EVALUATION OF THE IN VITRO ELUTION CHARACTERISTICS OF CARBOPLATIN-IMPREGNATED CALCIUM SULFATE BEADS

BY

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THESIS

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ABSTRACT

Pilot Study:

Objective: To characterize the elution of platinum from carboplatin-impregnated calcium sulfate hemihydrate (CSH) beads in vitro.

Sample: Sixty carboplatin-impregnated CSH beads and 9 CSH beads without added carboplatin (controls)

Procedures: Carboplatin-impregnated CSH beads (each containing 4.6 mg carboplatin [2.4 mg platinum]) were placed into separate 10 mL plastic tubes containing 5 mL of PBS in groups of 1, 3, 6, or 10; 3 control beads were placed in a single tube of PBS at the same volume. Experiments were conducted in triplicate at 37 °C and pH 7.4 with constant agitation. Eluent samples were collected at 1, 2, 3, 6, 12, 24, and 72 hours. Samples were analyzed for platinum content by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS).

Results: The mean concentration of platinum released per carboplatin-impregnated bead over 72 hours was 445.3 mg/L. Cumulative concentrations of platinum eluted increased as the number of beads per tube increased. There was a significant difference in platinum concentrations over time, with values increasing over the first 12 hours and then declining for all tubes. There was also a significant difference in percentage of total incorporated platinum released into tubes with different numbers of beads: the percentage eluted of platinum was higher in tubes containing 1 or 3 beads than in those containing 6 or 10 beads.

Conclusions and Clinical Relevance: Carboplatin-impregnated CSH beads eluted platinum over 72 hours. Further studies are needed to determine whether implantation of carboplatin-impregnated CSH beads results in detectable levels of platinum systemically and whether the platinum concentrations eluted locally are toxic to tumor cells.

Experiment 2:

Objective: To characterize the long-term elution of platinum from carboplatin-impregnated CSH beads *in vitro* using two distinct sampling methods.

Sample: Carboplatin-impregnated CSH beads containing 4.6 mg carboplatin/bead

Procedures: Method 1: Three carboplatin-impregnated CSH beads were placed into 10 mL plastic tubes with 5 mL of PBS at 37° C and pH 7.4 with constant agitation. PBS was sampled by evacuation of all 5 mL eluent at 1, 2, 3, 6, 9, and 12 hours and 1, 2, 3, 6, 9, 12, 15, 18, 22, 26, and 30 days. The fluid was then replaced with 5 mL of fresh PBS at each time point. Method 2: Tubes corresponding to each sampling time were established at time zero, with each tube containing 3 carboplatin-impregnated CSH beads and 5 mL PBS. PBS was sampled from only the assigned tubes at each time point by evacuation of all 5 mL eluent. Control beads without carboplatin were also evaluated using both methods. Samples were analyzed for platinum concentration by ICP-MS.

Results: Platinum was released from carboplatin-impregnated CSH beads for 22-30 days. There were significant differences in platinum concentrations and percentage of total incorporated platinum released over time and between methods.

Conclusions and Clinical Relevance: Carboplatin-impregnated CSH beads eluted platinum for 22-30 days. Sampling method significantly affected platinum release from carboplatin-impregnated CSH beads at nearly all time points. Results from sampling Method 1 and Method 2 provide estimations of the minimum and maximum platinum concentrations expected to elute from carboplatin-impregnated CSH beads *in vivo*.

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LIST OF ABBREVIATIONS

1. CSH — Calcium Sulfate Hemihydrate
2. PBS – Phosphate Buffered Saline
3. ICP-MS – Inductively Coupled Plasma Mass Spectrometry
4. OPLA – Open Cell Polylactic Acid
5. OPLA-Pt – Platinum-impregnated Open Cell Polylactic Acid
6. HAC – Hydroxyapatite Cement
7. IC_{50} – 50% Inhibitory Concentration
8. PPC – Peak Plasma Concentration
9. FISAS – Feline Injection Site Associated Sarcoma

CHAPTER 1
REVIEW OF THE LITERATURE

Carboplatin as a chemotherapeutic agent in veterinary patients:

