

## Reviewer Comments – Resident Intramural Grant Proposals:

The following are edited to protect anonymity (substituting “animal” for the species, “X” for a specific disease condition and so on), 2 reviewers per proposal

### Review criteria:

The reviewers are answering the following questions and allocating points, 100 max:

1. Significance/Clinical Relevance with adequate justification for the **stated** hypothesis (20).
2. Soundness of Methods (20):
  - a. Testable hypothesis
  - b. Specific aims or objectives that when achieved will be adequate to test the hypothesis
  - c. Experimental design that is adequately controlled and has well justified experimental units
3. Clarity of Presentation (20)
4. Project is likely to be completed within 1 year; the likelihood of publication (20)
5. Budget is adequate, well explained and justified (20)

### Proposal 1: (not funded)

1. The significance and potential clinical relevance of this study is high. To be able to demonstrate significant decrease in pain using a novel procedure is laudable.
2. The methods appear reasonably sound, however, they raise several concerns, to my reading. First, the description of how the hypothesis is to be tested is vague. I assume that lower stress hormones and lower pain scores will be compared, but no mention is made of the magnitude of those differences that might support the choice of X animals in the study. Second, a brief reference is made to previous work supporting the pain scoring, but it appears that differences were found in animals receiving different types of analgesia for full laparotomies. Did the work done here in castrated animals find differences using the pain score? If not, it must be wondered whether the differences in this study would be significant.

The specific aims section does not make a compelling argument for the methods or the outcome of this project.

While the experimental design presents a controlled study, it is not clear that the numbers are sufficient to achieve significant results.

3. The presentation is clear to convey the purpose of the study. What is lacking, from a grantsmanship standpoint, is a clear argument that the study will adequately test the hypothesis in question. What is a sufficient reduction in pain? Which parameters are most important? If the animal is not behaviorally in less pain, but one physiologic parameter is lower, have we demonstrated a clinically superior technique?
4. The project appears to be able to be completed in one year. The likelihood of publication is hard to gauge, as it is unclear whether significant results are likely or what the value of negative results would be.
5. The budget does not appear entirely adequate, as it uses department resources for surgery without any recovery of costs.

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1. **Significance/Clinical Relevance:** The basis of this proposal is related to current efforts in assessing and addressing pain in animals, specifically in this case regarding animals undergoing elective laparoscopic ovariectomy. The main thrust of this proposal is to compare the pain response in animals undergoing 2 different methods of mesovarian ligation. The authors state that while no objective assessment of differences in post-operative pain between techniques have been performed, anecdotally differences are believed to exist. The authors indicate that other researchers have demonstrated that some methods of ovarian pedicle and uterus ligation result in less pain postoperatively, however while the reference cited involves 2 different ligation techniques, it also involves 2 different surgical approaches (one via celiotomy and one via laparoscopy). Appreciation of the authors' anecdotal evidence is difficult since they make no comment regarding the level or degree at which differences may exist. It is therefore difficult to predict if differences in pain between ligation techniques will exist, can be measured, or will be clinically relevant.

2. **Methods:** This project will involve 20, healthy animals that undergo laparoscopic ovariectomy. A randomized comparison will be made of two different methods of hemostasis, including hand tied ligatures or a vessel sealing system. Ten animals will be employed per method. Assessment of pain will include recording of temperature, pulse and respiratory rates, and gastrointestinal auscultation at 1, 2, 4, 8, 12, 16, 18, 24, 32, 36, 40, 44, and 48 hours. A numerical rating scale for behavior will be employed from observations collected at the same time as those for pain assessment. Plasma cortisol levels will be collected for analysis (commercial RIA)

from an indwelling catheter at 0, 2, 4, 8, 12, 16, 24, 36 and 48 hours. The authors propose to use a 2-way repeated measures ANOVA to compare the 2 ligation methods.

This project does not mention the concept of negative controls which could arguably be necessary to consider the effects laparoscopic surgery, irrespective of the type of ligation protocol. The authors should provide a rational argument or at least discuss the reasoning behind not including controls in this study.

No rationale is provided with regards to sample size other than the group consists of a limited number of animals.

Minimal detail is provided with regard to surgical techniques or materials.

The authors do not mention any other factors aside from pain, which might be considered when choosing a particular ligation or hemostasis technique. One would assume that additional considerations exist.

The sampling time periods and duration for assessment of pain and behavior are at times random and unexplained.

No information is provided with regards to the radioimmunoassay for cortisol aside from the fact it is "commercial". It is unclear if the investigators will be conducting the assay themselves or if samples will be sent out. One must assume the latter. Either way, the methodology of the assay should be provided to ensure adequate interpretation and controls.

**Hypothesis:** Animals undergoing ligation with ligatures will exhibit more postoperative pain than those that undergo the sealing technique as evidenced by physical exam, hormone parameters, and behavioral scoring.

It would be helpful if the authors clearly stated the hypothesis in a stand-alone area of the proposal, ideally prior to the specific aims. Mentioning the concept of a hypothesis in several areas of the grant allows flexibility in interpretation, which is not generally advantageous to the authors. Aside from this, the suggested hypothesis is likely testable.

**Specific Aim:** Assess the pain response in animals undergoing two hemostasis techniques for laparoscopic ovariectomy.

Similar to the hypothesis, the specific aim(s) in this proposal should be provided as specific, stand alone statements. This particular specific aim basically summarizes the hypothesis and does little to actually reflect how the hypothesis will be tested. As mentioned earlier, there is no mention or consideration of controls with regard to experimental groups or even methods of analysis.

3. **Clarity of Presentation:** This proposal is written in a relatively clear and concise manner, however the content and design is at times superficial or incomplete. Most of the content issues have been previously mentioned. Overall, it appears that this grant may be more of an afterthought to take advantage of the fact 20 animals from a single source will be available for surgical sterilization.

4. **Project likely to be complete in 1 year & likelihood of publication:** It appears reasonable that this work could be completed within a year. It is unclear whether this proposal will result in a publication. If differences exist which are statistically significant, a publication could be submitted. Alternatively, if differences are not statistically different, it is unlikely a publication could be produced.

5. **Budget:** The budget lacks detail. For example, it is unclear why a boarding fee of \$5/animal/day will be charged when the current rate for hospitalization is \$37/animal/day. Some rationale or evidence of agreement must be provided when fees are proposed that are not in accordance with the existing fee structure.

No allowance is made for the radioimmunoassay costs.

## **Proposal 2: (not funded)**

The hypothesis is well stated, the research group proposes to determine if lap ovx will result in an increased cell count and tp in peritoneal fluid from preop. The objectives indicate that the animals will have lap ovx by either one of two methods, and that tp and cell count will be done before and after, and that temporal changes will be made. The justification for the hypothesis is that changes in peritoneal fluid parameters occur after lap ovx, and peritoneal fluid parameters can be used to diagnose changes in abdominal "pathology", such as abdominal pain. As I understand the justification, abdominal pain can occur after surgery, and thus it is important to know which changes of constituents of peritoneal fluid are a result of the surgery and which are due to abdominal pain. If the later is correct, then I am puzzled by the hypothesis and design of the study.

As it stands, the hypothesis can be effectively tested. However, I am not sure about the statistical modeling. Authors indicate that 2 way ANOVA will be used. The two factor analysis of variance is used to account for a

block effect, either in a completely randomized design or randomized block design. The current study does not fulfill either requirement since animal is her own block, with no replicates within block, and therefore nothing can be blocked on, and there is no randomization within block. Day and animal are linked. A more appropriate model would be a general linear, with parameter as the dependent variable and day and mare as independent variables. The authors feel that changes in the dependent variables from baseline will approach zero by day 7. I assume therefore that this is the value of most interest. I am assuming the values on Days 1-5 will be elevated as the authors suggest and will be of little utility in trying to differentiate between post surgery recovery and true abdominal pain. If this is correct, then, either the GLM or the 2 way ANOVA alone will not provide the analysis they desire. A GLM followed by a test of the means, such as a Duncan's Multiple Range Test, would be required. But I also wonder about the variation of the independent variable responses. The authors indicate that the pre-op values may deviate from normal due to the pre-op process itself. Thus the question has to be asked, is the single baseline value adequate for comparison? The authors never question this. Would the authors have been better off having included a control population and a treated population? I would argue yes.

Now I realize, the study is to piggy back on another study comparing 2 types of surgery procedures for lap ovs. So, the authors are tied to a model they can not change, and it would appear they are trying to obtain some additional data to be efficient. This in itself is a good thing. The problem is that it appears that by piggy backing on to an existing experiment the authors have created an awkward design that might not provide the information desired. If the authors find that there is a change in peritoneal fluid parameter concentrations, they will have provided evidence to accept their hypothesis. The real question becomes, of what value is that information? As I understand it, only the day 7 value is of critical importance and this is not indicated in the hypothesis.

