AMINOCAPROIC ACID FOR THE PREVENTION OF POSTOPERATIVE
BLEEDING IN GREYHOUNDS

THESIS

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By

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ABSTRACT

Delayed postoperative bleeding is common in retired racing Greyhounds (RRGs), despite normal results of routine hemostasis assays. The excessive postoperative bleeding in the RRGs is not due to primary or secondary hemostatic defects, and may be due to enhanced fibrinolysis or to a clot maintenance dysfunction. Providing a method to prevent or minimize the severity of postoperative bleeding in RRGs will not only have major economic impact for owners, but also will markedly decrease the associated complications of minor or major surgeries in the breed. Epsilon aminocaproic acid (EACA) is a potent inhibitor of fibrinolysis that also supports clot maintenance due to unknown mechanisms.

The objective of this double-blinded, prospective, randomized study was to evaluate the effects of EACA versus placebo on the prevalence of bleeding in RRGs, and to investigate its mechanism of action by using TEG.

We compared the effects of EACA and placebo in 100 RRGs that underwent elective ovariohysterectomy or orchiectomy at the Veterinary Medical Center, The Ohio State University during 2 years. The main endpoint was bleeding (prevalence and severity); minor endpoints included most TEG parameters.

Thirty percent (15/50) of the RRGs in the placebo group had delayed postoperative bleeding starting 36 to 48 hours after surgery, compared to 10% (5/50) in the EACA group (P= 0.0124).
On the TEG parameters, the slopes for R and K time were significantly different between treatment groups (P=0.050); the R and K time decreased over time in the EACA group after surgery, while they increased in the placebo group. The angle, MA, and G slopes were also significantly different between treatment groups (P=0.001, 0.001, and 0.006, respectively). The angle, MA, and G increased postoperatively over time in the EACA group, while they decreased in the placebo group. All these changes are supportive of hypercoagulability associated with EACA administration.

Therefore, the postoperative administration of EACA significantly decreased the prevalence of postoperative bleeding in RRGs by increasing the clot strength.
DEDICATION

To my parents, Gustavo and Luz, and my brother and sister, Gustavo and Linda for their unconditional support, love and strength; to my husband Joe for his patience and for giving me confidence and love.
ACKNOWLEDGMENTS

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Sincere thanks to Tim Vojt for his help with the figure of fibrinolysis and the mechanism of action of EACA, also to Gary Phillips for his help with the statistical analysis. Thanks to Morris Animal Foundation for their funding and to the Greyhound adoption groups for their collaboration.
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PUBLICATIONS

    Lord LK, Yaissle JE, Marin LM, Couto CG. RESULTS OF A WEB-BASED
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FIELDS OF STUDY

Major Field: Comparative and Veterinary Medicine
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The popularity of RRGs as pets has markedly increased in the United States in the past decade. Currently, the number of Greyhounds that have retired from racing around the world exceeds those actively racing, and there are now over 130,000 RRGs in North America.\textsuperscript{a} Research has shown that Greyhounds and other sighthounds have unique physiological traits that distinguish them from other breeds;\textsuperscript{1-5} Unfortunately those differences are frequently misinterpreted as abnormalities, unless Greyhound specific reference intervals are used.

In the complete blood count (CBC), these traits include higher packed cell volume (PCV), hemoglobin concentration (Hb), mean corpuscular volume (MCV) reported in one study,\textsuperscript{6} red blood cell count (RBC), whole blood viscosity,\textsuperscript{1-3} and shorter RBC circulating lifespan.\textsuperscript{7} Greyhounds also have lower white blood cell (WBC), neutrophil, and platelet (PLT) counts, and eosinophils (EOS) with a unusual morphology,\textsuperscript{1-3,6,8,9} low total serum protein concentration due to low α and β globulin concentration, low serum IgA and IgM, and acute phase protein (mainly haptoglobin) concentrations when compared to other breeds;\textsuperscript{10,11}

Normal Greyhounds also have high glomerular filtration rate,\textsuperscript{12} high serum creatinine concentrations,\textsuperscript{13} low serum T\textsubscript{4} and fT\textsubscript{4} concentrations,\textsuperscript{14,15} a functional aortic stenosis murmur (usually grade 1-2/6) with no apparent clinical relevance,\textsuperscript{16} high arterial blood
pressure compared to other breeds of dogs,\textsuperscript{17} a large heart,\textsuperscript{18} higher vertebral heart size\textsuperscript{19} and high serum cTnI.\textsuperscript{20} In addition, we recently demonstrated that RRGs have higher pH, PO\textsubscript{2}, oxygen saturation, oxyhemoglobin, total Hb, oxygen content and oxygen capacity, and lower deoxyhemoglobin and P\textsubscript{50} than non-Greyhound dogs.\textsuperscript{21} The above-mentioned characteristics emphasize the fact that veterinarians should be aware of the unique hematologic and biochemical idiosyncrasies of the breed.

1.1 Retired racing Greyhounds as patients.

The vast majority of Greyhounds who complete their racing careers are sexually intact and will be spayed or neutered at the time of adoption (some adoption groups make sure the pets are spayed/neutered prior to them being adopted); this represents as many as 15,000 to 20,000 surgeries a year.

