

# Investigation of the Anti-inflammatory, Antinociceptive Effect of Ellagic Acid as Measured by Digital Paw Pressure via the Randall-Selitto Meter in Male Sprague-Dawley Rats

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*Ellagic acid (EA), a dietary supplement, is purported to have anti-inflammatory, antinociceptive properties via cyclooxygenase (COX) inhibition. We measured the antinociceptive efficacy of EA alone and in combination with a nonselective COX inhibitor and a selective COX-2 inhibitor.*

*We assigned 54 male Sprague-Dawley rats to 1 of 6 groups to be given the following compounds: (1) vehicle, (2) ketorolac (nonselective COX inhibitor), (3) meloxicam (selective COX-2 inhibitor), (4) EA, (5) EA plus ketorolac, and (6) EA plus meloxicam. Inflammatory pain was induced in the right hind paw by injecting carrageenan. Rats were given study compounds via intraperitoneal injection 30 minutes after paw injections. Pain tolerance was assessed using*

*the Randall-Selitto instrument at 30 minutes and 4, 8, 12, and 24 hours. The highest pressure tolerated was recorded in grams.*

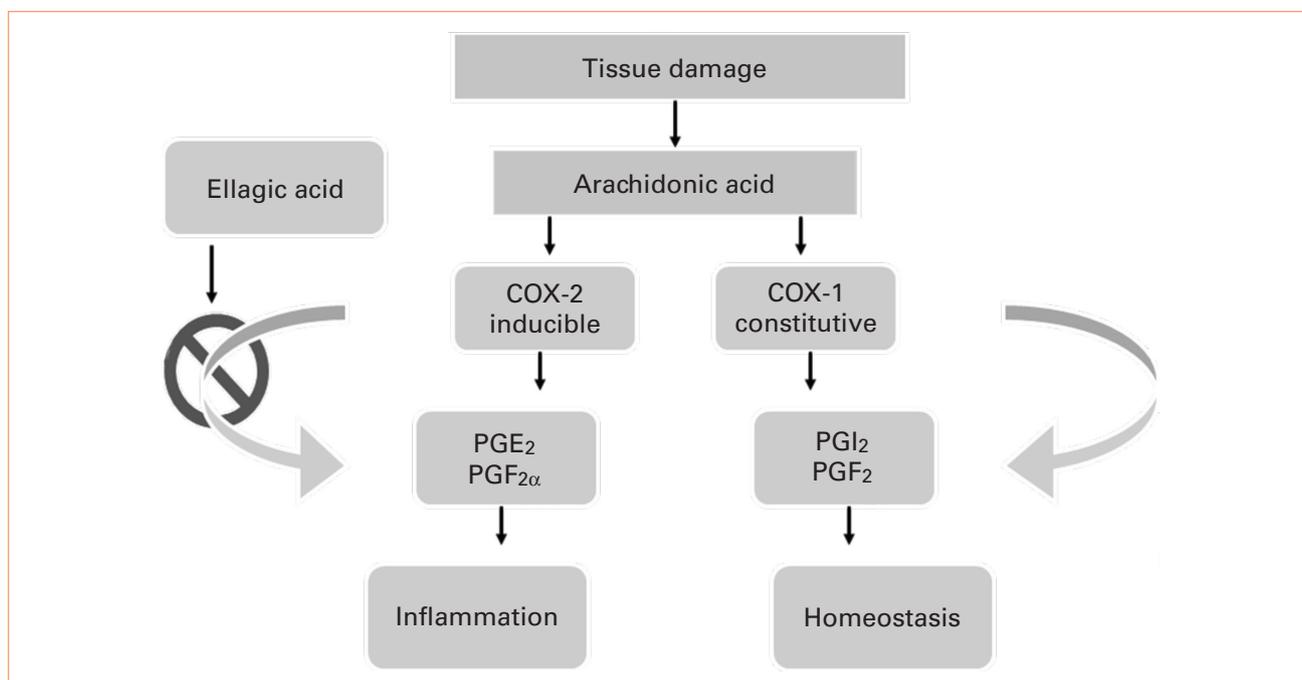
*The analysis of variance suggested a significant difference ( $F = 2.44$ ;  $P = .048$ ). The least significant difference post hoc analysis suggested that at 8 hours, EA plus ketorolac provided greater antinociception than all other compounds ( $P = .04$ ). Furthermore the combination of EA plus ketorolac provided longer antinociception than all other compounds ( $P = .03$ ) such that EA plus ketorolac was effective at 24 hours.*