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1. **Significance/Clinical Relevance:** This proposal focuses on investigating changes in peritoneal fluid parameters post laparoscopic ovariectomy. Normal parameters for peritoneal fluid analysis have been established and previous studies have indicated that these parameters can be altered after specified abdominal surgical procedures. However, to date no research has been conducted to specifically evaluate changes in peritoneal fluid parameters after laparoscopic ovariectomy. The overall goal of conducting this study is to determine if the changes in peritoneal fluid parameters observed post laparoscopic ovariectomy can be differentiated from values which may be observed during pathologic abdominal conditions resulting in clinically relevant peritonitis. This point could be strengthened in the grant.

2. **Methods:** This project will involve 20, healthy animals that undergo laparoscopic ovariectomy. Two different methods of hemostasis will be employed (10 animals per method), however, the authors state the study is not designed to compare changes in peritoneal fluid between methods. On the day of surgery, peritoneal fluid will be obtained prior to laparoscopy. After the procedure, peritoneal fluid samples will be obtained at 1 (24 hr), 3, 5, and 7 days post-operatively. Peritoneal fluid will be analyzed for total nucleated cell count and total protein. The proposed method for data analysis is a two-way ANOVA for each parameter using logarithmic transformation of cell count data.

The authors state that they will not examine differences in hemostasis technique because such a comparison is not clinically relevant and the number of samples too few. The present proposal is to be run in parallel with another study (same co-investigator) with the same animals, wherein differences in the two hemostasis techniques will be examined in relation to postoperative pain. It is unclear why two different hemostasis techniques are considered appropriate treatment groups when subjective scoring is used (pain score) but are considered a single treatment group when objective scoring (clinopathologic data) is employed that evaluates possible inflammatory changes in the body cavity undergoing treatment. The investigators should at least examine the data which is readily available rather than omit it and presume there is no clinical or other relevance.

The authors state that a previous study evaluating serial abdominocentesis did not show any variation in normal peritoneal fluid parameters, thus the lack of necessity for a negative control group. However, because the proposed study is conducted with different individuals, animals, location and time, a negative control is preferred. Furthermore, it is unclear if the same techniques for abdominocentesis as that stated in the earlier study will be used in the present study.

The authors state the method of analysis will involve a two-way ANOVA. It is unclear with the proposed sampling schedule, why a repeated measures ANOVA will not be used.

**Hypothesis** - "Laparoscopic ovariectomy will result in an increase in total nucleated cell count and total protein of peritoneal fluid when compared with pre-operative values".

While the hypothesis may be testable, the outcome is arguably predictable based on the nature of the procedure and earlier research. The authors state that previous investigations have determined that peritoneal fluid

parameters can be altered subsequent to surgical procedures and that even carbon dioxide insufflation during laparoscopy induces a significant increase in total nucleated cell count, even without manipulation of abdominal viscera. Results of the hypothesis as stated will have limited meaning and relevance. One suggestion would be to state in the hypothesis the fundamental purpose of the study; to be able to distinguish expected changes in peritoneal fluid parameters after laparoscopic ovariectomy from changes associated with clinically relevant peritonitis.

#### **Specific Aims:**

SA1. "To determine if there is a difference in peritoneal fluid analysis parameters before and after laparoscopic ovariectomy utilizing two different methods of hemostasis and subsequent tissue removal."

The specific aim can not adequately test the hypothesis as it is directed at only "determining a difference", wherein the hypothesis states an increase in various parameters. It is also unclear why this specific aim mentions utilization of two different methods of hemostasis when the body of the grant suggests that a comparison is not within the scope of the study, is not clinically relevant, and the number of samples too few.

SA2: "To determine the time course for changes in peritoneal fluid following laparoscopic ovariectomy utilizing two different methods of hemostasis and subsequent tissue removal."

Although the overall gist of this aim could test the hypothesis, as stated it is vague and makes the assumptions that changes will occur. This aim also mentions the utilization of two different hemostasis methods, which is not associated with the hypothesis and is discounted in the body of the grant.

Overall, the hypothesis of this grant is needs to be strengthened and more clearly defined. Whether or not the specific aims can test such a hypothesis is moot.

**3. Clarity of Presentation** The body of the grant is appropriately composed. In the Background and Significance, the authors build an adequate argument for the need to analyze peritoneal fluid changes during laparoscopy such that they can be distinguished from pathologic changes observed during peritonitis. That said, the authors would be advised to consider as well as mention the possibility of an overlap of data that could cloud any eventual interpretation. The Design and Methods section adequately states the experimental process. The investigators state that each animal will serve as its own control, however, as previously stated, no negative (non-treatment) control is included. The investigators indicate that preoperative evaluation will include a CBC, serum chemistry and ECG. However, no mention is made with regard to the analysis and use of this data. It is also unclear why these test might not be repeated after the procedure to further evaluate the effect of the surgery. A minor point, the investigators mention that a caudal epidural using detomidine will be employed, however what compound will be used to "q.s. to 10 ml) is not identified. The investigators predict that both the total nucleated cells and total protein of peritoneal fluid will increase post operatively then presumably return to normal values by day 7. However, it is not stated how such an increase might compare to pathologic changes associated with clinical peritonitis. While one might argue that the purpose of the study is to establish these values, this should be more clearly stated as a testable hypothesis. The literature cited appears adequate.

**4. Project likely to be complete in 1 year & likelihood of publication:** Although the authors do not provide documentation that an arrangement has been made for the use of the 20 animals, one can assume or hope that access to this number of animals will occur. Assuming animal access, there is no logical reason to think that 20 laparoscopic ovariectomies can not be completed within a 2-5 month period.

Data from this project may be publishable as a brief communication, observational study. It is unlikely that it can will provide sufficient data to serve as a stand-alone, research report. Combining the data from this study with that of the post-operative pain study (same co-investigator) utilizing the same animals, may enhance publication chances.

**5. Budget:** The budget provided in this proposal contains no detail and obviously rounded numbers.

#### **Proposal 3: (not funded)**

**1. Significance/Clinical Relevance:** X is a common condition of animals and is often a result of altered gastrointestinal motility. The author states that gastrointestinal motility is affected by disturbances in the interactions between hormonal, vascular and neuromuscular systems which stimulate intestinal contractions. Furthermore, alterations in motility can be caused by pathologic conditions as well as drugs, including alpha-2 adrenergic agonist sedatives.

Alpha 2 agonist sedatives are commonly used to chemically restrain animals during examinations for X. The goal of this project is to use trans-abdominal ultrasound to document changes in gastrointestinal motility in

animals administered alpha-2 agonists alone and in combination with opioids. The author reviews the literature regarding gastrointestinal motility physiology, studies documenting the effects of alpha-2 adrenergic agonists and opioids on gastrointestinal motility, and the use of ultrasound to document animal (and human) gastrointestinal motility. Overall, the author provides a broad, relevant review of the literature.

The author somewhat modestly states the overall importance, usefulness and relevance of this study. In future grants, it is suggested that modesty never replace candor, yet an increased level of literary enthusiasm is always a great advantage.

Ultrasound is one of many modalities currently used to examine the gastrointestinal tract of animals. The effects of alpha-2 agonists (including xylazine, detomidine, and romifidine) and butorphanol (alone and in combination with xylazine) on animal gastrointestinal motility have been described. The value of this study will therefore not lie in novel or unexpected results, but solely involve the "ultrasonographic characterization" of the effects of various alpha-2 agonists (with or without opioids) on gastrointestinal motility.

**2. Methods:** The study will utilize 5 animals administered 4 treatments (plus 1 negative control treatment) in a randomized, Latin Square design. The author has accounted for time of examination, recovery between treatments, optimal time for evaluating progressive digestive motility, ultrasound techniques, definitions of motility, and blinding of the examiner. Continuous variables will be analyzed using ANOVA with a defined minimum detection difference of 3+/- contractions per minute.

**Hypothesis:** "...there will be a significant difference in number of contractions of the gastrointestinal tract after administration of alpha-2 agonist sedatives (xylazine, detomidine, romifidine) when compared to the saline control with minimal effects on the gastrointestinal wall thickness. We also hypothesize that detomidine will have a longer duration of effect than either romifidine, xylazine or xylazine/butorphanol."

**Specific Aim:** "...document alterations in normal gastrointestinal motility, using transcutaneous abdominal ultrasonography, following administration of four different sedation protocols, including alpha-2 agonists alone and in combination with butorphanol."

The hypothesis should be stated as a clear, concise, testable statement. The specific aims should follow the hypothesis and, upon their completion, be designed (and stated) in such a way as to test (accept or refute) the hypothesis. While the gist of the authors "hypothesis" is apparent, the format is awkward and weak. Like the hypothesis, the gist of the specific aim is available.

The authors should always include thoughts regarding possible pitfalls or problems that may be encountered during the course of the project.