Delayed postoperative bleeding is a common complication in RRGs, even though most affected dogs have normal results of routine hemostasis assays, such as one-stage prothrombin time (OSPT), activated partial thromboplastin time (APTT), and platelet counts (PLT).\textsuperscript{22} In a recent study, we demonstrated that 26\% of RRGs bled 24 to 48 hours after routine gonadectomy, despite normal results of preoperative hemostasis panels.\textsuperscript{23} This prevalence is considerably higher than previously reported after ovariohysterectomy (OHE) or orchiectomy in other dog breeds (i.e.; 0 to 2\%).\textsuperscript{24-27}

Considering that a routine spay or neuter in a RRG often results in hemostatic complications leading to readmission to the clinic and transfusion of blood or blood components, identifying the patients at risk, and/or developing a simple protocol for
prevention of this complication will be extremely valuable. With a prevalence of bleeding of 26%, as many as 3,500 to 5,000 RRGs may be readmitted to the clinic and treated after routine surgery.

Furthermore, we recently reported that canine appendicular OSA was the most common form of cancer in RRGs, with a prevalence of 45% for dogs with cancer (42 of 94 RRGs). OSA is also the most common cause of death, accounting for approximately 25% of the deaths in the study period (28 of 113 RRGs). The treatment of choice for dogs with OSAs is limb amputation followed by postoperative adjuvant single agent or combination chemotherapy; thus, owners of RRGs with OSA who elect amputation may potentially face the associated complications and expenses related to blood component therapy and intensive care for hemostatic complications. From a monetary standpoint, this means that a routine surgical procedure may rapidly turn into very complicated situation, and expensive bills associated with transfusion therapy and intensive care for hemostatic complications, resulting in thousands of dollars of additional cost that could have been prevented.

Providing a method to minimize the severity or to prevent postoperative bleeding in RRGs will not only have a major economic impact, but will also markedly decrease the owners anxiety for having a very sick dog (and a high bill). Finally, due to the financial reality of veterinary medicine today, a high proportion of dogs with a variety of diseases are euthanized due to financial reasons; therefore, solving this hemostatic problem would also have a major impact on survival in clinical practice.
CHAPTER 2: HEMOSTASIS

The main function of the hemostatic system is to keep the blood flowing within the cardiovascular system; the integrity of the system in the event of vascular damage depends on the balance between coagulation and fibrinolysis. Any “imbalances” will lead to either hemorrhage or thrombosis.31

The coagulation mechanism is a very complex sequence of events described as enzymatic reactions initiated by a traumatic or surgical injury;31 these events result in formation of thrombin, which is responsible for the conversion of fibrinogen into fibrin, thus resulting in the formation of a blood clot at the site of the injury.31 The fibrinolytic process is in charge of terminating the coagulation phase once the clot is formed, followed by the elimination of fibrin deposits and reshaping of the thrombus, and the stabilization of the whole process while the endothelium is repaired.31

Hemostatic complications associated with ovariohysterectomy (OHE) or orchietectomy in dogs can be classified as “surgical”, those attributed to surgical technique and failure to control bleeding from the ovarian, uterine, or testicular vessels,32 or “non-surgical” (i.e.; failure of hemostatic pathways).31 The latter includes primary or secondary hemostatic defects; potential causes of failure of primary hemostasis include
thrombocytopenia, platelet dysfunction, or vonWillebrand disease (vWD); causes of secondary hemostatic defects include hypofibrinogenemia, hypoprothrombinemia, hemophilia A or B, factor VII deficiency, or combined clotting factor deficiencies, such as those associated with disseminated intravascular coagulation (DIC) or rodenticide toxicity. 

Sullivan et al, was the first to report in 1994 that RRGs have lower platelet concentrations (PLT) than other dog breeds; subsequently, several studies confirmed this finding. Currently there is no evidence that the low platelet count in RRGs has clinical relevance, and we believe that it is more likely the result of a breed-related variation without any pathologic consequences. Von Willebrand disease (vWD) has been identified in breed surveys in RRGs; however, it is uncommon in the breed. Less than 10% (22/216) of Greyhounds tested at The Ohio State University and at the Cornell Comparative Hemostasis Laboratory had concentrations of vWF below 30%, most of which could be attributed to sample handling (i.e.; preanalytical issues).

We recently investigated the potential pathogenesis of this postoperative bleeding tendency by evaluating primary and secondary hemostasis in RRGs after gonadectomy, the following tests were used to evaluate primary hemostasis: PLT, platelet function with PFA-100 closure time (CT), von Willebrand factor antigen concentration (vWF:Ag), and vonWillebrand factor activity with collagen binding assay (vWF:CBA). Secondary hemostasis was evaluated with activated partial thromboplastin time (APTT), one-stage prothrombin time (OSPT), fibrinogen concentration (FIB), antithrombin (AT) activity, and Factor XIII (FXIII) activity. The fibrinolytic pathway
was evaluated by measuring plasminogen (Plmg) and antiplasmin activities (AP), and D-dimer concentration.²³