Carboplatin (cis-diammine-1, 1-cyclobutane dicarboxylate platinum II) is a second generation, platinum-containing chemotherapeutic agent. Like other platinum agents, it works by binding and cross-linking DNA, resulting in non-cell-cycle-dependent tumor cell lysis. It was originally developed for humans in an attempt to mitigate the adverse effects of cisplatin, including nausea, vomiting, nephrotoxicity, neurotoxicity and myelosuppression. This decrease in toxicity is likely due to slower tissue binding and metabolism. The dose-limiting toxicity in dogs and cats is myelosupression characterized by neutropenia and thrombocytopenia.[1,2] The dose currently administered to dogs is 300 mg/m^2, and is based on the maximally tolerated dose with an acceptable level of toxicosis.[3] In cats, the maximally tolerated dose has been found to be 240 mg/m^2,[4] although dosing based on targeted area under the platinum concentration-versus time curve and individual glomerular filtration rate in cats may more accurately predict and minimize carboplatin-associated myelotoxicosis.[5]

In 1988, Gaver and others evaluated the disposition of carboplatin in the beagle dog. The authors determined that after administration of various doses of carboplatin between 3-24 mg/kg (65-580 mg/m^2), the peak plasma concentration of bound platinum occurred between 4 and 6 hours, and by 96 hours a mean of 70% ± 10% of the dosed platinum was excreted in the urine, with the majority of this excretion occurring within the first 24 hours.[2]

Carboplatin has been shown to be an effective therapy for delaying metastasis of canine osteosarcoma. In a study performed in 1996 by Bergman et al, 48 dogs were treated by limb amputation and up to 4 doses of carboplatin. In this study, the median disease-free-interval was 8.6 months and median survival time was 10.7 months. Thirty-four percent of dogs were alive at one year.[6] These results were not significantly different from previous studies using 2-4 doses of adjuvant cisplatin or a combination of cisplatin and doxorubicin.[7,8] A more recent study performed by Selmic and others in 2014 compared 5 different chemotherapy protocols following amputation for osteosarcoma (carboplatin every 21 days for 4 or 6 cycles, doxorubicin every 14 or every 21 days for 5 cycles, and alternating carboplatin and doxorubicin every 21 days for 3 cycles). There was no significant difference in disease-free interval or survival time among protocols, and the protocols with carboplatin as a sole agent resulted in a lower proportion of dogs experiencing adverse events, which could allow patients to maintain a higher quality of life during treatment.[9]

Melanoma is the most common oral malignancy in dogs. A study conducted in 2012 evaluated carboplatin as an adjuvant treatment for melanoma after surgical excision. The overall survival in this study was 440 days,[10] compared to 90-120 days following conservative surgical excision alone and 273-297 days following aggressive surgical excision (≥ 1 cm margin).[11-14] In a study evaluating carboplatin as the sole treatment for macroscopic oral melanoma, overall response rate was found to be 28%, with 24% of dogs having a partial response lasting a median of 165 days.[15] Another study from 2013 indicated that carboplatin may be an effective treatment for some patients with gross disease for which surgery is not an option.[16] These findings suggest that

carboplatin may be an effective single treatment or adjunctive treatment for oral melanoma in dogs.

A study completed in 2002 evaluated clinical presentation and response to therapy for dogs with anal sac adenocarcinoma. Partial remission was recorded in 30% of dogs treated with carboplatin, indicating that carboplatin likely has antitumor activity in cases of canine apocrine gland adenocarcinoma.[17] In this study, the effect of carboplatin treatment on overall survival was not evaluated. A later study conducted in 2013 did not find a statistically significant difference in overall survival between dogs with surgically excised anal sac apocrine gland carcinoma with or without adjuvant carboplatin; however, this was a retrospective study, and limitations and bias inherent to these types of studies may have skewed the results.[18]

Local delivery systems:

Sesame oil

One of the earliest attempts to utilize a sustained local delivery system for carboplatin administration involved combining carboplatin with sesame oil. This formulation was first used in veterinary patients to treat dermal tumors in horses. Four injections at a mean dose of 0.97 mg of cisplatin/cm^3 were administered directly into the tumor at 2-week intervals. Tumor regression was noted in all cases with minimal local toxicity. Mean relapse-free intervals were 21.6 months and 14 months for horses with sarcoid and carcinoma/papilloma, respectively.[19] In a study performed in 1996, a sesame oil-carboplatin emulsion was injected intralesionally in cats with squamous cell carcinoma of the nasal planum. Four weekly intratumoral chemotherapy injections of carboplatin (100 mg/m^2 of body surface area) injected with or without purified sesame oil, or

injections of carboplatin suspended in purified sesame oil (1.5 mg/cm^3 of tissue) were administered to cats with advanced stage tumors. It was found that formulations of carboplatin suspended in purified sesame oil significantly reduced systemic exposure to carboplatin and drug leakage from the site of injection.[20]

Plachitin

Another local delivery system, a combination of chitin and cisplatin (Plachitin), has been implanted in mice experimentally to treat Ehrlich tumor, a type of mammary adenocarcinoma. In this study, high cisplatin concentrations were measured in the tissue around the implant for greater than 8 weeks with no signs of nephrotoxicity in the subjects. Peak concentrations occurred at 4 weeks after implantation.[21] Local application of Plachitin also significantly improved survival.[22] This same delivery system was used intraperitoneally in humans with non-curative gastrointestinal neoplasia, and was considered to be safe and effective when used in this manner.[23]

Atrigel®

Atrigel® is a system that consists of a resorbable polymer in a biocompatible carrier. Upon administration, the polymer undergoes a phase change from a liquid to a formed implant. Release periods of 1 week to 4 months have been achieved.[24] The earliest study evaluating this delivery system combined the substrate with cisplatin in healthy beagle dogs and found variable local tissue toxicity, with 38% of injections resulting in some form of local tissue reaction. Only 3 of these reactions were severe, resulting in draining tract formation and tissue necrosis. No systemic

toxicity was noted, and serum platinum concentrations were seen to peak at 2 days following administration, gradually declining to day 30.[25]

One study evaluated the local and systemic toxicity as well as the platinum pharmacokinetics in dogs with stage IIb appendicular osteosarcoma after 4 subcutaneous injections of 70 mg/m^2 or 100 mg/m^2 of cisplatin in Atrigel®. Local toxicity was variable, and systemic toxicity was not noted. However, during this study it was discovered that dimethyl sulfoxide, which is the solvent used in the co-polymer system, may inactivate cisplatin and render the system ineffective. This supposition was supported by the fact that the disease-free interval was decreased in this study when compared with traditional chemotherapy.[26]

Nonetheless, this system was later tried for use with soft tissue sarcomas of the canine extremity. Nineteen dogs with histologically confirmed soft tissue sarcomas of the extremities were treated with a combination of marginal surgery and Atrigel® administration. The median dose of cisplatin was 52.1 mg/m^2. Wound complications were noted in 84.2% of dogs. Forty-seven percent of dogs were alive at the time of analysis (874 days post- treatment) with local recurrence being noted in 16.6% of dogs.[27] This may be considered a favorable result; however, recurrence rate of low-grade spindle cell sarcomas of the distal extremities with marginal excision and without adjuvant therapy has been shown to be low (10.8%), indicating that the administration of Atrigel® may have minimal effect on local recurrence.[28]

OPLA-Pt

Porous, solid, biodegradable polymer sponges, particularly open-cell polylactic acid (OPLA) sponges, have been impregnated with platinum-containing chemotherapeutic agents and implanted at various sites for local tumor control. Open-cell polylactic acid impregnated with cisplatin (OPLA-Pt) was first used in canine patients in a study conducted in 1992. Bilateral intercalary femoral allografts were implanted in 6 normal beagle dogs. OPLA-Pt was implanted adjacent to the allograft in one femur, and the polymer without cisplatin was implanted adjacent to the allograft in the other femur. The mean peak total serum platinum concentration was low when compared with that attained after a single intravenous bolus of cisplatin; however the area under the curve for total serum platinum concentration versus time for the first 21 days was large in comparison, indicating that sustained release of cisplatin can be delivered safely with this system. Local effects were minor, with moderate swelling and edema noted within 24 hours after surgery in 3 legs and resolving after 7 days. Radiographic healing was noted in all but one limb by 3 months after surgery.[29]