**Keywords:** Anti-inflammatory, antinociceptive, cyclooxygenase, ellagic acid, Randall-Selitto meter.

**N**ews of the purported anticancer activity of pomegranate juice is well published in the lay press, with CBS news reporting that pomegranate juice sales have dramatically increased.<sup>1</sup> Ellagic acid, one of the active compounds in pomegranate juice, has become a very popular dietary supplement, so popular that the American Cancer Society has a link to information regarding ellagic acid on its website.<sup>2</sup> A polyphenol compound found in pomegranates, raspberries, and other foods, ellagic acid represents one of hundreds of phenolic compounds found in plant foods. Most are flavonoids, but about one third are phenolic acids such as ellagic acid. Ellagic acid was first characterized in the 1960s by Bhargava et al,<sup>3</sup> who isolated it from the black walnut. Subsequently, ellagic acid was reported to affect blood clotting by activation of factor XII.<sup>4</sup> It was examined for use to control hemorrhage in animals<sup>5,6</sup> and humans<sup>7</sup> in the 1970s and 1980s,

but later was found to deplete high-molecular-weight kinins, prolong activated partial thromboplastin time, and produce prolonged hypotension.<sup>8,9</sup> Thus, researching the medical benefits of ellagic acid slowed.

With the more recent research focus on phytochemicals and nutraceuticals as antimutagenic agents, ellagic acid has been reexamined as a possible antimutagenic. Research suggests that it has antioxidant properties<sup>10</sup> and inhibits a number of cell-signaling pathways that are important to tumor growth, including inflammatory signaling such as tumor necrosis factor  $\alpha$ -induced cyclooxygenase-2 (COX-2) protein expression<sup>11</sup> and nitric oxide synthase inhibition.<sup>12</sup> For example, an intraperitoneal (IP) injection of ellagic acid in Ehrlich ascites carcinoma-transplanted mice enhanced the ability of radiation to eradicate tumor cells.<sup>13</sup> Two studies specifically examined COX inhibition<sup>11,14</sup> with regard to the antimutagenic properties of ellagic acid. Whereas Adams



**Figure 1.** Tissue Damage, Such as Caused by Chemical Toxins, Mobilizes Arachidonic Acid From Membrane Phospholipids via the Enzyme Phospholipase A<sub>2</sub>

Arachidonic acid is then oxygenated by the cyclooxygenase (COX) enzymes, which synthesize prostaglandins. Whereas COX-1 is constitutively expressed, COX-2 is inducible by painful stimuli. Prostaglandins (PGs, such as prostacyclin [PGI<sub>2</sub>] and PGE<sub>2</sub>) produced by the COX-2 enzyme are associated with inflammation, whereas prostaglandins (such as PGE<sub>2</sub> and PGF<sub>2α</sub>) synthesized by COX-1 are associated with homeostasis, such as platelet function and endothelial integrity. The mechanism of action of ellagic acid may be similar to that of COX-2 inhibitors such that it may inhibit the COX-2 enzyme.

and colleagues<sup>11</sup> found that extracts of pomegranate juice such as ellagic acid significantly suppressed tumor necrosis factor  $\alpha$ -induced COX-2 protein expression in human colon cancer cells, Stoner and colleagues<sup>14</sup> suggested that *N*-nitrosomethylbenzylamine-induced esophageal tumorigenesis was inhibited by 31% to 64% when ellagic acid was administered in the diet of Fischer 344 rats. The reduction in tumor growth was due, in part, to COX-2 inhibition. Although recent studies suggest that ellagic acid may be a COX inhibitor and there is evidence that polyphenolic herbal supplements have antinociceptive properties,<sup>15</sup> few studies were found regarding the anti-inflammatory, antinociceptive efficacy of ellagic acid.