Twenty-six percent of the RRGs developed postoperative bleeding 36–48 hours after surgery. Preoperatively, there were no significant differences between bleeders and non-bleeders for any of the following factors: PLT, hematocrit (HCT), platelet function using the PFA-100®, vWF:Ag, OSPT, APTT, FIB, F-XIII, Plmg and D-dimer.²³ Historically, Greyhounds with spontaneous bleeding have had normal platelet counts for the breed, vWF, FIB, OSPT, and APTT at the time of postoperative hemorrhage, making common bleeding disorders such as thrombocytopenia and clotting factor or vWF deficiencies unlikely causes of the bleeding.²³

In the previously mentioned study, the AP and AT activities, although within the reference range, were significantly lower in the dogs that bled than in those who did not bleed.²³ These results ascertain that the postoperative bleeding is not attributable to a primary or secondary hemostatic defect, and that they may be due to enhanced fibrinolysis.²³

Thromboelastography (TEG) is an in vitro technique that allows global evaluation of the blood coagulation process;³⁸ it is a novel device that evaluates the primary and secondary hemostasis, and the fibrinolytic pathway via assessment of the speed and strength of clot formation.³⁹ According to the manufacturers, native whole blood is the most sensitive method for analysis; however, the testing has to be initiated within 4-6 minutes of sample collection, and is thus impractical in veterinary medicine. An alternative is to use citrated blood, and recalcify the sample before analysis; the required
time to equilibrate the sample at room temperature with this method is 30 minutes,\textsuperscript{40} which is more practical. In order to facilitate clot formation and obtain additional information, blood samples can be treated using activators such as kaolin or tissue factor.\textsuperscript{40}

The TEG instruments consist of a pin attached to a torsion wire that is introduced into a cup containing the blood sample to be analyzed.\textsuperscript{38} The blood clotting in the cup is what initiates the movement, and the tension on the wire is translated into an electrical signal that generates a tracing\textsuperscript{38} (Table 1, Figure 1). The descriptions of the TEG parameters are:

**TABLE 1-TEG STANDARD PARAMETERS.**

<table>
<thead>
<tr>
<th>Clotting time</th>
<th>R</th>
<th>The period of time of latency from the time that the blood was placed in the TEG® analyzer until the initial fibrin formation.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clot kinetics</td>
<td>K</td>
<td>A measure of the speed to reach a specific level of clot strength.</td>
</tr>
<tr>
<td>alpha</td>
<td></td>
<td>Measures the rapidity of fibrin build-up and cross-linking (clot strengthening)</td>
</tr>
<tr>
<td>Clot strength</td>
<td>MA, G</td>
<td>A direct function of the maximum dynamic properties of fibrin and platelet bonding via GPIIb/IIIa and represents the ultimate strength of the fibrin clot.</td>
</tr>
<tr>
<td>Hemostasis profile</td>
<td>CI</td>
<td>Coagulation Index, which is a linear combination of the above parameters.</td>
</tr>
<tr>
<td>Clot stability</td>
<td>LY30</td>
<td>Measures the rate of amplitude reduction 30 minutes after MA</td>
</tr>
</tbody>
</table>
We recently reported that Greyhounds have slower clot kinetics and weaker clot strength when compared to non-Greyhounds\textsuperscript{39}, consistent with the previously described increased tendency to bleed after minor trauma or surgical procedures.

In a recent retrospective study we showed that 13 of 46 RRGs (28\%) that had limb amputation, developed delayed postoperative cutaneous/subcutaneous or external bleeding originating in the surgical site, requiring additional administration of blood components starting 36 to 72 hours after the surgery. (Manuscript undergoing review) In an attempt to prevent postoperative bleeding, decrease transfusion requirements and related costs, the fibrinolytic inhibitor epsilon aminocaproic acid (EACA) was
administered to 25 of the 46 RRGs that underwent amputation; only four of the 25 RRGs (16%) that received EACA developed bleeding complications, whereas nine of the 21 RRGs (42%) that did not receive EACA bled postoperatively. None of the dogs receiving EACA required transfusion of blood components. RRGs that did not receive EACA were 5.7 times more likely to bleed than the RRGs that did receive EACA. (Manuscript undergoing review)
CHAPTER 3: MANAGEMENT OF POSTOPERATIVE BLEEDING.

The most commonly used blood components to manage postoperative bleeding in veterinary medicine include fresh frozen plasma (FFP) and cryoprecipitate (CRYO). Fresh frozen plasma contains clotting factors, albumin, alpha2-macroglobulin, and immunoglobulins, which can be useful in the treatment of a variety of disorders in dogs, such as bleeding episodes after surgery, or as replacement of clotting factors in animals with congenital or acquired hemostatic disorders. Cryoprecipitate is rich in factor VIII and von Willebrand's factor, and it is therefore used primarily for the treatment of hemophilia A and von Willebrand’s disease.

Pharmacologic options to manage postoperative bleeding include aprotinin, desmopressin (DDAVP), recombinant factor VIIa, and lysine analogs. Aprotinin is a protease inhibitor used in humans as an antifibrinolytic; to our knowledge, its use in veterinary medicine is limited to canine research models for bypass surgery, and as a blood collection preservative for laboratory analysis. DDAVP is a synthetic analogue of the antidiuretic hormone vasopressin, of which it retains the capacity of increasing the plasma levels of factor VIII (FVIII) and vWF. DDAVP is used to decrease the prevalence and severity of bleeding in human patients with hemophilia A or von Willebrand’s disease, and in veterinary medicine to improve platelet function, increase vWF plasma concentrations, and minimize bleeding in dogs with VWD.
Recombinant factor VIIa is a novel treatment for human patients with hemophilia and a variety of disorders that cause significant bleeding. Its use in veterinary medicine is limited by its cost and potential hypersensitivity reactions.