The first clinical trial performed in dogs to evaluate the effect of OPLA-Pt on overall survival evaluated its use in conjunction with radiation therapy to treat malignant nasal tumors. Thirteen dogs were treated with a combination of OPLA-Pt implanted intramuscularly at a distant site and megavoltage radiation. No systemic toxicity was noted, and local tissue reactions were seen in only 2 dogs. The overall survival time was longer (580 days) in this study group when compared with a group of historical controls that received radiation alone (325 days). This difference was found to be significant on multivariate analysis, indicating that this combination treatment may favorably affect survival of dogs with nasal tumors.[30]

A study performed in 1997 evaluated the effect of OPLA-Pt placed in the wound bed of marginally resected soft tissue sarcomas in 30 dogs. The implant was removed from 28% of sites due to local wound complication, and the rate of recurrence (31%) was comparable to previous reports of soft tissue sarcoma treated by marginal resection and radiotherapy, indicating that this system may not be ideal for this application.[31]

Later, this system was used clinically in conjunction with limb-sparing surgery to treat canine osteosarcoma. In this study, 80 dogs with osteosarcoma were treated by limb-sparing surgery and were randomized to receive an OPLA implant either with or without cisplatin. The dogs also received 4 doses of adjuvant cisplatin chemotherapy. Although the difference did not quite reach statistical significance ($p=0.071$), dogs in the OPLA-Pt group were 53.5% less likely to develop local recurrence than dogs in the control group, indicating that local tumor recurrence may be decreased after limb-sparing surgery by use of OPLA-Pt.[32]

PMMA

Polymethyl methacrylate (PMMA) is a tissue-compatible cement that has been evaluated for use as a depot for sustained release of cisplatin. In one study, PMMA was fashioned into cylinders, impregnated with 20 mg of cisplatin, and implanted into subcutaneous tissue over the thorax in healthy dogs. Cylinders without cisplatin were implanted on the contralateral side as negative controls. Plasma samples were obtained before implantation and at various time points during the study. Tissue chamber samples were also obtained for platinum determination. Clinical laboratory testing was performed to evaluate for systemic toxicity, and necropsy was performed at the termination of the study. Platinum concentrations at the treated sites and plasma platinum

concentrations were found to be significantly greater than control and pretreatment samples at the majority of the time points evaluated; however, no local or systemic adverse reactions were noted. [33]

There have been more extensive studies evaluating PMMA as a method of providing local delivery of antibiotics to chronically infected wounds. In 1998, Dernell and others evaluated the use of PMMA beads implanted with tobramycin and vancomycin to treat severe infections associated with limb sparing surgery. In 67% of dogs, clinical signs of infection resolved in a median of 4 weeks.[34] In 1997, PMMA impregnated with amikacin, gentamicin, tobramycin or cefazolin was used in horses with open or infected fractures repaired with internal fixation or external coaptation devices. Fracture union was achieved in 15 of 19 horses in this study, indicating that these beads may have been helpful in controlling local infection.[35] In people, PMMA has been used for decades as a means of providing local antibiotic therapy to compound fractures. A study conducted in 1993 by Ostermann and others found that fractures treated with systemic antibiotic prophylaxis alone had an overall infection rate of 17%, while fractures treated with both systemic and local antibiotic therapy had an infection rate of only 4.2%. Thus, use of antibiotic-laden PMMA beads in addition to systemic antibiotic may prevent infectious complications in compound fractures.[36]

Calcium sulfate hemihydrate:

Calcium sulfate hemihydrate is a biocompatible material that is also biodegradable, inexpensive, readily available, and sterilizable by γ- radiation. A study performed in 2000 evaluated the rate of degradation of calcium sulfate hemihydrate beads both with and without cisplatin in equine

tissue. In this study, control beads and beads containing cisplatin were implanted into the cervical subcutaneous tissues of the necks of 6 healthy horses. One control bead was harvested every 7 days for a total of 35 days. Mild swelling and edema were noted over and around the bead until the 5th post-operative day. The matrix material was evident on histopathology up to 7 days in 1 horse, 14 days in 3 horses, and 21 days in 2 horses. The lesions surrounding the beads progressed from necrosuppurative inflammation to granulomatous inflammation and fibrosis, with the most severe inflammation being present at 7 days. Degradation of the beads appeared to occur through phagocytosis by multinucleated giant cells and macrophages. At 28 days, only residual inflammation and focal areas of fibrosis remained.[a]

In 2006, cisplatin-containing calcium sulfate beads were implanted in horses for the treatment of cutaneous neoplasia. Types of tumors treated included sarcoid, fibrosarcoma, fibroma, peripheral nerve sheath tumor, squamous cell carcinoma, melanoma, lymphosarcoma, adenocarcinoma, and basal cell tumor. Eighty-three percent of animals for which long-term follow-up was available were relapse free 2 years after treatment and adverse effects were minimal, suggesting that implantation of these beads may be an effective treatment for various equine cutaneous neoplasms.[37]

Calcium sulfate beads impregnated with platinum-containing agents have also been evaluated in the treatment of canine soft tissue sarcomas. An unpublished study conducted by Hess and others evaluated outcome after implantation of cisplatin or carboplatin-containing calcium sulfate beads at the site of wide resection of soft tissue sarcomas. Local reactions were mild and consisted of seroma formation, cellulitis, swelling and erythema. Overall disease-free-interval was not

reached in this study, and only 5 tumors recurred locally, indicating that these beads, in addition to being well tolerated, might improve local control for canine soft tissue sarcoma.[b] More recently, a study was published by Bergman and others evaluating cisplatin-impregnated calcium sulfate bead placement after marginal excision of soft tissue sarcomas in dogs. Local reactions in this study occurred in less than 50% of dogs and were most often classified as mild or moderate. Twenty-nine percent of tumors recurred. Median disease-free interval was not reached for grades 1 and 2 soft tissue sarcomas and was 148 days for dogs with grade 3 sarcomas. These results were favorable and further support that calcium sulfate beads containing platinum agents may play a role in local control of soft tissue sarcomas.[38]

Elution studies in veterinary medicine:

Elution is the extraction of a solute from a material by washing with a solvent. Elution studies are useful in characterizing the *in vitro* release characteristics of local delivery systems. Although no studies to date have been performed evaluating the elution of carboplatin from either PMMA or calcium sulfate, there have been numerous studies evaluating the elution of various antibiotics from these materials.

In 2000, Ethell and others evaluated the elution of gentamicin, amikacin and ceftiofur from PMMA and hydroxyapatite cement (HAC). Historical sampling methods were utilized, wherein all of the eluent fluid was sampled and replaced at each time point. The authors found that the rate of elution for all beads was greatest within the first 24 hours, and the total antibiotic concentration released from beads over 30 days was significantly greater from HAC than from

PMMA. However, both gentamicin- and amikacin- impregnated PMMA and HAC released bacteriocidal concentrations of antibiotic for at least 30 days, indicating that this method allows for sustained local release of therapeutic levels of antibiotic locally. Ceftiofur-impregnated beads eluted reasonable concentrations of antibiotic for the first 3 to 7 days, but concentrations then decreased rapidly.[39]

Later, Phillips and others evaluated the release of amikacin and cefazolin, both separately and together in the same bead, from PMMA. The authors found that when amikacin or cefazolin were incorporated into beads independently, they each eluted concentrations greater than the MIC for selected bacteria over the 30-day study period. However, when these compounds were combined into a single bead, a significantly shorter duration of elution resulted, indicating that co-elution of amikacin and cefazolin from PMMA cannot be recommended for sustained treatment of infection. When individual beads of each antibiotic are used alone, however, each is likely to be clinically effective. [40]