The COX isoenzymes, COX-1 and COX-2, regulate prostaglandin synthesis by converting arachidonic acid into the various prostaglandins. Phospholipase A<sub>2</sub> is activated by painful stimuli, such as occurs with tissue inflammation, and cleaves arachidonic acid from the cell membrane. Arachidonic acid is then available as a substrate for COX enzymes. The oxygenation of arachidonic acid by the COX enzymes begins the complicated pathway of prostaglandin synthesis. Prostaglandins subsequently alter the activities of cells in which they are synthesized. Whereas COX-1 is constitutively expressed and important for certain homeostatic functions such as adequate platelet activity, the expression of COX-2, the inducible enzyme, is significantly increased during

inflammation. Prostaglandin synthesis via COX-2 is integral to pain transmission. Conversely, inhibition of COX-2 promotes analgesia. Based on our review of the literature, we postulate that ellagic acid may inhibit the COX-2 enzyme, resulting in inhibition of inflammatory pain. Figure 1 broadly illustrates the COX pathway.

Until recently, pharmaceutical COX inhibitors, the nonsteroidal anti-inflammatory drugs (NSAIDs), were nonselective and inhibited COX-1 and COX-2 enzymes, causing side effects such as decreased platelet function. The newer COX-2 selective agents have no effect on platelet function but may increase the risk for myocardial infarction.<sup>16</sup> Therefore, the selection of an NSAID is patient-dependent. Nonselective and COX-2 selective NSAIDs are widely used as analgesics for acute and chronic painful inflammatory conditions, and many are sold over the counter. The considerable increase in nutraceutical use in the United States increases the likelihood that people may be taking NSAIDs and herbal supplements that may be COX inhibitors. There is no research regarding synergistic or additive effects that may occur when nutraceuticals and NSAIDs are combined.

The purpose of this study was to investigate the antinociceptive, anti-inflammatory effects of ellagic acid when administered alone and when administered in conjunction with ketorolac, a COX-nonspecific inhibitor, or meloxicam, a COX-2-specific inhibitor. Hence,

Group	No. of rats	Compound	Dose (mg/kg)
1	9	Vehicle (negative control, saline)	0
2	9	Ketorolac (positive control, nonselective COX inhibitor)	10
3	9	Meloxicam (positive control, COX-2 selective inhibitor)	7
4	9	Ellagic acid and vehicle	75 and 0
5	9	Ellagic acid and ketorolac	75 and 10
6	9	Ellagic acid and meloxicam	75 and 7

**Table.** Group Assignments of Rats and the Compounds and Doses Given

Abbreviation: COX indicates cyclooxygenase.

this study was directed by the following questions: (1) Is ellagic acid an efficacious anti-inflammatory, antinociceptive agent in male Sprague-Dawley rats as measured by the Randall-Selitto mechanical hyperalgesia instrument? (2) Are there interactions between ellagic acid and the anesthesia adjuvant ketorolac or the COX-2 inhibitor meloxicam as measured by the Randall-Selitto mechanical hyperalgesia instrument compared with control substances and ellagic acid alone?

If ellagic acid indeed inhibits the COX-2 pathway or is a COX-nonspecific inhibitor, rats given ellagic acid should exhibit an ability to tolerate greater pressure from the mechanical stimulus compared with rats given vehicle. Furthermore, by combining ellagic acid with other COX inhibitors, this study assessed and revealed possible antinociceptive interactions that may exist.