Lysine analogs include EACA and tranexamic acid. Fibrinolytic inhibitors have proven to be effective in human patients and horses where complications are associated with enhanced fibrinolysis, but they have also been beneficial in patients with systemic bleeding due to other mechanisms. EACA was found to be a potent inhibitor of fibrinolysis in 1957 and was reevaluated in 1959. The in vitro and in vivo actions of EACA have been extensively studied in the 50s and 60s; interestingly, EACA neutralizes bleeding states created experimentally in dogs by infusion of plasmin or a plasminogen activator.

EACA prevents activation of plasminogen into plasmin on the fibrin surface, by preventing the binding of plasminogen to C-terminal lysine residues on partially degraded fibrin, thus blocking reversibly the plasminogen binding site, which is essential for efficient plasmin formation. (Figure 2)

EACA can either block enhanced fibrinolytic activity, or rapidly restore hyperfibrinolytic states to normal, thus impeding the dissolution of fibrin clots, and thereby decreasing RBC transfusion requirements in human patients undergoing surgery. EACA has a wide therapeutic index; no relevant adverse effects were reported in toxicologic studies in dogs, rabbits, and rats, with doses as high as 0.5 g/kg.
FIGURE 2: SCHEMATIC REPRESENTATION OF THE FIBRINOLYTIC SYSTEM AND THE MECHANISM OF ACTION OF EACA.

The top figure shows how plasminogen activator (PA) and plasminogen bind to fibrin at the lysine residues, thus generating plasmin, which degrades the clot into fibrin.
degradation products (FDPs). The bottom figure shows how EACA prevents activation of plasminogen into plasmin on the surface of the fibrin clot, by blocking the plasminogen lysine-binding site.

To our knowledge there are no prospective studies evaluating the effect of EACA in spontaneously occurring fibrinolytic disorders or other hemostatic abnormalities in dogs. The purpose of this study was to evaluate the prevalence and severity of postoperative bleeding in RRGs undergoing OHE or orchiectomy and receiving either EACA or placebo in a prospective, double-blinded, randomized study. In addition we aimed to evaluate the effects of EACA on selected TEG and fibrinolysis parameters (i.e.; R, K, angle, MA, G, LY60, and routine coagulation tests).

3.1 Materials and methods

One hundred RRGs from a local adoption group (www.greyhoundadoptionofoh.org), were spayed or neutered as part of a third- and fourth-year veterinary students operative practice curriculum at The Ohio State University-Veterinary Medical Center (OSU-VMC) over a 2-year period. The study had a current animal use protocol approved by the Institutional Animal Care and Use Committee at OSU. Blood samples in all dogs were collected after signed consent from the director of the rescue group.

All dogs were evaluated preoperatively by means of a physical examination; blood samples (20 ml) were collected before surgery for CBC, serum biochemistry profiles, rapid SNAPb test for some common vector borne diseases and TEG analysis. The CBCs were performed using a LaserCyte,d biochemistry profiles were performed using a
COBAS analyzer and hemostasis panels (OSPT, APTT, FIB, Antiplasmin, D-dimer and Plasminogen) were done using an Stago compact analyzer and commercially available reagents.

Before surgery, all RRG were premedicated with buprenorphine (0.05mg/kg) and acepromazine (0.5mg/total dose) intramuscularly; in some dogs (those with pyoderma) a prophylactic dose of intravenous cefazolin sodium (22mg/kg IV) was administered. Anesthesia was induced with ketamine (5mg/kg) and diazepam (0.25mg/kg) IV, and maintained using isoflurane in oxygen. Breathing was supported with intermittent positive-pressure ventilation; intraoperative fluid therapy consisted of lactated Ringer’s solution (10mL/kg/hour IV). The dogs were monitored during surgery with pulse oximetry, respirometry, measurement of peripheral arterial pressure, and body temperature. After surgery, a single intramuscular injection of carprofen (Rimadyl - 2.2mg/kg) was given for additional analgesia. The dogs were monitored postoperatively until recovery, and then transferred to a boarding area.

After the surgical procedure (spay or neuter), dogs were kept at the OSU-VMC for 4 days. All dogs underwent daily physical examinations and the standard of care at the OSU-VMC, bleeding scores were recorded once a day by the same person (LM), and the surgical areas were photographed digitally.

Dogs were randomized (using a randomization table) to receive either EACA (500 mg PO q8 hours for 5 days starting the night of the surgery) or placebo, which consisted of lactose. Both the EACA and placebo were packaged into gelatin capsules of identical size by the Veterinary Medical Center Pharmacy, and labeled as drug “A” and “B”. The
clinicians were blinded as to the type of drug administered.