**3. Clarity of Presentation:** Overall, this proposal is clearly written. However, as previously mentioned the hypothesis and specific aim are the weakest parts of the document. The author must recognize that a clear and effective hypothesis and specific aim(s) will serve as a focal point of a proposal. A clear and concise hypothesis and aims sets the stage for a compelling and persuasive grant.

**4. Project likely to be complete in 1 year & likelihood of publication:** There should be no difficulty in completing this proposal in a year. That said, it is always prudent to allow for the possibility of pitfalls, delays or other problems. The data from this proposal should reasonably result in a publication.

**5. Budget:** The budget does not account for any cost of animals (board, housing, hospitalization, transportation, etc.). In the event there are no such costs for experimental animals, the authors should clarify this. The authors simply include the cost of bottles of experimental drugs, rather than approximate the amount which will be used for the study. Aside from these points, the budget appears adequate.

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The investigators propose to evaluate gastrointestinal motility of sedated animals using transcutaneous ultrasound. The hypothesis is that sedation will alter GI motility, as measured by ultrasound. The subtext is that ultrasound will be able to adequately measure this, establishing baselines for future ultrasound exams.

Evaluation

1. The significance and potential clinical relevance of this study was difficult to understand as the proposal is written. The beginning of the proposal states the hypothesis, then cites much data showing that one can both measure motility with ultrasound and that motility is altered by administration of sedatives. What was not clear until very late in the proposal is that it is important to show that this can be detected by ultrasound, which appears to be the more relevant question in this proposal.

2. The methods appear reasonable. The hypothesis is testable by the nice Latin square design of the study. I would strongly suggest that the investigator consider redesigning the Latin square such that each treatment follows every other treatment at some time in the study. This would eliminate bias generated by any holdover

effect of a drug. Also, the way the hypothesis is stated, a two-tailed test is necessary. Frame the question as a one-tailed test. Assuming the calculations are correct and the expected difference and variance are correct, the sample size appears adequate. Having said that, the work really appears to propose evaluation of ultrasound's ability to measure already demonstrated differences in motility by the drugs listed. Given the previous work as the standard, then, the study will evaluate whether ultrasound can detect these differences.

The specific aims section did not engender enthusiasm for the project. With so little preceding background, it was unclear what the benefit of this study would be. This was then followed by description of the changes seen in GI motility with sedatives, causing me to wonder what the use of the study was. It was not until late in the proposal that it was clear that the experiment will confirm transcutaneous ultrasound as a method to measure the known GI alterations.

The design is adequately controlled and fairly well conceived with the Latin Square. See above for comments about modification. The methods would be enhanced by a discussion of potential anticipated problems.

3. The order of the proposal is not at all clear. More description prior to the specific aims to frame the problem would be extremely helpful to clearly understanding the proposal. The writing is otherwise precise, but not adequate to sell the idea.

4. The project appears to be able to be completed in one year. The likelihood of publication is moderate, unless the findings are strikingly concise.

5. The budget appears adequate, although it is not clear if the exams are being performed at a discounted price.

#### **Proposal 4: (funded)**

The proposal is well written. The ideas, justification, and hypothesis are clearly stated. There is no doubt as to what will be studied, why and how. No assumptions have to be made. However, improvements are indicated.

There are a few typos or grammatical errors. The hypothesis that is stated in the abstract is not the hypothesis that is stated in Section B. The former is more detailed and suggests proteoglycans will be measured, uterine pH will be measured, etc. I understand why there is mention of these points, but they should be restricted to a discussion or introduction or justification, not inserted into a hypothesis. The hypothesis should be more clear in Section B by indicating that the change in uterine "pathology" in response to enrofloxacin will be studied in clinically normal females.

The significance is well stated and discussed. Some background is missing, especially some discussion on the study by XX. This later study seems pivotal. Obviously the author of the proposal does not believe the results of the XX study are based on a well designed, impeccably tested, study. Otherwise, the author would not intend to repeat the study in some fashion. So, what are the weaknesses of the XX study? How will the current study build upon the errors of the past and provide more convincing evidence, yea or nay? I recognize the author clearly states that the experience of practitioners in the field are not consistent with the XX study. But what is the author's assessment of the XX study? Why would the results by XX be inconsistent with field practitioners? Is it the single dose of drug given, as opposed to the 3x regimen proposed? Is the later regimen more consistent with what is done in the field? Answers to these questions would be helpful.

The research methods are well outlined, although again some detail would be helpful. The histo grading scale is clear, but deficient. How is inflammation assessed, by the number of leukocytes present? Is there an assessment of necrotic tissue, how?

What is the effect of biopsy itself on inflammation, can this be controlled? There is a paragraph on ultrasonography exam, this unnecessary since it is not part of the proposed study. The statistical design could be improved, or the description could be improved. I am not sure what term will be used to analyze the repeated measures effect in an ANOVA. The author does not specify. I believe a mixed model, using female(day) as a random effect would be more appropriate. Additionally, one mixed model could be performed, using data from all days as the independent variable in the model with histo score as the dependent variable.

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**1. Significance/Clinical Relevance:** Bacterial endometritis is a significant cause of reduced fertility in females. Intrauterine infusion with antimicrobial drugs is commonly employed during treatment of equine endometritis. A recent study suggests that the fluoroquinolone enrofloxacin is the only antimicrobial to which common endometritis pathogens are not resistant. However, despite positive in vitro sensitivity data, little information is available regarding the pharmacokinetics and safety of many drugs that are used via an intrauterine route. Therefore the use of these drugs, including enrofloxacin is often empiric.

The author cites a 2002 report which demonstrated an insignificant inflammatory response to the one-time intrauterine infusion of enrofloxacin in females. In another study, females treated intravenously with enrofloxacin demonstrated adequate endometrial MIC concentrations of enrofloxacin and ciprofloxacin (its metabolite). Yet a different study demonstrated intravenous enrofloxacin attained only moderate uterine levels and may not have been sufficient for more severe infections. The author cites the last study as a reason for considering the intrauterine use of enrofloxacin. The author goes on to state that intrauterine use of enrofloxacin is often contemplated by veterinarians as demonstrated by the messages on practitioner list servers. The reasoning of these last two points is somewhat obscure.

There appears to be a rational argument for investigating the use of enrofloxacin as an intrauterine infusion in females. Chief among these is not necessarily the level of messages on the internet, but rather the safety, sensitivity pattern and efficacy of the drug as it seems to be used widely in an extralabel manner. The authors state that field observations suggest that intrauterine enrofloxacin has deleterious effects on the endometrium and these could be related to its high pH (pH 10.4), inhibition of cell proliferation, and induction morphological changes.

Results of this project may elucidate the risks of intrauterine enrofloxacin and help put an end to the empiric, extra label use of this drug in animals.

**2. Methods:** The study will utilize 9 adult, healthy females. The females will undergo physical examinations, clinical parameter measurements, and initial transrectal ultrasonographic reproductive examination. Transrectal ultrasound and uterine biopsy will occur on day 0, prior to infusion with enrofloxacin. Enrofloxacin will be administered daily for 3 consecutive days, during which daily transrectal ultrasound will be conducted. On day 4, (24 hrs post last enrofloxacin infusion), females will be ultrasounded and biopsied again. This second biopsy will be compared to the pretreatment biopsy to evaluate acute changes in uterine histologic structure. On days 14 and 60, females will again undergo ultrasonographic examination and biopsies. These latter biopsies will be compared to pretreatment biopsies to evaluate chronic changes in uterine histologic structure.

For practical purposes, normal, healthy females will be evaluated. The author should however mention possible differences that might exist when comparing the effects of enrofloxacin in normal and animals suffering metritis/endometritis, since animals with disease are most likely going to be receiving treatment. This simply demonstrates that the author is aware and has considered the limitations of an experimental model.

It is unclear how the author arrived at a total of 9 females to examine. The reasoning for the experimental unit size should be stated.

The authors state that enrofloxacin will be administered at a dose of 2.5 mg/kg in "15 ml of solution" for infusion. It is unclear what this "solution" consists of. At a dose of 2.5 mg/kg of standard stock drug for typical females, the total volume would be 12.5 to 13.75 ml of solution. To obtain a total volume of 15 ml, something else must be added. This information needs to be provided or the situation clarified. Furthermore, what ever this other solution is, it may also serve as a control medium (see next comment).

No control groups are provided, either positive or negative. Failure to include a control is a significant flaw when attempting to evaluate the effects of any particular treatment.

The author states that ultrasonographic examinations will be videotaped for later interpretation by an individual (rater) blinded to the treatment. It is unclear what the rater is being blinded to, since there is only one treatment (i.e., no controls). One might assume that it is the time period after treatments? This needs to be clarified.

**Hypothesis:** "...daily intrauterine infusion of enrofloxacin in the mare induces acute uterine endometrial inflammation and chronic fibrosis."

**Specific Aim 1:** "...determine if intrauterine infusion of enrofloxacin in the female induced significant acute endometrial inflammation."