## Methods

This study used a prospective, experimental design. The groups and doses were based on the results of an experiment by Beltz and colleagues,<sup>17</sup> in which ellagic acid was found to be as effective as NS398, a selective COX-2 inhibitor similar to meloxicam, in alleviating inflammatory pain, measured by the modified hotplate assay in rats. The dependent variable, the amount of paw pressure tolerated, was examined using 6 discrete drug-compound conditions, the independent variable. Institutional Animal Care and Use Committee approval was obtained from the 59th Clinical Research Division, Wilford Hall Medical Center, Lackland Air Force Base, Texas. All animals were handled according to institutional animal protection protocols.

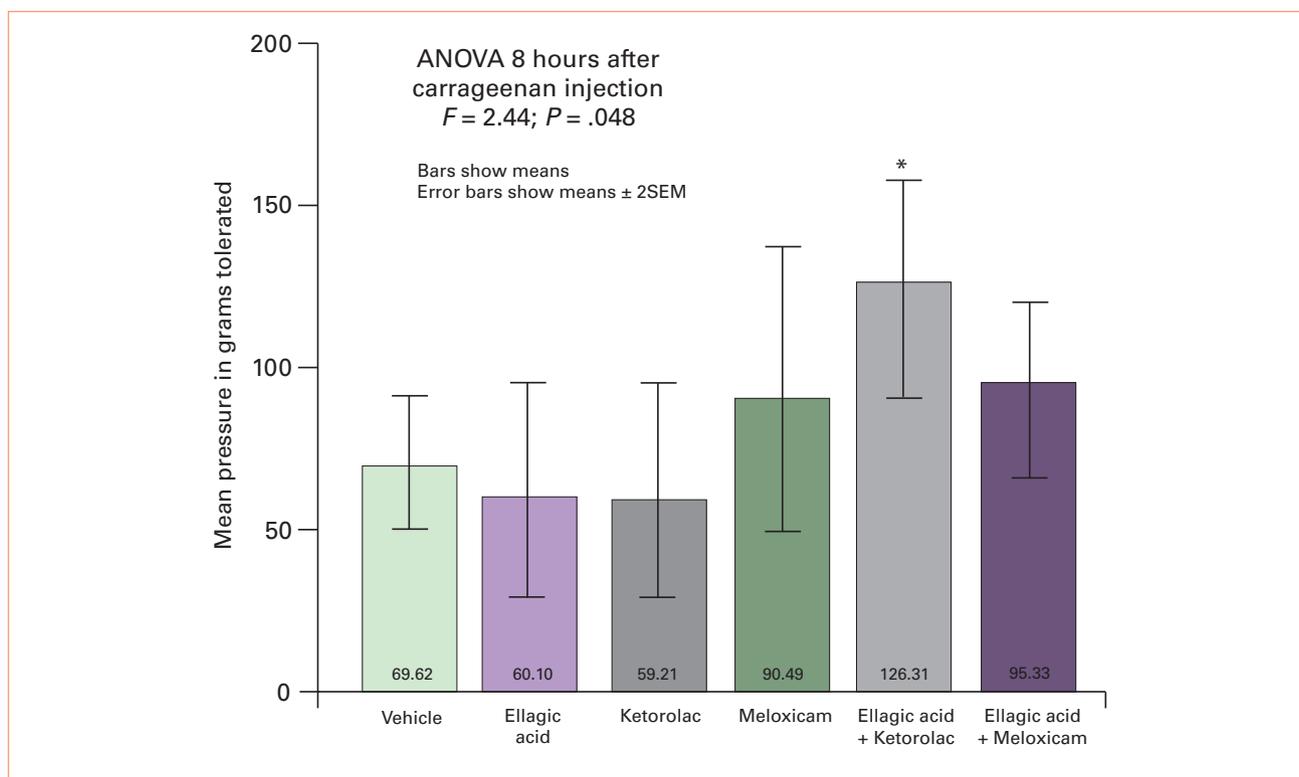
Mature male Sprague-Dawley rats weighing 200 to 225 g were used for the experiment. All rats were acclimated to the vivarium per the 59th Clinical Research Division protocol. The Table displays the drug doses for each group. Group 1 was the negative control group. Group 2 was given ketorolac. Group 3 was given meloxicam. Group 4 was the experimental group and was given ellagic acid, and groups 5 and 6, used to study possible interactions, were given ellagic acid and ketorolac or

ellagic acid and meloxicam, respectively. All rats were given the compounds by IP injection in equivalent volumes.

