then analyzed by the flow cytometer by passing the platelets through a focused laser. The platelets are then assessed for their positive fluorescence as well as their characteristic light scatter.

We assessed the similarities of ellagic acid to known COX-2 specific and nonspecific COX inhibitors by looking at their effects on platelet activation by flow cytometry. If ellagic acid is COX-1 specific, there would be little or no subsequent expression of P-selectin and the washout of fluorescein antibodies would occur. Conversely, if the experimental compound inhibits COX-2, the amount of fluorescence would be high. This would be due to no inhibition of the platelets with the positive expression of P-selectin and the adherence of the fluorescein antibodies (Figure 2).
Figure 1. Administration of Ketorolac Showing a Decrease in Platelet Activation Because of Blocking of Both Cyclooxygenase (COX-1 and COX-2) Pathways
Administration of meloxicam should show no change in platelet activation because of its COX-2 specific effects. PG indicates prostaglandin.

Figure 2. Process of Flow Cytometry
If the experimental compound is a cyclooxygenase 1 (COX-1) specific inhibitor, P-selectin will not be expressed, with no attachment of the fluorescein antibody, and no fluorescence seen. ADP indicates adenosine diphosphate.
Materials and Methods

Our prospective, nondirectional research was conducted using 34 male Sprague-Dawley rats weighing between 200 and 225 g. The Sprague-Dawley rat is widely accepted in the research community as dependable for use in general research, including pharmacology, and is a general model for the study of human health and disease. The use of laboratory rats in this protocol was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and received approval from the Institutional Animal Care and Use Committee. All animal research was conducted at the 59th Medical Wing Clinical Research Squadron of Wilford Hall Medical Center, located at Lackland Air Force Base in San Antonio, Texas.

To minimize the effects of a stress-induced catecholamine response on platelet activation, the rats were brought in 10 days before experimentation for habituation. Laboratory conditions were maintained in a temperature-controlled environment (22 ± 1°C, 60% humidity) on a 12-hour light-dark cycle in which there was free access to food and water. The rats were involved in a parent study researching the effects of ellagic acid as an anti-inflamatory and antinociceptive for 10 days with a 2-week washout period before this trial.

All experiments occurred between 6 AM and noon to ensure control. On day 1, each rat was briefly anesthetized with isoflurane. Blood was drawn via cardiac puncture, not to exceed 1.5 mL, into a syringe containing 50 µL of 3.8% sodium citrate kept at room temperature, to assay baseline platelet activation. Platelet activation using the platelet activation marker P-selectin was assayed according to Wilford Hall Medical Center’s animal laboratory protocol. Beginning on the second day, 24 hours after the initial blood draw, each group was injected intraperitoneally daily for 7 consecutive days with its preassigned interventional medication. All animals received 1 injection, and the total volumes administered were equal to allow for control of number of injections and injection effects. On the eighth day, a terminal anterior vena cava draw was collected, again with the animal under isoflurane anesthesia, for postintervention platelet activation.

The rats were divided into 6 groups using a Latin square, with 9 rats in each group. Each rat was assigned to 1 of the following groups: (1) the negative control group (vehicle), (2) the nonselective COX inhibitor positive control (ketorolac), (3) the COX-2 selective inhibitor positive control that was administered meloxicam and vehicle, (4) an experimental group administered ellagic acid and vehicle, (5) rats administered ellagic acid and ketorolac to investigate interactions between the nonselective COX inhibitor and ellagic acid, and (6) rats administered ellagic acid and meloxicam to investigate interactions between the COX-2 selective inhibitor and ellagic acid (Table).

### Table. Treatment Group Assignments

<table>
<thead>
<tr>
<th>Group</th>
<th>Intervention</th>
<th>Dose (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle (negative control)</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Ketorolac (positive control)</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>Meloxicam (positive control)</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>Ellagic acid and vehicle</td>
<td>75</td>
</tr>
<tr>
<td>5</td>
<td>Ellagic acid plus ketorolac</td>
<td>75 (ellagic acid), 10 (ketorolac)</td>
</tr>
<tr>
<td>6</td>
<td>Ellagic acid plus meloxicam</td>
<td>75 (ellagic acid), 4 (meloxicam)</td>
</tr>
</tbody>
</table>

- **Drug Dosages.** Given that ellagic acid is selling on the Internet in doses as high as 25 mg/kg, that rodent metabolism is nearly twice that of humans, and that there are 2 in vivo experiments in which ellagic acid was found to be effective at 75 to 100 mg/kg without toxicity, we administered the lower dose of 75 mg/kg of ellagic acid intraperitoneally. This should enable us to discern any difference in platelet activation while preserving any COX-2 selectivity of the compound.