Calcium sulfate has also been shown to be an effect depot of antibiotic release *in vitro*. In 2003, Santschi and McGarvey evaluated the elution of gentamicin from CSH beads. Gentamicin was released over the entire 14-day study period, with 80% of release occurring over the first 48 hours. The eluent fluid from the beads was evaluated against cultures of *E coli* bacteria, and was found to inhibit bacterial growth at all time points. The authors also determined that the beads retained bacteriocidal activity after ethylene oxide sterilization and storage at room temperature for up to 5 months.[41]

In 2010, Atilla and others evaluated the release of amikacin and vancomycin from CSH beads and the effect of eluent fluid on inhibition of growth of *Staphylococcus* spp. In this study, amikacin elution was rapid, and only inhibited bacterial growth for <24 hours. However, vancomycin elution occurred more slowly and inhibited growth for 56 days when eluting alone, or 5 days when eluting with amikacin. The authors concluded that local treatment with vancomycin-impregnated CSH beads would likely be clinically effective, while treatment with amikacin- or amikacin *and* vancomycin-impregnated beads likely required further study due to concerns over efficacy.[42]

Finally, in 2015, Phillips and others evaluated the elution of clindamycin and enrofloxacin from CSH beads. Based on MIC values for clindamycin against bacteria commonly infecting wounds in dogs and cats, clindamycin did not elute concentrations sufficient to inhibit growth of bacteria. However, enrofloxacin eluent concentrations were maintained sufficiently above the MIC for common wound pathogens of dogs and cats, indicating that enrofloxacin-impregnated CSH beads may be an effective local treatment for susceptible bacterial infections. An additional interesting finding was that enrofloxacin exhibited a unique release pattern resulting in steady, sustained release of the antibiotic. Such a release pattern is different from most others seen in previous studies with other antibiotics, where release initially occurs rapidly, and then continues at a lower rate for the duration of the study period.[43]

Fluid exchange in tissues and sampling methods:

In humans, approximately 2/3 of the extracellular fluid volume is contained in the skin and skeletal muscle. It has been found that this fluid is likely completely exchanged every 24-48 hours in healthy individuals.[44] However, fluid exchange is altered substantially by processes such as inflammation, neoplasia, fibrosis or surgical intervention. [45,46] To date, elution studies have employed a sampling method in which the entirety of the eluent volume is exchanged at each sampling time.[40,42,43,47-60] However, it is likely that this sampling method dose not accurately mimic the fluid dynamics of the altered *in vivo* conditions in which impregnated beads are typically implanted.

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Footnotes:

[a] Matrix III, US Patent 6391336, Royer Biomedical Inc, Frederick, MD

[b] Hess T, Miller J, Fettig A, et al. "Treatment of Canine Subcutaneous Soft Tissue Sarcomas With Surgical Excision and Intraoperative Placement of Platinum-Containing Biodegradable Beads", Veterinary Cancer Society Annual Conference (Las Vegas, NV). October 18-21, 2012.

CHAPTER 2

ELUTION OF PLATINUM FROM CARBOPLATIN-IMPREGNATED CALCIUM SULFATE HEMIHYDRATE BEADS: PILOT STUDY

Introduction:

Carboplatin (cis-diammine-1,1-cyclobutane dicarboxylate platinum II) is a second-generation, platinum-containing chemotherapeutic agent that has been safely used in dogs and cats and is reported to have less severe nephrotoxic and emetogenic effects than cisplatin. [1-3] Additionally, recent studies[1,4-11] have demonstrated cytotoxicity of carboplatin against appendicular osteosarcoma and oral malignant melanoma in dogs as well as oral and cutaneous squamous cell carcinoma in cats.