Venous blood samples (6 ml) were obtained at 24, 48, and 72 hours after the surgical procedure from the external jugular vein using a 20-gauge needle and a 6 ml plastic syringe; 4.5 ml of blood were immediately placed into a 3.2% buffered sodium citrate glass tube. They were stored for 30–45 minutes at room temperature in a tube rack and analyzed with the TEG within 30-45 minutes of collection. After TEG analysis the residual blood samples were centrifuged (1,380 g for 10 minutes) within 45 minutes of sampling, and plasma was stored at -80 Celsius for other hemostasis assays (OSPT, APTT, FIB, plasminogen and antiplasmin). CBCs were performed with 0.8mL of EDTA blood in a LaserCyte, and duplicate packed cell volume (PCVs) were run from the remaining blood.

Although there is no standardized scale to evaluate the severity of bleeding in dogs, a system with scores ranging from 0 to 4, was adapted from the one proposed by Buchanan and Adix for children with idiopathic thrombocytopenic purpura and recently validated in Greyhounds (Table 2).67,68

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Definitely no new bleeding</td>
</tr>
<tr>
<td>1</td>
<td>Questionable new petechiae or bruising</td>
</tr>
<tr>
<td>2</td>
<td>Definite new cutaneous and/or mucosal hemorrhagic lesions</td>
</tr>
<tr>
<td>3</td>
<td>Moderate to severe cutaneous or mucosal bleeding without measurable decline in hematocrit (HCT)</td>
</tr>
<tr>
<td>4</td>
<td>Severe external bleeding of sufficient magnitude to decrease HCT by ≥6% points</td>
</tr>
</tbody>
</table>

TABLE 2-BLEEDING SCORE MODIFIED FROM BUCHANAN AND ADDIX 68
Dogs were classified as non-bleeders if they had a bleeding score of 0 or 1, and as bleeders if they had a score of $\geq 2$ (Table 1). The final bleeding score assigned to each RRG corresponded with the highest score recorded during the postoperative period.

**3.2 Statistical analysis**

The primary statistical analysis was to test if there is an association between the hematological and hemostatic parameters measured and the drug treatment (EACA vs. placebo), whether or not the dogs bleed after surgery, and the time (day 1 to 3). All two-way interactions between treatment, bleeding, and time were included in the model and were kept in the model, if significant. Due to longitudinal nature of the data, we used a random-effects (slope and intercept) linear regression model to test the main effects and interactions.

Random-effects models take into account the variability within and between dogs in order to estimate the standard error used to test model coefficients. The model also adjusted for the baseline hematological parameters. This model allowed us to test main effects, interactions, and whether slopes (outcome per time) over treatment or bleeding were significantly different from each other and whether the individual slopes were significantly different from zero. Outcome differences across treatment and bleeding on day 3 were also tested using this model. A bleeding prediction model using baseline parameters was developed using logistic regression. All analyses were run using either Graph Pad Prism software or Stata 11.1.
The variables that were included in the model were treatment, gender, weight, age, color, month of surgery, CBC (RBC, HCT, Hb, MCV, MCH, MCHC, RDW, Reticulocytes, PLT, WBC, NEU, LYM, Mono, EOS) and TEG (R, K, angle, MA, G, LY30, LY60).

3.3 Results.

3.3.1 Signalment and prevalence of postoperative bleeding

One hundred Greyhounds were evaluated. The EACA group included 32 females (64%) and 18 males (36%), with a median age of 3 years (range 2 to 4 years), and a median weight of 28.5 kg (range 26.8-32 kg). The placebo group included 32 females (64%) and 18 males (36%), with a median age of 3 years (range 2 to 5 years), and a median weight of 27.8 kg (range 26.6 to 31.7 kg).

None of the dogs experienced intra-operative or immediate postoperative bleeding; however, 15/50 RRGs (30%) in the placebo group had delayed postoperative bleeding 36 to 48 hours after surgery, compared with only 5/50 RRGs (10%) in the EACA group. (P=0.0124)

In affected dogs, bleeding consisted of cutaneous bruising that extended from the area of the surgical incision toward the periphery (Figure 3). There was no bleeding from mucosal surfaces or in areas distant from the surgical site. None of the dogs required transfusion of blood components and the bleeding was self-limiting; bruising was still present at the time the dogs were discharged, 4 days after the surgery.
FIGURE 3: SURGICAL SITE IN A FEMALE AND A MALE GREYHOUND

(A) Image of the surgical site in one female and one male Greyhounds of the EACA group 36 hours post-surgery (bleeding score 0). (B) Image of the surgical site in one female and one male Greyhounds of the placebo group 36 hours post-surgery (bleeding score 3).

The estimated probability of bleeding based on the logistic regression model was 29.1% (95%: 17.4% – 44.4%) with placebo and 7.4% (95%: 2.7% - 18.8%) with EACA. The odds of bleeding increased 19% (OR = 1.18, P=0.050) for every 1 kg increase in the body weight of the dog, after adjustment for treatment; also the odds of bleeding
increased 18% (OR = 0.82, P=0.058) for every 0.1 unit increase in the baseline eosinophil count (EOS), after adjustment for treatment. Finally the use of EACA decreased the odds of bleeding by 79% (OR = 0.21, P=0.011) after adjustment for baseline weight and EOS, assuming that the average dog weight was 29.4 kg and the average EOS is 0.80k/µL. None of the other variables included in the logistic regression model were predictors nor had an association with the bleeding status.

3.3.2 Comparison of preoperative hemostatic and TEG parameters between EACA vs. Placebo groups and between bleeders vs. non-bleeders.