**Specific Aim 2:** "...determine if intrauterine infusion of enrofloxacin in the female induces significant chronic endometrial inflammation and fibrosis."

Once the hypothesis and specific aims are separated from the text, it becomes apparent that they are very similar. The author should note that specific aims should be designed and articulated such that, when achieved, they will successfully test the hypothesis. Terms like "measure", "score", etc. are more appropriate than "determine".

The author does include an "expected results" section. This section should not only communicate what the investigators expect to happen, but also potential pitfalls or alternate events. Doing so indicates that the investigator is aware of the limitations of experimental designs and procedures and that data from 9 animals may not necessarily represent what will happen in all females.

3. **Clarity of Presentation:** The proposal is clearly written, utilizes an appropriate literature review, and has a defined end point. The hypothesis is clear, defined and testable. The specific aims, while understandable, could be restated in a manner that test the hypothesis when finished. The most significant shortcoming with the proposal is therefore not in its presentation, but rather with the design (lack of controls), and to a degree, the justification for the study.

4. **Project likely to be complete in 1 year & likelihood of publication:** Assuming that animals are available, there should be no difficulty in completing this proposal in a year. It is possible that data from this proposal can result in a publication, however, the lack of experimental controls may be an impediment.

5. **Budget:** The author does a good job detailing the various costs of that would be included in conducting this project. The information is very thorough and complete. It is unclear however, how the final monetary request is arrived at. Specifically, if the experiment will cost approximately \$15,500, and the request is for approximately \$5500, where will the other \$10,000 come from or be covered by? This needs to be clarified prior to consideration for funding.

### Proposal 5: (funded)

1. **Significance/relevance:** The applicant has provided adequate rationale for the proposed work.

2. **Soundness of Methods:** How was the decitabine concentration and treatment duration determined? It seems prudent to include more than one concentration or adequately justify the use of a single concentration and treatment duration.

\*\* see #4 below regarding student's participation in this project

3. **Clarity of Presentation:** Overall this is a well written proposal. Specific aim 1 contains a typo or grammatical error (the first sentence after specific aim 1 is described would read better without the word "the" between 'animal' and 'disease').

The second specific aim is vague—this could include everything from altered expression of these genes, other genes, changes in cell viability, etc.

Figures 1 and 2: These are not able to be interpreted by the reviewer. The applicant should either provide color copies of the proposal or format figures such that color copies are not necessary (for example, striped bars or the use of white, gray and black).

The authors describe treatment of cells with decitabine (which the reviewer had to research to determine if this was the same drug as "5-aza" as the drug had been referred to in the specific aims section).

4. **Project likely to be completed in 1 year:** It is unlikely that the applicant would be able to complete the project in one year as a clinical resident. It is not clear how much of the project the applicant will be conducting herself and how much will simply be performed by a technician.

5. **Adequate/justified/complete budget:** No concerns—budget is detailed and adequately justified.

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1. **Significance/Clinical Relevance:** This proposal focuses on the regulation of cell-cycle control genes, specifically the finding that hypermethylation of cytosines in promoter regions causes silencing of these genes. Importantly, these epigenetic alterations are potentially reversible by drug therapy, unlike genetic mutations.

The authors possess data from a pilot study which demonstrates hypermethylation within the promoter regions of 2 genes (Lhx2 and POU3F3) from cases compared to normal tissues. These same genes have been found to be hypermethylated and silenced in human patients where they appear to function as suppressor genes.

Based on their clinical findings and preliminary data, the authors propose to investigate the biological significance of hypermethylation and determine the effects on gene expression using cell lines. The authors have established 2 such cell lines. These will be used to determine if hypermethylation decreases or silences expression of the Lhx2 and POU3F3 genes, and if treatment with a demethylating drug increases expression of these genes. The authors state that this work would be the first functional examination of hypermethylation induced dysregulation of these two genes. If the authors data support their hypothesis, this work may serve as a foundation for further work devoted to diagnostic screening tools for X, novel treatment options, and support for utilizing animal X as a model for human X.

The authors clearly state the significance and relevance of this project and build a cogent argument for not only it, but future work as well.

**2. Methods:** Similar genetic dysregulation of hypermethylated genes have been described in human X patients and preliminary studies by the investigators has demonstrated increased hypermethylation in Lhx2 and POU3F3 in animal X compared to controls. While gene expression in cell lines may differ from that of biopsied, in situ lymph nodes, the preliminary data provides a strong rationale for the direction and success of this project.

The investigators appear to have gathered the appropriate knowledge, techniques, equipment and supplies to successfully conduct and complete this project. The investigators have obtained the necessary cell lines for the project and have assembled and trained a team in the necessary methodology.

The authors indicate that triplicate samples of cells from cell lines and normal animal controls will be analyzed. While the sampling of the cell lines in triplicate appears adequate, it is unclear if sampling of the control tissues means 3 samples from the same site, or 3 different sites. It is also unclear what is meant by, "a non-parametric Mann-Whitney will be performed to establish the methylation in the case samples". This statement clearly needs to be re-written. Finally, the authors do not provide thoughts or considerations with regard to possible pitfalls or unexpected results. Doing so demonstrates a more thorough and pragmatic approach to any research project.

**Hypothesis** - "hypermethylation decreases expression of Lhx2 and POU3F3 in cell lines compared to tissue samples", and that "suppression can be reversed by treatment with the demethylating agent,...(5-aza)."

**Specific Aims:**

SA1. Determine the relationship between hypermethylation and gene expression of animal Lhx2 & POU3F3 in two NHL cell lines (OSW and CL-1).

SA2: Determine the effects of application of the demethylating agent 5-aza in Lhx2 & POU3F3 gene expression in cell lines and normal samples.

The hypothesis is clear, concise and testable. The specific aims, when completed will adequately support or refute the hypothesis.

While the experimental design includes negative control tissues, no mention is made considering positive controls within the assays. The investigators state that samples will typically be assayed in triplicate and that 3 normal animal tissue samples will be evaluated as controls. The reasoning for choosing this sampling protocol is not provided. Finally, the investigators state that, "cell line genes with methylation proportions over 40% and expression four-fold lower than tissue, or increased by four-fold by decitabine treatment will be considered hypermethylated and down-regulated in a four quadrant plot analysis with confidence set at 95%". This statement is awkward, unclear and the rationale for the statement is not provided.

**3. Clarity of Presentation:** This grant is very well written, flows appropriately, and provides most needed information in a clear and concise manner. Most of the shortcomings have been identified previously including the legitimization of sampling, and how potential problems of pitfalls might be handled. Nonetheless, these issues have a marginal effect on the overall quality of the grant.

**4. Project likely to be complete in 1 year & likelihood of publication:** Assuming things flow as predicted, it appears reasonable that this work can be completed well within a year and result in the submission of a novel and interesting publication.

**Budget:** The budget is detailed and appears to cover the costs of the project adequately

**Proposal 6: (funded)**

The investigators propose to evaluate the difference in the effect on intrasite pressure (ISP) of two drugs at two levels. The hypothesis of this experiment is that agent 1 will result in a better ISP than agent 2. The proposal is overall well-written and straightforward in its presentation.

**Evaluation**

1. The significance and potential clinical relevance of this study is high. Determination of the agent with the least impact on ISP would have great potential relevance in management of clinical cases.

2. The methods appear sound. The investigator clearly states the methods to be used. What is lacking is a discussion of potential limitations and any plan to minimize the impact of those limitations. The statistical analysis appears adequate for the study.

The specific aims section is well introduced by the abstract. More discussion of the position of this proposal in an ongoing investigation of the drugs in the specific aims section would be useful to understanding the full impact of this research.

The lack of discussion of potential pitfalls in the research design makes me wonder what they might be. The fact that a standard deviation for ISP is known suggests that there are some problems with accuracy or precision of measurement that could confound the interpretation of this data.

3. The overall organization of the proposal is easy to follow. The significance and clinical relevance is clearly supported. The methods section bogs down in excessive use of acronyms, however. I would recommend for future grants to state the purpose of each measurement and calculation in clear English, as well as provide the equations themselves. For a non-clinician, this section is hard to follow.

4. The project appears to be able to be completed in one year. The likelihood of publication is difficult to judge. A company appears willing to make a large contribution to this project, but there is no mention of any potential publication limitations placed by the funding company. Do such limitations exist?

5. The budget appears adequate for the scope of the work. The large company contribution would allow a large project to be performed with less contribution from the department.

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1. **Significance/relevance:** This section was too generalized and did not convince the reviewer of the importance of this research. The proposal could be improved with specific examples of how important increased ISP is to the management of patients. The applicant discussed basic physiology but did not describe how results of this research would be beneficial. For example, which patients would benefit from this information? For how many patients is this a problem? How often do clinical complications occur in patients receiving drug 1?

2. **Soundness of Methods:** It is not clear why animals will be treated 4 times. The applicant describes two treatments (drug 1 – group 1 and drug 2 - group 2).