The adverse effects associated with platinum-based chemotherapeutics include nephrotoxicosis, myelosuppressive effects such as neutropenia and thrombocytopenia, and gastrointestinal effects such as nausea, vomiting, and inappetence.[4,12-14] To decrease the incidence of toxicosis and adverse events in treated patients, local, intralesional, and targeted chemotherapeutic protocols for use of cisplatin and carboplatin have been developed to treat local disease.[4,12-20] Results of several investigations suggest that direct chemotherapy, or intratumoral injection of a single, systemic dose of chemotherapeutic agent, may be associated with short duration of action and substantial uptake into the bloodstream. [1,21-23] In contrast, delivery systems allowing sustained, local release of a chemotherapeutic agent for the treatment of local disease offer the advantage of achieving high concentrations at the tumor site with minimal to no systemic toxicosis.[12-21,24] In dogs, some sustained-release delivery systems have efficacy against appendicular osteosarcoma

and nasal tumors (cisplatin) as well as soft tissue sarcomas (carboplatin).[15,20,24-27] However, these delivery systems are not commercially available, have been associated with unacceptable regional toxic effects including development of wound dehiscence and local infection, or have resulted in equivocal clinical improvement.[15,18,20,24]

Carboplatin-impregnated calcium sulfate hemihydrate (CSH) beads are a commercially available drug delivery system for sustained release of carboplatin and can be implanted at sites of grossly evident tumor or of marginal tumor extirpation. A proven depot for drug release, [28-33] CSH is biodegradable, biocompatible, inexpensive, readily available, and sterilizable by γ-irradiation. Published studies[22,34] and anecdotal reports[a] have shown promising results for cisplatin-and carboplatin-impregnated biodegradable beads as treatment for various tumors in horses and soft tissue sarcomas in dogs. However, to the authors' knowledge, no published data exist regarding the rate, pattern, and duration of elution of platinum from commercially available carboplatin-impregnated CSH beads.

The purpose of the study reported here was to evaluate whether platinum elutes from carboplatin-impregnated CSH beads and, if so, to determine the initial pattern of release of platinum. We hypothesized that platinum would elute from carboplatin-impregnated CSH beads into PBS at concentrations greater than the peak plasma concentration reported in dogs following administration of a single IV dose of carboplatin (300 mg/m^2 of body surface area).[2,4] We also hypothesized that the concentration of platinum in the eluent would be positively associated with the number of carboplatin-impregnated beads placed together in a sample tube.

Materials and methods:

Carboplatin-impregnated beads were created at an accredited compounding pharmacy.[b] Briefly, a forged metal bead mold with a synthetic polytetrafluoroethylene-based coating[c] was used to create chains of uniform, 3 mm diameter beads[d] containing either 4.6 mg of carboplatin (2.4 mg of platinum) with 18.4 mg of CSH or 23.0 mg of CSH (used as a control); both formulations included dextran (at a final concentration of 0.67 mg/bead; added to slow release of the agent).

All beads were formed and evaluated in triplicate (groups A, B, C). For each experiment, carboplatin-impregnated CSH beads were placed in individual 10 mL plastic tubes in groups of 1, 3, 6, or 10 with 5 mL of PBS, and 3 control beads were placed together in another tube with the same volume of PBS. The tubes were maintained at 37° C and pH of 7.4 with constant agitation. The eluent was sampled by evacuation of all 5 mL of the PBS solution at 1, 2, 3, 6, 12, 24 and 72 hours (with the initial placement of the beads in solution considered time 0). The evacuated fluid was replaced with 5 mL of fresh PBS at each time point. Eluent samples were analyzed for platinum content by inductively coupled plasma-mass spectrometry (limit of detection, 0.1 ppm).[e 35] Beads were monitored for signs of dissolution, including grossly discernible changes to the surface of the bead, opacity of the eluent, or accumulation of particulate matter in the eluent.