Preoperatively, there were no significant differences between EACA vs. Placebo groups nor between bleeders vs. the non-bleeders for any of the following parameters: RBC, HCT, Hb, MCV, MCH, MCHC, RDW, Reticulocytes, PLT, WBC, NEU, LYM, MONO, EOS from the complete blood count. R, K, angle, MA, G, LY30, LY60 from the TEG and OSPT, APTT, fibrinogen, antiplasmin, and plasminogen.

3.3.3 Comparison of preoperative and postoperative hemostatic parameters within EACA or Placebo group.

When the hemostatic variables were compared before and after surgery within the EACA and the Placebo groups, a random-effect regression model showed that there was a significant decrease in HCT, Hb, and reticulocyte count in both groups. HCT significantly decreased 2.2% (95% CI: -3.69 to 0.69; P=0.004), Hb decreased 0.5 g/dl
(95% CI: 0.77 to 0.199; P=0.001) and reticulocyte count decreased 2.8k/µl (95% CI: -4.47 to -1.15; P=0.001) after surgery. There were no significant differences between EACA and Placebo groups.

There was a significant increase in WBC and neutrophil counts after surgery in both groups. The WBC increased 0.5 k/µl (95% CI: -0.93 to -.080; P=0.020) and the neutrophil count increased 0.4 k/µl (95% CI: -0.74 to -.060; P=0.021). There were no significant differences in WBC or neutrophil counts between the two treatment groups.

3.3.4 Comparison of preoperative and postoperative TEG parameters within EACA or Placebo group.

When the TEG variables were compared before and after surgery, a random-effect regression model showed that there was a significant decrease in the K time and Ly60 in both groups after surgery. The K time drops 0.21 minutes per day in both groups. (95% CI: -0.32 to -.10; P<0.001). The Ly60 drops 0.30 units (%) per day in both groups (95% CI: -0.55 to -0.64; P=0.013). Neither K time nor LY60 were significantly different between EACA and Placebo groups.

3.3.5 Comparison of hematological and coagulation test slopes (Rate Change) over 24, 48, and 72 hours after surgery between EACA and Placebo

There were no significant postoperative differences between the slopes of EACA and Placebo for any of the following hemostatic parameters: RBC, HCT, Hb, MCV, MCH,
MCHC, RDW, PLT, WBC, NEU, LYM, MONO, EOS from the complete blood count, and OSPT, APTT, D-dimers, fibrinogen and antiplasmin.

The only hematologic variable that had significantly different slopes between EACA and Placebo group was the reticulocyte count (P=0.028); in both groups the reticulocyte count increased over time after surgery. There was not significant difference between EACA and Placebo reticulocyte slopes at day 3. The only coagulation variable that had significantly different slopes between EACA and Placebo group was the plasminogen (P=0.022) in both groups the plasminogen increased over time after surgery. There was not significant difference between EACA and Placebo plasminogen slopes at day 3.

3.3.6 Comparison of TEG slopes (Rate Change) over 24, 48, and 72 hours after surgery between EACA and Placebo

There were significant differences in the TEG slopes for R, K, Angle, MA, G and LY60 when compared at 24, 48, and 72 hours after surgery. The slopes for Ly30 were not significantly different between EACA and Placebo groups.

The slopes for R and K time were significantly different between treatment groups (P=0.050). The R and K time decreased over time in the EACA group, while it increased in the placebo group. The R time of the EACA group was 0.9 minutes shorter at day 3 compared to the placebo group (P=0.033). The K time of the EACA group was 0.7 minutes shorter at day 3 compare to the placebo group (P=0.019). (Figure 4)
Figure 4: TEG R and K time slopes of bleeders and non-bleeders in EACA and placebo group.
The angle, MA, and G slopes were significantly different between treatment groups (P=0.001, 0.001 and 0.006). The angle, MA, and G increased postoperatively over time in the EACA group, while they decreased in the placebo group. The angle of the EACA group was 4.7 degrees higher at day 3 compared to the placebo group (P=0.010). Although not significantly different, the MA of the EACA group was 1.9 millimeters larger at day 3 and the G value was 424 d/sc higher at day 3 compared to the placebo group (Figure 5).

The LY60 slopes were significantly different between treatment groups, (P=0.028). In both groups LY60 decreases over time after surgery, the EACA group was 0.7 units (%) greater than the placebo group at all time points. The slopes are parallel for treatment and placebo.
Figure 5: TEG Angle and MA slopes of bleeders and non-bleeders in EACA and placebo group.
3.3.7 Comparison of hemostatic and coagulation tests slopes (Rate Change) over 24, 48, and 72 hours after surgery between bleeders and non-bleeders.

We tested the effect, interaction, and differences in the slopes of hematological parameters between bleeders vs. non-bleeders at 24 (day 1), 48 (day 2) and 72 (day 3) hours after surgery, additionally we tested the differences between variables at day 3 (72 hours after surgery).

The only hematological variables that had significantly different slopes between bleeders and non-bleeders were the HCT, platelets, WBC, and neutrophil count.

The slopes of the HCT were significantly different between bleeders and non-bleeders (P=0.014), after an initial decrease after surgery, the HCT in the non-bleeders remained stable for 72 hours after surgery, while the HCT in the bleeders increased over time. Although not significantly different the HCT of the bleeders was 3.1 units higher at day 3 (72 hours after surgery) compare to the non-bleeders (Figure 6).