The applicant describes invasive measurement of cardiac output (and a number of other parameters such as CVP, PCWP, etc) yet there is no rationale for this procedure—it is not a part of the hypothesis or specific aims.

3. **Clarity of Presentation:** The title is too broad and does not reflect the proposal. The title implies that the study will compare the safety/efficacy of the two drugs in clinical cases. The actual proposal compares only one measurement (ISP) in healthy animals.

After looking at the budget it appears that there may actually be 2 studies going on—one that will determine drug levels (which explains the 4 treatments episodes) and one that involves the study proposed here. A company will be paying for part of this study.

4. **Project likely to be completed in 1 year:**

5. **Adequate/justified/complete budget:** The cost of the special catheters is not justified (see methods section review)

## **Proposal 7: (not funded)**

The author proposes to study two methods, X and Y, for assessing Z of an animal. The claim is that X is the standard test, but with increase availability of Y, it might be a suitable replacement and thus a comparison needs to be made. The justification is clear and straight forward but without sufficient support. The author indicates that the cost of X is less, how much, and is X is so accurate that it is needing replacement? What will be the full advantage of using Y? Would those clinics using Y not use X - does it save money by not using X if it uses Y? A stronger justification would have been helpful.

The hypothesis is clearly stated but not testable using the statistical model proposed. But more on that in a bit. The background and significance are also clearly stated, but lacking depth. This is a section where the author could expand and better state the case for the significance, reviewing the literature, and making the case that Y would ultimately be a better method of analysis than X. A question that came to my mind, is: Has X ever been compared to Y in other situations? This question and others could have been addressed in this section. The Research Design and Methods are well stated, but there are some weaknesses to address. I assume there is a fair bit known about the variation between multiple tests of Z using X and perhaps YI. Thus, with the knowledge of the variance, the authors could compute a power of the test and then calm the anxious reviewer wanting to know if 30 animals will be sufficient to determine significant differences if they were to exist. I recognize the authors speculate that the variability will be small given the same test will be used, last paragraph of the proposal. However, what is the variation between successive tests? Any? Has anyone measured this? Would this be something that should be evaluated in this proposal. Secondly, how can intra and inter test variability be determined using the paired T test? How can correlation be determined using the paired T test? Now I realize that the author has budgeted money for statistical consult. However, the consult should be done up-front, during

the experimental design phase of the proposal. No statistician, no matter how excellent, can rectify a failed statistical design after the study has been completed.

Moving from the pure statistical critique. A couple of other design questions: How will the authors determine which side to test? It appears X will always follow Y, will that have an effect, the order of testing?

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The investigators propose to compare measurements between Y and Z. The hypothesis is that the two are highly correlated. The clinical utility of the study is to establish a dataset of normal animals measured by both tests.

Evaluation

1. The significance and potential clinical relevance of this study is questionable. Failure to correlate would raise questions about which is most accurate or precise, but the study is not designed well to understand this. As neither represents a gold-standard, which will be considered to be less accurate if the null hypothesis, that they are not correlated, is accepted? Perhaps a better study is to look at cadaver animals by both methods and then using the gold standard method, which can only be done on dead animals.

2. The testing methods are reasonable, but the statistical methods are entirely inappropriate to the goal of the study. A paired T-test seeks to find differences, when correlation is what the investigator wishes to show. The need for triplicate measurements suggests inaccuracy in the measuring process and only tests the reproducibility of the methods. These would not be independent values, and cannot be assessed as such. A mean could be evaluated, but then the variability of the measuring process must be examined. A linear correlation would be a better test with single values for each measurement. Rights should be compared to rights and lefts to lefts, as each right/left pair is not independent. There is little discussion about the potential variability of X in measuring other parameters.

The specific aims section did not engender enthusiasm for the project. With so little preceding background, it was unclear what the benefit of this study would be. There are quite a few grammatical errors in the paper that are distracting as well. ("The measurements... is)

The study is of normal animals. Although X is well established as a testing means, it is not nearly the gold standard comparison.

3. The order of the proposal is not at all clear. Further, descriptions are confusing and don't match. For example, the parameters to be measured by each test method appear to be different, although I suspect this is not the intent. More explanation prior to specific aims to develop enthusiasm for the project would be very helpful.

4. The project appears to be able to be completed in one year. The likelihood of publication is moderate, unless great correlation is demonstrated.

5. The budget appears adequate, although it does not include additional hospitalization to complete the testing, which should not be borne by the clients.

### **Proposal 8: (not funded)**

The thrust of the proposed project is to determine if a PCR method of differentiating polyclonal from monoclonal populations of cells will improve the differential diagnosis of animal X and Y. The hypothesis is that standard HandE histology misclassifies the diseases, and that HandE is the gold standard. It is argued that standard HandE histology can not differentiate polyclonal vs. monoclonal cell populations. The authors reference HandE histology as the gold standard, which is confusing since one normally considers the gold standard as the best method. The authors reveal that immunochemistry will be done to determine if the cell populations are mixed or of one type. Clearly a mixed cell population would not be distinguished by immunochemistry, and certainly not by HandE staining, but possibly by PCR. Thus it seems that the standard of comparison should be immunochemistry. If this is true, and Boolean variables are considered (either X or Y), then one could simply make the comparison using a kappa statistic, either they match or do not match.

The justification for the research is clear and may be the strongest part of the proposal. The specific aims are a bit muddled as Hand E is referred to as the gold standard and immunochemistry method as the basis of comparison. The authors do consider the pitfalls in the current research section. The methods are a bit jumbled and lack some specificity. For example, the PCR is a critical component, but description of the primers, and the gene involved, is left entirely to a couple of references. Kudos to the authors for trying to calculate a sample size providing reasonable power. I am not sure they have calculated this correctly, but to be honest, I am a bit confused on how power would be evaluated using a kappa test. However, using their data, and the understanding of the potential variance I can see how they came up with a sample size of 10. One of my biggest

concerns is what will be done by the lead investigator. Apparently a pathologist will do the histopath, the PCR at another university, will the immunochemistry be done by the primary investigator, or by pathology? A tech is called for DNA extraction and biopsy. So again, what is the lead investigator to do? The investigator is not required normally to do any lab work in the grander scheme of things. However, a young researcher often is involved in various aspects of the “trenches” of research. The budget is adequately detailed and explained.

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1. **Significance/relevance:** This section of the grant could be improved. While the applicant does make a case for the importance of distinguishing between X and Y, the information could be provided more clearly. The applicant appears to have written the proposal assuming that the reader has a background in such diseases of this animal. This is particularly the case with the first few sentences of the 2nd paragraph in the background and significance section.

2. **Soundness of Methods:** There is a great deal of information missing from this section. The applicant clearly had enough room left to go into greater detail about the methods of PCR—what is/are the target gene(s), for instance? It is inappropriate to simply list a reference. PCR products are typically run on agarose gels, not polyacrylamide gels; the applicant should provide more detail here.

It is extremely disappointing that the applicant will simply be collecting samples rather than performing any of the work, particularly since some of these methods could be performed here at WSU (by the applicant) rather than shipped off.

3. **Clarity of Presentation:** Generally clearly presented and easy to read, but some sentences are not well worded (in abstract, “The cells types present in a biopsy sample are determined by using immunohistochemistry, which clarify the type of process occurring except when the cells appear identical.”).

4. **Project likely to be completed in 1 year:** WSU caseload unlikely to be high enough to accumulate the required number of samples. In one of the references, (5), “32 cases of X between 1984 and 2001” were studied—17 years to accumulate 32 cases.

A quick search of the WSU records for Jan 1 thru Dec 5 2008 (X OR Y) yielded:

ClinicPrefix	ClinicNum	ClinicExt	DiagDate	Diagnosis	Doctor
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(4 WSU cases are cited)

5. **Adequate/justified/complete budget:** \$800 seems too high for DNA extraction from 20 tissue samples. This could easily be performed in one day (not 5 days), and should actually be performed by the applicant. There are no indirect costs associated with this grant.

The amount for shipping is excessive: Why is overnight shipping needed? Formalin fixed samples do not need to be shipped overnight. Samples would also not need to be shipped individually but could be grouped.

## Proposal 9: (not funded)

The proposal is well organized and describes in sufficient detail the protocol with the animals. However, I should note that you did not follow the proposal format, no title page, font size, and the copy we received was one with all the editorial changes, comments, etc., included. That is, you did not accept the changes in word before attaching the file to your email. We expected to receive a final version.

The hypothesis is that the old method of determining cardiac output, Frick, is no better than a new method, pressure volume catheter, PVC. It is argued that the PVC method is much less difficult and could replace the Frick method, especially important for work with these animals as it is argued. The author appears to be very familiar with the literature and methods needed to achieve measures of each test variable. Moreover, the experimental design considers cardiac output under 3 different conditions: resting, dobutamine and xyaline. The major shortcomings of the proposal I find are; 1) the lack of experimental units with expression of the variances in the measures, 2) deviations in the statistical modeling described by Bland and Altman; and 3) the budget explanation for costs not charged.