Several rat studies have examined the analgesic efficacy of ketorolac. A very thorough dose-response study using the rat formalin pain model suggested that the peak response of inflammatory pain was at a dose of 5 mg/kg, with higher doses not producing any increased response. This dose of 5 mg/kg was used in conducting our research.

 Plasma concentrations of meloxicam (a COX-2 inhibitor, approved for humans since 2004) have been shown to be comparable in rats, dogs, and humans, with plasma levels of humans most equally reflecting that of rats. Meloxicam is known to be 300 times more selective for COX-2 than COX-1, with varying dosages being used. The dosage used for the purpose of our experiment was 4 mg/kg injected intraperitoneally to ensure effect and to preserve its COX-2 specific effects.

- **Statistical Analysis.** Because no other studies have been done concerning the effects of ellagic acid on platelet activation, the effect size of 0.5 was calculated from work done by Rogerio and colleagues, who found ellagic acid to have a large effect size using analgesic type assays. Using a general power analysis program (G*Power 2.1.2, Softpedia.com), an effect size of 0.5, a power of 0.80, and an α of 0.05, a sample size of 9 rats was calculated for each group. An analysis of covariance was used, with the preintervention platelet activation results as a covariate.

Results

Figures 3 and 4 show the results from flow cytometry. Figure 3 consists of 2 graphs derived from blood samples obtained from a vehicle-injected (control) rat. Note the increased platelet activation with ADP in the blue box in the right graph compared with the left graph. Figure 4 shows
the flow cytometry results for a rat injected with ketorolac and ellagic acid. Note the significant suppression of platelet activation with ADP activation (right graph vs left graph). Data from the flow cytometry were converted to percent change and then analyzed using analysis of variance, which suggested a significant difference ($F = 2.86; P = .026$) existed between all groups. Ellagic acid or ketorolac exhibited a significant decrease in platelet activation compared with the vehicle group. The least significant difference post hoc analysis suggested that platelet activation of animals administered ellagic acid had an additive or synergistic effect when combined with ketorolac ($F = 2.86; $P = .026$).

**Figure 3.** Flow Cytometry With Vehicle
Left graph, blood before activation with adenosine diphosphate (ADP); right graph, results after ADP activation. This rat received vehicle. Area in the blue box shows the fluorescent activity of platelets with both the primary antibody and fluorescein antibody attached. Note the greater number of activated platelets in the blue box in the right graph vs the left graph.

**Figure 4.** Flow Cytometry With Ellagic Acid and Ketorolac
This rat was administered both ellagic acid and ketorolac. Results are before (left graph) and after (right graph) activation with adenosine diphosphate (ADP). Note the few activated platelets in the right graph. Platelet activation was suppressed, P-selectin was not expressed, and the antibodies did not attach to the platelets, with limited fluorescence noted.
These findings suggest that ellagic acid may be a nonspecific COX inhibitor and in combination with a known nonspecific COX inhibitor may cause a significant decrease in platelet activity.

**Discussion**

Our findings demonstrated that the effects on platelets due to ellagic acid were similar to those of the nonspecific COX inhibitor ketorolac and when combined with ketorolac have a synergistic effect. This effect included a decrease in platelet activation that was significantly different from the vehicle group as well as an amplified decrease in platelet activation when combined with ketorolac. These findings are suggestive that ellagic acid may have nonspecific COX inhibitory effects similar to those of ketorolac.

The effect on platelet activation seems to be contradictory to studies showing a hypercoagulable state with the administration of ellagic acid. The original studies on ellagic acid’s effects on coagulation attributed the properties to Hageman factor and not to effects on the COX pathway. In these studies, it was also found that ellagic acid had produced a long-lasting thrombocytopenia. In at least one study, it was found that with the addition of specific nonsteroidal anti-inflammatory drugs (NSAIDs), there was a further increase in the thrombocytopenia. Those studies looked at the effect on coagulation over a shorter time span versus our experiment. Also of note is that our data collection was at one specific time interval and did not take into consideration any variability in the effects of time on platelet activation.

This decrease in platelet activation has numerous consequences. The risk of increased postoperative bleeding with ellagic acid use can be exacerbated if combined with nonspecific COX inhibitors. However, the decrease in platelet activation has the possibility of being used as an adjunct therapy in situations that demand a decrease in inflammation as well as platelet activation. Patients with a history of major cardiac disease or events may benefit from a decrease in platelet activation.

However, before the use of ellagic acid as a therapy, further studies must be performed to quantify the amount of platelet activation seen in humans. It would also be necessary to determine the appropriate dosage and time intervals to achieve the correct therapeutic level. Further studies would also be required to specifically study the
effects of both isoflavones of ellagic acid. Our research is merely suggestive in this area, and further identification and confirmation of this compound’s effect on the COX system is warranted.

REFERENCES


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ACKNOWLEDGMENT

The AANA Foundation, Park Ridge, Illinois, helped support this research.

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