The slopes of the platelet counts were significantly different between bleeders and non-bleeders (P=0.025); the platelet count in both bleeders and non-bleeders increased over time after surgery, but the platelet count in the bleeders was 30.3 k/µl lower at day 3 compared to the non-bleeders (P=0.025).

The slopes of WBC and neutrophils were significantly different between bleeders and non-bleeders, (P= 0.002, and 0.023 respectively). The WBC and neutrophils were not significantly different at day 3 between bleeders and non-bleeders.

There were no significant postoperative differences between the slopes of bleeders
and non-bleeders for any of the following hemostatic parameters: RBC, Hb, MCV, MCH, MCHC, RDW, Reticulocytes, LYM, MONO, EOS from the complete blood count.

The coagulation variables that had significantly different slopes between bleeders and non-bleeders were OSPT (P=0.033) and APTT (P=0.026). The OSPT in both bleeders and non-bleeders decreased over time after surgery, the APTT in both bleeders and non-bleeders increased after surgery, there were no significant differences in OSPT and APTT between the EACA and placebo groups.

There were no significant postoperative differences between the slopes of bleeders and non-bleeders for any of the following hemostatic parameters: D-dimers, fibrinogen and antiplasmin.

**HCT SLOPES**

Figure 6: HCT slopes in bleeders and non-bleeders in the EACA and Placebo groups.
3.3.8 Comparison of TEG slopes (Rate Change) over 24, 48 and 72 hours after surgery between bleeders and non-bleeders.

None of the slopes of TEG variables evaluated were significantly different between bleeders and non-bleeders.

3.4 Discussion

None of the dogs included in the study experienced intraoperative or immediate postoperative bleeding; however, 30% of the dogs in the placebo group had delayed postoperative bleeding 36 to 48 hours after surgery, compared to only 10% of the dogs in the EACA group (P=0.0124). These results demonstrate that postoperative administration of aminocaproic acid (EACA) significantly decreases the prevalence of delayed postoperative bleeding in RRGs.

The HCT and HGB decreased after surgery in all dogs; however, the HCT slopes were significantly different between bleeders and non-bleeders (P=0.014). The rate of change of the HCT in the non-bleeders remained stable after surgery, whereas it increased in the dogs that bled (Figure 6). In normal dogs, the degree of intraoperative bleeding during spay and neuter surgeries is minimal; therefore, volume expansion with fluid therapy and splenic distention due to preanesthesia and anesthesia, as it has been described in dogs after administration of barbiturates, were likely the primary causes of the mild HCT and reticulocyte count decrease 24 hours after surgery in all dogs.

After the initial decrease, the HCT of the non-bleeders remained stable over time; in
contrast, in the bleeders there was mild, yet sustained increased in HCT over time (Figure 6). This is likely due to mild splenic contraction caused by the pain and discomfort from the subcutaneous edema, inflammation, and bruising; Stress and pain have been reported as causes of transit and mild increases in HCT in rabbits, people and dogs\textsuperscript{71-73} in addition, the resolution of the drug-induced splenic distention may have contributed to this phenomenon.

The slopes of the reticulocyte and platelet counts increased over time in all dogs, suggesting a bone marrow response to the mild blood loss during surgery; however, the fact that the reticulocytes increased within 48 hours of the surgery also suggests premature splenic release as a mechanism for these hematologic changes. These findings were statistically significant, yet not likely clinically relevant.

Although the WBC and neutrophil counts significantly increased 24 hours after surgery, they remained within the reference range for the breed. The slopes for WBC and neutrophil counts decreased over time in all the dogs, likely due to the administration of nonsteroidal anti-inflammatory drugs (NSAIDs) and decrease in the surgery-induced stress response.

The TEG parameters used in the present study to quantify the velocity of clot growth include R, K, angle, MA, and G. We confirmed that RRGs that received EACA for the prevention of bleeding in spay/neuter surgeries had significant differences in the slopes of these parameters 72 hours after surgery when compared to the dogs that did not receive EACA.

The significantly shorter slopes of R and K times 72 hours after surgery in the dogs
that received EACA, suggest that the initial clot starts forming more rapidly, and the clot strength is reached faster in dogs that received EACA. The differences in the angle, G, and MA slopes 72 hours after surgery in the dogs that received EACA, suggest that administration of EACA amplifies the strengthening of the fibrin clot. The rate changes in the R, K, angle, G, and MA suggest that dogs that receive EACA tend to become hypercoagulable, as has been demonstrated in human patients. 74,75

Our results are similar to what Hamada et al described in 1995 in 30 human patients undergoing upper abdominal surgery, that randomly received placebo or carbazochrome sodium sulfonate (CS) and tranexamic acid (TA), a drug similar to EACA. 76 Similarly to our study they found significant differences between presurgical and postsurgical TEG variables in both groups, showing that all the patients became hypercoagulable after surgery; similar to us, they did not find significant differences in the TEG parameters evaluated between the Placebo and the treatment group. However, their single analysis was done only 2 hours after administration of TA, which limits the interpretation of the results, since a single TEG analysis does not necessarily represent the coagulation status of the patient.