Regarding the budget, I note that there are no clin-path charges, no charges for personnel time (anesthesia) nor any anesthesia costs, associated with the trial. The other charges are well described, especially for expendable items. Some explanation is needed as to why there are no charges for these line items.

The author references Bland and Altman’s explanation of statistical method for assessing agreement between two methods of clinical measurement. This is an appropriate reference but I should note that the author did not correctly reference Bland and Altman, the volume of the 1986 paper is not “1”. This misrepresentation of the journal volume caused difficulty for this reviewer’s effort to retrieve the article. Thus the author should strive to

be more careful. Bland and Altman carefully explain the method for statistical analysis to compare 2 methods is to look at the level of agreement, not differences. Yet the statistical methods proposed (paired t tests, Tukey's comparison of means, one way ANOVA) would all examine differences in the mean values. Thus I am not sure why the author proposes to measure differences, but references Bland and Altman who argue for an analysis of agreement. Also, Bland and Altman discuss clinical differences that although may be statistically significant, are not clinically relevant. Will such differences be considered by the author? Bland and Altman have a small segment of the paper discussing repeated measures. The author proposed to employ a repeated measure on the same animal to assess cardiac output under 3 different conditions: resting, dobutamine and xyaline. These conditions will be applied in succession. I wonder if there is any carry over effect, from dobutamine to xyaline administration for example. Is 30 minutes enough time for the dobutamine effect to subside? The author does assure the reviewer that 30 minutes is sufficient. If the author is to truly follow Bland and Altman and adjust the calculation of standard deviation for the repeated measure on each animal, could they account for a treatment by animal interaction? Would a better approach have been to have designed a trial to make the measures on three different days of sufficient interval duration to be free of any carry over effects?

The above comments on design could be easily changed to accommodate a proper analysis. But I am not certain of my last critical comment: The number of experimental units, 6, could be the biggest problem. The authors indicate they believe the 2 measures will yield identical differences, where  $\mu - \mu_0 = 0$ . Yet no information on the variation between animals in terms of Frick measures, and no estimate of the variation using PVC, is presented. It is very difficult to get enough power to truly test the effect of no difference, hence agreement. In which case, the authors would truly have to follow the technique described by Bland and Altman. My calculations suggest that if  $\sigma$  is two thirds of differences of the mean, then 6 animals are adequate. But if the coefficient of variation is much smaller, the number of experimental units are pitifully small. What type of variation is expected? Ten percent on some tests is noted. Is this applicable? Some discussion on how the number of experimental units was selected would ease my curious mind. I am not sure you have nearly enough to test for the way you propose. Perhaps following Bland and Altman would have been a much better choice.

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1. **Significance/relevance:** The applicant does a good job of describing rationale and clinical relevance of the proposed research. One drawback is that the applicant describes the technique as "novel", yet several references from the 1980's appear to describe studies using this technique in other animals and humans.
2. **Soundness of Methods:** Specific aims are never stated in this proposal, which significantly detracts from the reviewer's enthusiasm for this proposal. Another critical omission is a power calculation—this is particularly important for a validation study when finding no significant difference would imply that the "novel" method is equivalent to the gold standard method. If there are not a sufficient number of animals, then the results are not valid.
3. **Clarity of Presentation:** A number of grammatical errors and typos were present throughout the application. References are missing for several important statements throughout this paper. For example, the specific aims section contains 2 sentences that describe previous research on the pressure-volume method in animals, but these statements are not referenced. The research time table is split on 2 different pages.
4. **Project likely to be completed in 1 year:** The applicant states that the animals to be used will have had a special procedure performed at least 2 years before the proposed study begins. It is not clear when this will occur, or if these animals are currently available.
5. **Adequate/justified/complete budget:** The applicant should state how the per diem is being paid for the animals. Another major concern is what would happen if the one special catheter breaks before the study is complete (are these not disposable human products?). It is not clear what the room and equipment charge listed under 'catheters' is for.

#### **Proposal 10: (funded)**

1. **Significance/relevance:** The applicant provided good rationale for the proposed study.
2. **Soundness of Methods:** A power calculation was performed to determine the number of animals needed. It would have been optimal to demonstrate (if a reference is available) that the method of determining volumes has been validated in animals, preferably, or even humans.
3. **Clarity of Presentation:** Hypothesis and specific aims are very straightforward. A typo/grammatical error is present in an important part of the proposal—page 2, first specific aim.
4. **Project likely to be completed in 1 year:** Time table provided appears reasonable.

**5. Adequate/justified/complete budget:** Adequately described and justified.

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The investigators propose to evaluate the difference between insufflation and a mechanical lift on working volume for laparoscopy. The study would use CT to compare the volumes in question using image analysis software. The investigators argue that the results would be significant, as the question has never been asked in veterinary species, and it would allow a new group of procedures to be accomplished that are not currently possible. This study is part of a larger, ongoing effort.

**Evaluation**

1. The significance and potential clinical relevance of this study is high. Evaluation of a novel method of visualization in veterinary laparoscopy would clearly expand the utility of the technology.
2. The methods appear sound. The investigator clearly states the methods to be used, potential limitations and a plan to minimize the impact of those limitations. The use of repeated measures to maximize statistical power appears reasonable in this case. It is possible that post-mortem artifacts will be more problematic than anticipated.

The specific aims section is well written first grant submission. It is clear what the significance of this project is in context of a larger research program. The aims are clearly stated and serve to test the overall hypothesis.

In reading this, I am left with the question of whether the mechanical lift would interfere with the CT scan, making some of the borders for volume calculation difficult to visualize.

3. The overall level of grantsmanship displayed is high. The organization of the proposal is easy to follow and supports its significance and clinical relevance. The project is nicely couched in the context of the larger scope of work of the lab. One recommendation I would make is to eliminate the use of words such as “hope” and “believe”, using instead words like “expect” and “anticipate.” The former words undercut the impact of the propose research and introduce a sense of doubt about the likelihood of success of the project.
4. The project appears to be able to be completed in one year. The likelihood of publication is high, based on the human experience of the differences between these procedures and the novelty of this proposal.
5. The budget appears somewhat undersized, given the number of scans each subject will require. Is the variance from the standard scan charge reasonable?

**Reviewer Comments – Resident Intramural Grant Proposals:**

(edited to protect anonymity, 2 reviewers per proposal, **funding status not recorded**)

**Proposal 1:**

The Background and Significance section is the strongest section of the grant. There is good justification for the study. However, this section could be written more concisely.

The hypothesis is testable but is vague. It is unclear what type of results might be expected or exactly how those results would be used to formulate a new hypothesis.

Although somewhat broadly stated, the specific aims appear appropriate for the hypothesis.

Experimental design is the weakest section of this proposal. There is a very detailed explanation of statistical methodology but insufficient detail paid to the broader outlines of the study. The investigator states that sample size is calculated by PASS software, but there is no indication of what that sample size is. A clear explanation of the number of herds (not less than 93 or more than ?), animals/herd, total number of animals, etc is needed at the beginning of this section. Additional details regarding the format of the owner interview is needed. Is there a formal survey with specific questions that could be included as an appendix? Will all herds be visited in the same season of the year? Will individual infected animals be identified? How might this happen (it is included as an independent variable with no information on methodology). In the budget, the average distance per trip is 4,000 miles. Where are you going? Will each herd be visited only once? The Expected Results section does not state what is expected.

This proposal would benefit markedly from closer review and attention to grammar. At times the proposal was difficult to follow. This was especially true within the Research Design and Methods section (as described above). It was almost impossible for this reviewer to discern exactly what is proposed to happen in the project.

This seems doable if herd visits can be completed in a timely fashion. The information gained from the study is likely to be publishable, regardless of exact results.

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Significance/Clinical Relevance with adequate justification for the stated hypothesis. The applicant presented a reasonable case for studying the disease.

Culturing is difficult—with low sensitivity (as mentioned in methods section), particularly with a 10 microliter sample! One could miss a great deal of positives and therefore miss some important risk factors. Wouldn't a PCR approach be a more sensitive method? The information regarding the herd survey lacked detail (herd status, general management, herd biosecurity, method to identify infection problem, infection control program).

Generally well-written, but there were many grammatical errors that made the grant somewhat difficult to review. Budget is poorly justified--\$1,500 for publication costs? No budget for cultures?? Transportation states an average distance of 4000 miles per trip?

The proposed work appears to be do-able in a 1-year time period and it is likely that the results will be publishable.

## **Proposal 2:**

The applicant does a very good job of demonstrating the significance and clinical relevance of the proposed research. A major weakness of this proposal is that the specific aims do not address the hypothesis. The hypothesis is very clearly stated while the specific aims are quite vague.