To study new compounds purported to have NSAID-like activity, the appropriate animal model must be used. Injection of carrageenan into the hind paw of rats releases the various mediators of inflammation, such as prostaglandins, and represents the classic model used to study inflammation and inflammatory pain. Carrageenan injection causes edema and an exaggerated sensitivity to mechanical stimuli. Mechanical pressure is the classic method by which to evaluate pain sensations from inflammatory agents. The Randall-Selitto IITC 2500 Digital Paw Pressure Meter (IITC Life Science Inc, Woodland Hills, California) assesses mechanical hyperalgesia, an increased response to a stimulus that is normally painful.<sup>11</sup> The Randall-Selitto mechanical hyperalgesia instrument allows comparison of the healthy paw (nonedematous paw) with the inflamed paw.<sup>18</sup> It is uniquely suited to testing the efficacy of anti-inflammatory compounds because those compounds are inactive in the healthy paw but show efficacy in the inflamed paw.<sup>19</sup> Moreover, the Randall-Selitto instrument has been described as the most predictive of animal models of acute pain.<sup>20</sup>

To habituate animals to the testing procedure, on days 6 through 10, rats were moved to the room in which testing took place and held with paws dangling. Each back paw was manipulated with the Randall-Selitto instrument. The day immediately before experiment, the rats were rested. The next day, data collection took place.

First, inflammation was induced in the right hind paw of each rat by injection of 0.1 mL of a 3% carrageenan (Sigma-Aldrich, St Louis, Missouri) solution using a 26-gauge needle. The injection was placed in the subcutaneous space just proximal to the foot pads. The same volume of sterile 0.9% saline was administered to the left hind paw to act as the negative control. Rats were given the compound 30 minutes later, based on group assignment by IP injection. To test throughout the expected time course of all compounds, animals were tested at 30 minutes and at 4, 8, 12, and 24 hours after injection.



**Figure 2.** Findings 8 Hours After Intraperitoneal Injection

The x-axis represents each of the groups, whereas the y-axis represents the mean pressure tolerated by the rats as measured in grams. At 8 hours, rats given ellagic acid plus ketorolac demonstrated significantly greater tolerance to mechanical pressure compared with all other groups. The numbers in boxes above the substances are the exact mean pressure tolerated, in grams.

\*Indicates significant finding.