The trend towards hypercoagulability in the postoperative period has been described since 1977 77 and was supported with TEG analysis in 1987; 78 however, these studies were limited to a single TEG measurement and to a short postoperative period. Therefore, serial TEG evaluation was necessary to detect the postsurgical hypercoagulable changes over time in humans. A more recent study 79 demonstrated a continuous increase in clot firmness 2-6 days after surgery; Similarly to our study they
also established that OSPT and APTT did not reflect hypercoagulability after mayor surgery. Our results corroborate the fact that surgery triggers the coagulation process and dogs also develop a hypercoagulable state; they also support the fact that administration of EACA enhances the already established hypercoagulable state, therefore decreasing the prevalence of the bleeding in RRGs.

The proposed mechanism of hypercoagulability after surgery is associated with the local tissue trauma, release of tissue factor from damaged vessels, decreased blood flow, activation of inflammation, and compromised fibrinolysis. Tuman et al, demonstrated an association between hypercoagulability (determined by TEG) and the risk of arterial and venous thrombotic events in human patients; interestingly, in our study the TEG values remained within the reference ranges for Greyhounds and none of the dogs developed clinically detectable thromboembolic events.

The fact that the postoperative bleeding in the RRGs is not associated with abnormalities in routine hemostasis and coagulation assays, that it is delayed, and that there appears to be a low antiplasmin activity in the breed, suggest that the cause of the bleeding is not likely attributable to a primary or secondary hemostatic defect. Enhanced fibrinolysis has been the proposed mechanism responsible for the delayed postoperative bleeding in RRGs. We have demonstrated that RRGs that developed delayed postoperative bleeding had significantly lower activities of antiplasmin and antithrombin. However we have been unable to document increased fibrinolytic variables on TEG in our patients.

Surgical trauma, deficiencies of antiplasmin or plasminogen activator inhibitor type
1 (PAI 1), hyperactivity of fibrinolytic enzymes, and iatrogenic are among the causes of hyperfibrinolytic syndromes in people.\textsuperscript{80} In general enhanced fibrinolysis occurs when the balance between fibrinolytic activators and inhibitors is disturbed. In humans, increase levels of fibrin degradation products (or D-dimer) and low fibrinogen concentration are used to establish a diagnosis of hyperfibrinolysis.\textsuperscript{80} Although in our previous and it our current study we did not find differences in D-dimer concentration between bleeders and non-bleeders postoperatively\textsuperscript{23}, preliminary data using TEG had suggested enhanced fibrinolysis as a possible mechanism for the bleeding.\textsuperscript{23}

In 1961 Fichera et al using TEG in people demonstrated that EACA inhibits fibrinolysis, \textsuperscript{71,75} In 2007 another study demonstrated the \textit{in vitro} efficacy of EACA in human patients with severe hemophilia, showing that administration of EACA normalized the TEG patterns in affected patients.\textsuperscript{74} Interestingly, in our study we did not find evidence of enhanced fibrinolysis; LY60 decreased over time after surgery in both treatment groups.\textsuperscript{79}

We (and others) have been unable to document in dogs the association between increased D-dimer concentration, fibrinogen/fibrin degradation products (FDPs), and increased fibrinolytic variables on TEG that has been seen in humans.\textsuperscript{38,81} Based on these and other data, we propose that the TEG may not be a valuable tool to assess fibrinolysis in dogs.

Recently, a study using human plasma showed that derived TEG variables could be used more accurately to evaluate fibrinolysis and clot stability.\textsuperscript{82} The rationale is that the TEG assessment of fibrinolysis has been based on determinations based on the
maximum amplitude of the clot (MA), which is thought to be a subjective non-parametric measure,\textsuperscript{82} therefore Nielsen et al. used a methodology, referred to as the ‘elastic modulus’, to measure the velocity of clot growth and disintegration based on an equation determined by changes in amplitude. \textsuperscript{82} Further studies are needed in veterinary medicine to establish if elastic module evaluation will be a more effective method to assess fibrinolysis.

We concluded that administration of EACA significantly decreased the prevalence of delayed postoperative bleeding in RRGs undergoing surgery by amplifying the strengthening of the fibrin clot.

b. SNAP 4DX-IDEXX Laboratories, Westbrook, ME
c. Thrombelastograph, TEG Haemoscope, Niles, IL
d. LaserCyte, IDEXX Laboratories, Westbrook, MD
e. Cobas Mira Plus, ABX Diagnostics, Montpellier, France.
f. Diagnostica Stago Parsippany, NJ.
g. Buprenorphine HCL, Bedford Laboratories, Bedford, OH
h. Aceproject, Butler Animal Health Supply, Dublin, OH
i. Cephazolin sodium, Sandoz Inc, Princetown, NJ
j. Ketaset, Fort Dodge Animal Health, Fort Dodge, IA
k. Diazepam, Hospira Inc, Lake Forest, IL
l. Isosol, Vedco Inc, St Joseph, MO
m. Rymadil, Pfizer Inc, New York City, NY
n. Xanodyne pharmaceuticals, Inc, Newport, KY.
o. Vacutainer, BD, Franklin Lakes, NJ
p. Prism version 4.0, GraphPad Software Inc, San Diego, CA.
q. Stata Corporation, College Station, Texas.
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