The majority of the methods do not address the hypothesis, and it is difficult to tell if they address the specific aims because the specific aims are so vague. It is not clear why the applicant intends to spend so much time/effort looking for an insertion sequence in an animal that is infection free. There is no justification for many of the methods proposed—why is the applicant proposing to perform flow cytometry for B-cells, and T cells other than CD4 and CD8 cells? The applicant proposes to necropsy the animal at 6 months post inoculation, yet there is no mention of ever inoculating it. In fact, the applicant justifies the use of a non-infected animal.

There were several poorly worded sentences in the proposal, making it seem put together at the last minutes (example: 2<sup>nd</sup> paragraph of Background section-sentence 3). The applicant refers to a figure 1 to explain the inflammatory pathway—yet there is no figure 1. To a non-immunologist, more than one paragraph is necessary to describe all of the cell types and 8 or so cytokines that are involved.

A great deal of time/space was spent discussing (“selling”) the model, while little time was spent discussing the rationale for investigating CD4 and CD8 function.

It was very difficult for this reviewer to find out what gene the PCR was supposed to detect, and the proposal did not state what gene (mRNA) the real time RT PCR was to be used to detect.

The proposed work appears to be do-able in a 1-year time period as long as the animals “cooperate” in the disease course. It is not clear whether or not the proposed work will be publishable.

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The project proposal is divided into 2 specific aims, sequential, that are targeted to study the hypothesis that infection leads to CMI immunosuppression, CD4 and CD8 function. The author does a good job of describing problem with the infection and the justification of the hypothesis. The background and significance of the study are well described. The proposal suffers from weak experimental design. In work described under the first specific aim, only 1 animal will be used to determine whether the model worked out for young animals will work in an older animal. The animal will be infection-free. Thus the author will have to extrapolate to data collected in this animal to what would be found in an infected animal. One would imagine that inflammation in an infected animal might influence the model, and thus what seems to work in an infection-free animal does not work well in an infected one. The author indicates that the infection free animal is readily available and easier to handle from a biosecurity standpoint. But is this better from a research standpoint? The author alludes to the fact that perhaps this animal will be used as an age adjusted control, I am assuming for specific aim 2. There are problems with this concept, but I will get to that in a few lines. But also of importance, the reader is left to imagine which variables will be tested to determine if the model works. The author indicates that PCR will be used to identify the infectious agent. But why do such analysis in an infection free animal? How does this help achieve the goal of the specific aim? The author indicates that culture will be done after exteriorization, again why would this be part of the test if the animal was infection free? What is the point of RT PCR in work under this aim, ditto ELISA? The flow cytometry analyses are clear. But there are problems inherent in using a historical control. How can one be sure that changes are normal, and not necessarily variation due to changes

in environment, pregnancy, diet, etc. Besides, an n of 1 is a bit dicey, there is no way to measure variation in a response if there is only one animal.

Now in Specific Aim 2 methods, the animal numbers are doubled and it becomes clear why the variables mentioned above that relate to infected, but not uninfected, animals. Yet 2 animals, from different genetics, of different ages, stages of production, duration of disease, etc., but have very different immune responses. Thus even though with 2 animals one can have an estimate of variation, the source of the variation is huge, and may not relate to the infection. I believe it will be a stretch for the author to convince a scientific audience that the baseline information obtained for the animal in the Specific Aim 1 of the trial relates to that obtained in Specific Aim 2 cows. It may make for some acceptable pilot data for an abstract, but not a publication in a journal with a rigorous review process.

The proposal might have been more competitive if it had focused on just establishing the exteriorization model through a hypothesis that doing so will not alter the GALT and immune response. Perhaps some tests and challenges could be done and compared to animals not exteriorized. A larger sample size would have been helpful and would have made for a stronger proposal.

### **Proposal 3:**

This is a problem of high significance to clinical medicine. The Background/Significance section is well-written and contains adequate justification for the proposal. Justification for the hypothesis is based on studies in other species. However, sedation for collection of blood samples is essential for this type of study and therefore the question being asked is an important preliminary step in the overall research program.

The preliminary data includes evidence that the human enzyme ELISA cross reacts in animals. However, the range of results was quite wide and there is no data that these results correlate with cell activation. There is no estimate of sensitivity or specificity of the assay for activated cells. This validation could be strengthened by measuring enzyme levels after *in vitro* activation of cells.

The hypothesis could be more specific and succinct by stating exactly which sedation protocols are likely to affect cell activation and how they will affect it – increased or decreased activation. Be more clear with the hypothesis statement and don't intermingle it with background/ideas that were important in the development of the hypothesis.

The lack of strong validation of the enzyme assay may ultimately be problematic.

The objectives are written as descriptions of the overall idea and hypothesis and expected outcomes rather than as specific aims that directly test the stated hypothesis. Therefore, they are not appropriate and do not help further the understanding of the experimental approach and the structure of the proposal.

There is no justification of sample size (5 animals) using power analysis. Given the wide range of enzyme levels observed in preliminary data, it seems unlikely that data from 5 animals will provide data that can distinguish effects of sedation on cells. Will treatments be randomized? What evidence is there that alternate day sedations will provide a sufficient wash-out period between samplings? Will repeated venipuncture result in *in vivo* cell activation unrelated to sedation (randomization of treatment becomes critical here). At what time is the blood sample for enzyme testing obtained in relation to administration of sedation? Is a pre-sedation sample or a sample from an animal on a day that it is not sedated included as a negative control? Are sedatives administered IV or IM? Could route of administration influence cell activation regardless of drug used (because of venipuncture rather than as a drug effect)?

There is no budget for this proposal.

Overall, the grant is clearly presented. However, the owner consent form (Appendix 1) does not appear to be the correct form for this project. The first page should include a proposal title and indication of the names of the investigators. There does not appear to be a budget included with the proposal.

The project is highly likely to be completed within a year. However, this reviewer is doubtful, from the information presented, that the resultant data will be sufficient to result in publication.

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It appears that the author hypothesizes that enzyme levels can be used to predict animals at risk for morbidity. One later reads that sedation may affect the enzyme. This explains why the objectives focus on sedative affect on the enzyme and false elevation of it. But I hope the author recognizes the disconnect, that the objectives do not relate to the hypothesis. The relationship between morbidity and the enzyme will not be studied. The disconnect confused this reviewer, who was looking for a logical and well justified hypothesis, and objectives or

specific aims that when fulfilled, would provide evidence to test the hypothesis. The way to improve this fundamental aspect of the proposal would be to state clearly the over-arching hypothesis. Then to explain that in this proposal, the specific test of a hypothesis that sedation would alter enzyme levels was to be made. It could be easily justified why knowledge of this effect was important in ultimately determining how morbidity could be predicted by the enzyme.

Justification for the hypothesis is critical. I thought the author did a good job of discussing the situation in humans. Yet the discussion of enzyme in animals was lacking. Also, the statistical methods needs more detail. The principle dependent variable, cell aggregation, is clear. What is not clear is what independent variables will be tested. Essentially with each animal receiving all 4 treatments, on successive days, there is a split plot design. Are 5 animals enough to handle this design? Are there any carry-over effects from day to day that would affect the outcome? What are the controls? What error term will be used to correct a day by animal confounder? These are real concerns that could clearly affect the interpretation of the data.

Lastly, I am not sure the author realizes this, but the document came to us without a title and author. I believe the email made it clear as to the author, but the document was initially saved before having been read.

#### **Proposal 4:**

- Overall a good proposal but critically missing a budget!!
- Well written hypothesis and specific aims.
- It is not evident in the proposal why 1.5 mins will be a critical time point for the procedure and where this number actually came from. Does the author mean that she believes the new procedure will be 1.5 times faster then the traditional procedure?
- Although the author states that this will be a cross over designed study, it is not clear in the proposal what the recovery time (if any) will be between techniques for the animals.
- If there is going to be only one endoscopic procedure used to assess the condition and if there is not recovery time allowed or endoscopic examination immediately after each procedure is, how will the investigator know what technique caused the least or greatest trauma or other complications?
- It is not adequate to just state "analysis of variation" in your data analysis section. You should state if you're going to use a 1, 2, or 3-factor analysis of variance or repeated measures ANOVA, etc. Also, what test will you perform if you have ANOVA- based significant differences? You will have to use a post-hoc test for sure! Please be sure to consult a statistician ASAP!
- Where's the budget????? It really is not acceptable to submit a proposal without a detailed budget. So the bottom line question is, "will the funding available for these grants be adequate to fund your project?"