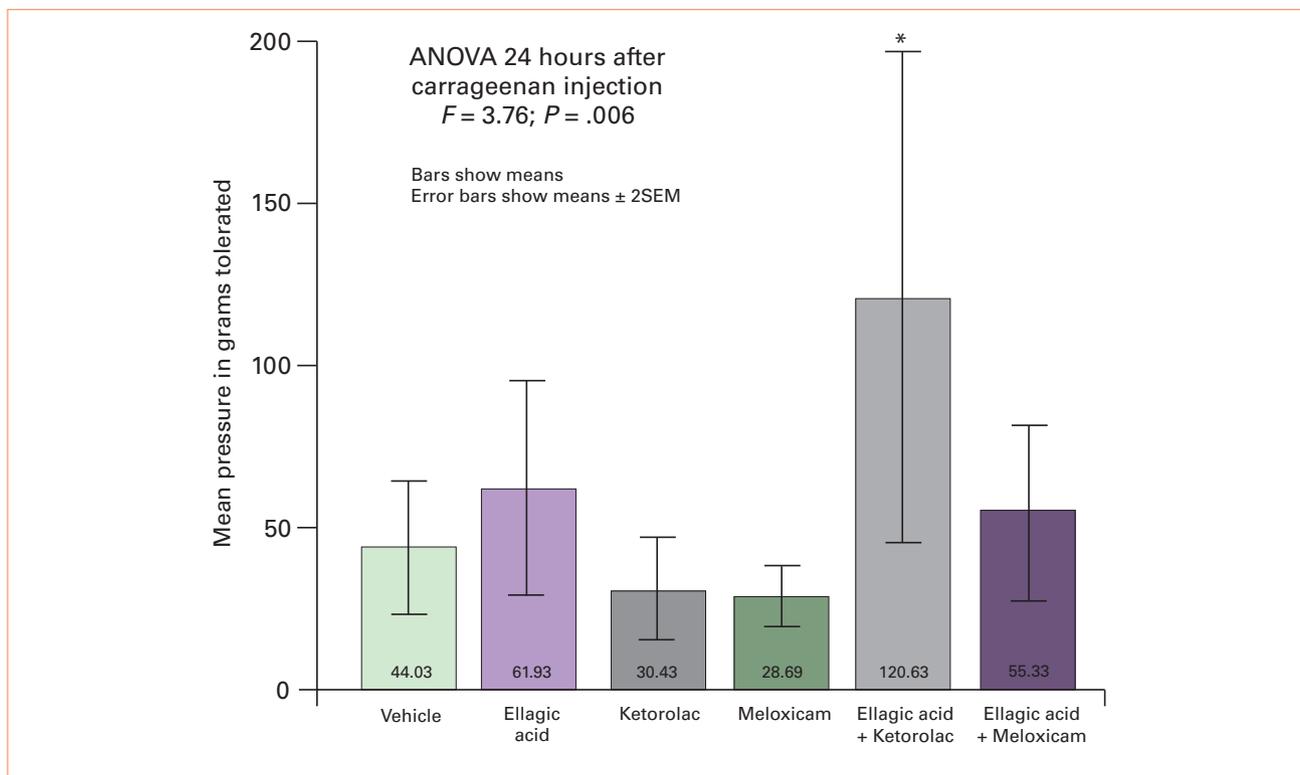
Abbreviations: ANOVA indicates analysis of variance; SEM, standard error of the mean.

Data were collected in a systematic manner combined with another research group examining the effects of ellagic acid against edema. A single rat paw was stimulated with the Randall-Selitto IITC 2500, and a reading was taken and recorded. The paws were then measured for inflammation for the other research group using a water displacement device. On completion of the inflammation measurement, the other paw was measured for pain using the Randall-Selitto device. During the testing procedure, the paws were alternated at each testing interval to ensure that the animals did not become sensitized to the testing procedure. For this experiment, pressure was applied to each paw until the animal withdrew or vocalized pain, at which time the instrument recorded the maximal amount of pressure withstood by the rat to within 0.2 g.

• *Compounds/Drugs and Doses.* We used ellagic acid (Sigma-Aldrich). Teel<sup>21</sup> found that after IP injection of ellagic acid, peak plasma levels occurred at 0.3 hours with  $\alpha$  and  $\beta$  half-lives at 1 hour and 5 hours, respectively. Most of the recent studies regarding ellagic acid have been in vitro experiments; however, one in vivo dose-response study using a carrageenan-induced inflammation model similar to that used in the present study suggested that whereas 1 and 10 mg/kg of IP ellagic

acid inhibited paw edema and the writhing reflex (a pain measure) up to 3 hours, 100 mg significantly inhibited both up to 4 hours.<sup>22</sup> We found no other studies that had examined the analgesic effects of ellagic acid in vivo. The only other in vivo rodent study we found suggested that 75 mg/kg of ellagic acid is effective in reducing tumor cell growth in experimentally induced Ehrlich ascites carcinoma.<sup>13</sup> Given that ellagic acid is selling on the Internet for human use at doses as high as 25 mg/kg,<sup>23</sup> that rodent metabolism is nearly twice that of human metabolism,<sup>24</sup> and that there are 2 in vivo studies that found ellagic acid to be effective at 75 and 100 mg/kg without toxic effects, we administered the lower dose of 75 mg/kg of ellagic acid intraperitoneally so that the dose would be sufficient to detect a difference in analgesia and platelet activation should one exist and so that any COX-2 selectivity of the compound would be preserved.

Ketorolac (Syntax Corporation, Palo Alto, California) is a nonselective COX inhibitor that has been approved for human use since the 1990s. Several rat studies have examined the analgesic efficacy of ketorolac.<sup>25-27</sup> The studies that used a carrageenan- or formalin-induced inflammation model found that IP doses of 1 to 10 mg/kg of ketorolac were effective in attenuating inflammatory pain in rats.<sup>28-30</sup> Because the study by Beltz et al<sup>17</sup> found



**Figure 3. Findings 24 Hours After Intraperitoneal Injection**

The x-axis represents each of the groups, whereas the y-axis represents the mean pressure tolerated by the rats as measured in grams. At 24 hours, rats given ellagic acid plus ketorolac continued to demonstrate significantly greater tolerance to mechanical pressure compared with all other groups. The numbers in boxes above the substances are the exact mean pressure tolerated, in grams.

\*Indicates significant finding.

Abbreviations: ANOVA indicates analysis of variance; SEM, standard error of the mean.

that 7 mg/kg of ketorolac was ineffective using an assay similar to that used in the present study, we selected a dose of 10 mg/kg of ketorolac.

Meloxicam (Sigma-Aldrich) is a well-known selective COX-2 inhibitor used frequently in veterinary medicine and research as an analgesic in rats and mice.<sup>31</sup> The routine dosage is 1 to 2 mg/kg every 12 to 24 hours. The present study used a 2-mg/kg dose.

- **Data Analysis.** Only 1 previous published study evaluated the antinociceptive efficacy of ellagic acid. Therefore, the determination of effect size for this experiment was based on this previous work by Rogerio and colleagues.<sup>22</sup> In the study by Rogerio et al,<sup>22</sup> ellagic acid was found to have a significant effect against pain induced by IP administration of acetic acid. By using the data reported in the study by Rogerio and colleagues,<sup>22</sup> our calculations showed that treatment with ellagic acid produced a large effect size. By using G Power 2.1.2 (a free online power analysis program; <http://www.psych.uni-duesseldorf.de/aap/projects/gpower/>), an effect size of 0.5, a power of 0.80, and an  $\alpha$  of .05, it was determined that a sample size of 9 rats per group was needed for the experiment. Hence, data were collected from 54 rodents.

Because there was no significant difference found

in paw pressure tolerated between the groups in the nonedematous paw, it was unnecessary to use those data as a covariate or an explanatory variable.<sup>32</sup> Analysis of variance was used for analysis of the dependent variable data—paw pressure tolerated in the carrageenan-injected paw. The analysis of variance suggested a significant difference among groups; therefore, the least significant difference post hoc test was used to assess specific group differences. There were no significant differences at 30 minutes or at 4 and 12 hours.

## Results

Figure 2 graphically represents the experimental data obtained from the Randall-Selitto digital paw pressure meter. The y-axis represents the mean paw pressure in grams that the animals tolerated on the carrageenan-inflamed paw, whereas the x-axis represents each of the 6 groups. Data analysis by analysis of variance at 8 hours after IP injection suggested a significant difference among the groups. The post hoc analysis suggested that ellagic acid plus ketorolac provided significantly greater anti-inflammatory analgesia compared with all other compounds (post hoc  $P = .01$ ).

Figure 3 illustrates that ellagic acid prolonged the

effect of ketorolac at 24 hours such that the combination of ellagic acid plus ketorolac provided significantly longer anti-inflammatory analgesia compared with all other compounds.