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The thrust of the study proposed is to contrast 2 methods: traditional and new. This reviewer appreciates the simple and straight-forward design, a classical treatment and control procedure. The justification is more than adequate, it is clear what why the test should be done and how. The hypothesis is clearly stated, but herein is a flaw. The author hypothesizes that the new procedure will be 1.5 minutes faster than with the traditional. The author is very specific on the 1.5 minutes. Yet the author does not justify, or even discuss, why the period of 1.5 minutes is so critical. Is 1.45 minutes worse or 1.55 minutes better? A better hypothesis would have been to state that the new method would be significantly faster. Alternatively, if more applicable, it would have been better to state that it needed to be at least 1.5 minutes, or n minutes whatever n is, faster in order to achieve status of a more improved practice because of these x reasons, whatever number x is. Although for the purpose of this RFP, this is no where near a fatal flaw, I would like to leave the author with the understanding that design of the hypothesis is critical, and the specific aims must logically follow the hypothesis and lead to a test of the hypothesis. Lastly, the hypothesis has to be very well justified, down to the smallest detail. In a more competitive pool, what might seem a small thing, such as a statement of 1.5 minutes faster without justification for the specific time, might be a fatal flaw that kills the proposal. However, I in no way want to leave the author with the impression that the proposal was not well written and thought out, to the contrary.

One more suggestion, you indicate one of your independent variables will be the number of people handling the animal, and you indicate the statistical analysis will be by ANOVA. What is not clear if the number of people is a dichotomous or continuous variable. Also, how will the cross over be handled in the ANOVA? You create a split plot design with the crossover, and I am not sure what error term will be used to test the main effects in your

statistical model. I know there are different ways to handle the analysis. However, again, it is just as important to detail the statistical procedures as it is to describe the procedures. Leave nothing to the imagination of the reviewer. Often the reviewer in a competitive field will look more for faults to weed out proposals during the first screen, than strengths.

Good job.

#### **Proposal 5:**

- Overall a very good proposal
- We written hypothesis and specific aims
- It is not clear to the reviewer why splenic injections are favored over IV injections. There is a good risk (even under US) for splenic trauma with this technique.
- I concerned your dose will be too low to obtain the level of sedation you are looking for with regard to nuclear scintigraphy and intrasplenic injections. The usual dose used is between 8-10 micrograms/Kg. Also, your other drug dose may be too low as well. Please consult with an anesthesiologist regarding these doses.
- Also, be sure to note in your manuscript that you used isoflurane in oxygen and not by itself!
- Your budget is not accurate in that you did not use current VTH full prices for the services required to do your study. Also, you did not include VTH charges for keeping the animals in radiation isolation before returning them to the vivarium. Please recalculate your budget accordingly.

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The project as outlined is a first part of a larger project, and is well described at the onset. Thus the hypothesis is targeted at the larger project and the proposed project is merely a specific aim of the larger project. To that end, the background and significance is well stated and it is clear that some animals are at risk for neurotoxicity following certain standard therapies. In this project the procedures will be “worked-out” to classify the animals. The endpoints are clearly established, preliminary results are presented, and methods to be used are clear and with enough detail. The proposal could have been improved with some discussion of expected results. Perhaps a figure with an extinction curve could have been presented which would have made it clear what effects were likely to be seen. It is not clear if completion of Phase 1 will result in a publication, but it is clear that Phase 1 and 2 should result in publication. The budget may be low, reflecting research discounts that do not exist currently. But there is confidence that the project can be carried to fruition within a year. Overall, well written.

#### **Proposal 6:**

- Overall an interesting proposal
- Although the aims are there is not stated hypothesis
- The proposal was awkward in that it did not flow smoothly. The author needs to eliminate the use of “Aim 1” and “Aim 2” throughout the body of the proposal.
- When you state “independent radiologist” do you mean a “blinded” radiologist?
- The statement concerning data analysis is imbedded into the proposal and not a separate heading. What is the P value you are establishing as significance for this study? It is not clear to me how you were going to analyze your radiographic scores either
- I did not see any reference to having a study specific client consent form that would be required for this kind of clinical study
- The budget needs to be itemized in a detailed way. You also need to plan ahead for the specific amount of money you intend to give each client as an honorarium to allow them to animals to participate and complete the study

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Hypothesis: none. The greatest weakness of this proposal is that it lacks a stated hypothesis. An implied hypothesis is that 1 of the 2 proposed methods for catheterization is superior to the other. It appears that Aim 1 attempts to address this hypothesis. Aim 2, however, does not address this hypothesis. The applicant has done

a reasonable job of justifying the reasons/importance of investigating anatomical morphology in animals. However, one of the supporting statements regarding health problems in animals is not referenced (first paragraph, page 2). Additionally, there seemed to be a lack of information about normal anatomical morphology in this species.

In the methods section, the applicant states a slightly different specific aim (to determine the least invasive catheter placement method). The criteria for determining "least invasive", and occurrence of side effects are not adequately stated.

The paired approach (using the same animal to evaluate each procedure) is a strength of this proposal.

Aim 2 is quite vague and significantly detracts from the proposal because it is simply observational/descriptive information. The applicant needs to develop a hypothesis and specific aims regarding this "survey".

The proposal is generally well written.

It appears likely that the project can be completed within 1 year; it is likely that a descriptive publication could result from this work.

### **Proposal 7:**

No specific aims are stated. While a hypothesis was stated, there were no specific aims identified to test that hypothesis. This is a major weakness of the proposal. An additional weakness is the lack of data to justify the clinical relevance of this research. In one of the applicant's references, one University Veterinary Teaching Hospital performed 7 procedures in 7 years. It is difficult for this reviewer to see the importance/clinical relevance for the proposed study.

A critical weakness of this proposal is that the applicant lists no criteria with which to compare the experimental approach to the traditional approach. Are there historical controls? What experience does the applicant or faculty sponsor have in performing the "traditional" surgery if, indeed, historical controls are to be used for comparison?

Several poorly worded sentences detract from the clarity of the proposal (examples: sentences beginning on line 4, page 4; line 6 page 5).

It is not clear how much of the work the graduate student will be doing and how much will be performed by the faculty mentor(s). Likelihood of publication is moderate.

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The investigators adequately explain the problems with existing surgical methods. However this appears to be an uncommon procedure and it is unclear the significance of this research in relation to the larger field of surgery. In other words ... how many animals is this technique likely to benefit. How commonly is the procedure needed for this species?

If this procedure has been done on 7 clinical cases at WSU and the manuscript is in progress (page 3), how will the currently described project add to the information from the clinical retrospective study?

The hypothesis is too broadly stated and is not testable with this proposal. There is no control population of animals undergoing the procedure by the traditional approach so it will be impossible to show definitively that one procedure is superior to the other. Alternatively, data obtained from the study could be compared to historical information collected from animals undergoing the traditional surgery (recognizing that historical control data is never as good as contemporaneous comparative data).

Specific aims are not stated and therefore, it is impossible to tell how they will test the hypothesis.

Why are 6 animals being used? What is the justification for this sample size? The surgical procedure is well described. However, there is no discussion of data analysis. How frequently will postoperative physical examination data be collected. How will data be analyzed? Why will endoscopy be performed at 24 hours, 7 and 28 days after the procedure? How were these time points chosen? How will postoperative pain be monitored and what are the criteria for administration of additional analgesia? It appears that this will be a strictly descriptive study with no true hypothesis or comparative/mechanistic outcomes to be expected.

The proposal is fairly well-written. There is no budget included with the proposal.

This project is quite likely to be completed within 1 year. The descriptive information is likely to be published.

### **Proposal 8:**

The Significance/Clinical Relevance section is generally well written and presents logical and well-supported arguments in support of the proposal. This section would be strengthened by discussion of the "gold standard" for identification of the infectious agent species and a more direct explanation of the potential misinterpretation of culture results

The hypothesis is adequate but was a little confusing as written.

Experimental design that is adequately controlled and has well justified experimental units

The experimental design was easy to follow as related to each specific aim. The number of herds and the number of samples to be analyzed was insufficiently justified. How will the herds to be sampled be chosen? Are there large differences expected in the number of infections between herds? How does the culture system definitively identify the agent? How were the virulence factor genes to be analyzed chosen? Is there any preliminary data related to this type of analysis in this lab?

Overall the presentation was sufficiently succinct and clear. The proposal would benefit from more precise editing for grammar, spelling, etc. The expected results section was well-presented. The separate section for detailed methods was appreciated.

Collection of sample should be fairly straightforward; however, the number of analyses to be performed on this large sample set seems quite ambitious for a one year project. Additional funding is necessary to complete analysis of all 800 samples.

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A clearly stated hypothesis was stated, and there appears to be adequate justification. However, many important references were not peer-reviewed (Proceedings articles/newsletters). For individuals that are not familiar with control of the infection, it would be helpful to have additional information about how an owner would actually USE the information. How would this change treatment or control?

The methods appear to be appropriate for the stated aims. For individuals who are not familiar with infection control and treatment, it is not clear why antibiogram data is not included.

The proposal is reasonably well written, however there are a few poorly worded sentences (i.e., 2<sup>nd</sup> sentence on page 3) that should be corrected if this proposal is sent to other agencies. The budget is adequately justified.

It is not clear how much of the work the graduate student will be doing and how much will be performed by staff or faculty. Therefore it is difficult to evaluate the likelihood of completion of this project in 1 year. Likelihood of publication is high.