## Discussion

The experimental results suggest that ellagic acid may have enhanced the effect of ketorolac. This possibility of an enhanced effect is supported by the work of Kim and Chung,<sup>33</sup> who found that the combination of ibuprofen and epigallocatechin-3-gallate (EGCG), a green tea extract with COX-inhibitory properties, acted synergistically to inhibit the growth of prostate cancer cells. Although neither ibuprofen nor EGCG was evaluated in our study, each of the drugs has similar mechanisms of action to the drugs we examined, making the studies comparable to some extent. Ibuprofen is a COX nonspecific inhibitor, as is ketorolac, and EGCG is purported to have COX-inhibitory properties similar to those of ellagic acid. The findings of Kim and Chung<sup>33</sup> suggest that the administration of 2 COX inhibitors may have additive or synergistic effects. Moreover, Adhami and colleagues<sup>34</sup> suggested a possible synergism against prostate cancer cells when EGCG and NS398, a COX-2-specific inhibitor, were administered concurrently.

Second, the continued effect of the combination of ketorolac and ellagic acid at 24 hours after injection suggests that ellagic acid may prolong the effects of ketorolac, which has a half-life of 4 hours in rats, according to the research by Mroszczak et al.<sup>35</sup> While examining the efficacy of ellagic acid, Corbett and colleagues<sup>36</sup> demonstrated a similar prolonged effect of ketorolac combined with ellagic acid in reducing paw edema in carrageenan-injected rodents. Their research showed that at 8 hours after IP injection of 10 mg/kg of ketorolac plus 75 mg/kg of ellagic acid, rats given the combination of compounds showed significantly reduced paw edema as measured by a plethysmometer. Active metabolites of ellagic acid such as hydroxy-6H-benzopyran-6-one derivatives (uro-lithins) may contribute to this finding. For example, the work of Seeram and colleagues<sup>37</sup> suggests that active metabolites of ellagic acid can be detected in the plasma of humans for up to 48 hours and that these metabolites may contribute to the beneficial effect of ellagic acid.

Finally, the experimental finding that the first statistically significant effect of ketorolac plus ellagic acid occurred at 8 hours after injection suggests that the onset of ellagic acid may be greater than 4 hours. Although the study by Teel<sup>21</sup> suggests that the onset of ellagic acid is within 1 hour, our finding that ellagic acid had a delayed onset of action is congruent with the finding of Corbett and colleagues<sup>36</sup> that ellagic acid alone had no significant effect on paw edema until 8 hours after injection.

Several possibilities for future research are recommended based on the results of the present study. First,

the doses for the compounds in this research protocol were based on a literature review of studies with similar research conditions. We therefore recommend continued investigation of the dose-response curves for all compounds replicating this specific research method. Second, although the group size for the present study was based on the calculated effect size in the research by Rogerio and colleagues,<sup>22</sup> replication of the present study after calculation of effect size using current data to determine sample size under these conditions is also recommended. A third area of future research is to further investigate the combination of ellagic acid with other known anti-nociceptive, anti-inflammatory agents such as ibuprofen and aspirin to elucidate the potential effects. Future research aims may include determination of the minimum required amount of oral ellagic acid (such as that found in pomegranate or grape juice) that will produce a clinically significant effect or the possible synergism that may exist between orally administered ellagic acid compounds and other known COX inhibitors administered orally or by other routes. Finally, investigations that examine side effects associated with the administration of ellagic acid may elucidate limitations for use.

## Conclusion

The research findings suggest that ellagic acid, a commonly used herbal supplement, may enhance the effects of ketorolac in reducing or eliminating inflammatory nociception for up to 24 hours, based on a rat model. The affordability of ellagic acid and its potential to enhance other COX inhibitors such as ketorolac has the potential to make healthcare for all persons with inflammatory pain more cost-effective and obtainable. This finding has implications for improving healthcare while lowering healthcare costs nationwide.

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