Statistical analysis:

Distribution of continuous data was evaluated by means of a Shapiro-Wilk test, assessment of

skewness and kurtosis, and Q-Q plots. Data that were normally distributed (hour 1through hour 24) were reported as mean, standard deviation (SD), and minimum-maximum (range). Nonnormally distributed data were reported as median, 10^{th} to 90^{th} percentiles, and range. Estimated marginal mean ± SEM data and 95% confidence intervals were reported for normally distributed data over time. Nonnormally distributed data were log-transformed for parametric analysis. A repeated measures general linear model was used to determine whether there was a difference in platinum concentrations in the eluent fluid over time (within subjects), by triplicate group A, B, or C (between subjects), and by number of beads per tube (between subjects). This was also done for evaluation of the percentage of total incorporated platinum eluted by the beads. A Mauchly test for sphericity was used to evaluate the homogeneity of covariance. Because the homogeneity of covariance was violated, the Greenhouse-Geisser method was used to interpret the results. The total amount of platinum released by each bead was calculated by adding the total milligrams of platinum released over 72 hours and dividing by the number of beads. A one-way ANOVA was then used to determine if there was a difference in the amount of platinum released per bead among groups containing different numbers of beads. A commercially available statistical software program[f] was used to analyze the data. Values of $P < 0.05$ were considered significant.

Results:

There was a significant ($P < 0.001$) difference in platinum concentrations in the eluents for carboplatin-containing beads over time, with amounts increasing over the first 12 hours and then declining thereafter for all tubes (Figure 2.1). Control beads did not elute detectable levels of

platinum at any time point. There was also a significant ($P < 0.001$) difference in the total amount of platinum released over 72 hours when results were compared for tubes containing different numbers of carboplatin-impregnated beads, with mean platinum concentrations in the eluent increasing significantly with increasing number of beads per tube (estimated marginal means: 1 bead, 63.4 mg/L; 3 beads, 201.3 mg/L; 6 beads, 377.7 mg/L; 10 beads, 609.3 mg/L) (Figure 2.1). There was no significant ($P = 0.974$) difference in platinum concentrations of the eluents among triplicate groups A, B and C.

The mean ± SD concentration of platinum released per bead over 72 hours was 445.3 ± 31.5 mg/L (range, 390.0 to 509.1 mg/L). There was no significant ($P = 0.488$) difference in amount of platinum released per bead when results for all beads over all time points were compared. There was, however, a significant ($P < 0.001$) difference in the percent of total incorporated platinum released over time, with the values for all time points significantly different from one another (Table 2.1). There was also a significant ($P = 0.001$) difference in the percentage of total incorporated platinum released into tubes with different numbers of beads, with the percentage of platinum eluted significantly higher in tubes with 1- or 3-beads than in tubes with 6- or 10-beads (Table 2.2). After 12 hours, 89%, 87%, 79%, and 67% of the incorporated platinum had been released in the tubes with 1, 3, 6, and 10 beads, respectively (Figure 2.2). At 72 hours, 92%, 97%, 91%, and 88% of the incorporated platinum had been released in the tubes with 1, 3, 6, and 10 beads, respectively (Figure 2.2). Control beads did not elute detectable levels of platinum at any point in time.

Grossly, the beads showed minor changes consistent with dissolution over the study period. By 24 hours, the surface of the beads appeared slightly roughened and the eluent appeared slightly cloudy on aspiration. These changes had subjectively progressed slightly by 72 hours, but the beads did not completely dissolve nor disintegrate substantially over the study period.

Discussion:

Carboplatin exerts its antitumor effects by binding and cross-linking DNA, resulting in non-cell cycle dependent tumor cell lysis.[3,12] It was developed as a safer alternative to cisplatin, and is an effective agent against solid tumors in dogs and cats.[2,4,5] Specifically, it has been found to be useful in treating osteosarcoma, oral melanoma, and anal sac adenocarcinoma.[1,4-10,36,37] Carboplatin is typically administered IV, but it can also be given subcutaneously, intraperitoneally and intratumorally. The primary dose-limiting toxic effect of the drug is myelosuppression characterized by neutropenia and thrombocytopenia.[1,3,4,12-14]

The doses of carboplatin administered to dogs and cats are determined on the basis of the maximally tolerated dose (ie. the highest dose associated with an acceptable degree of toxicosis).[4] For dogs, the typical dosage is 300 mg of carboplatin/m^2 of body surface area, IV, every 21 days. This dose achieves a peak plasma concentration of approximately 80 mg of carboplatin (42.1 mg platinum)/L 4 to 6 hours after IV administration.[2,4] In the present study, carbopatin-impregnated beads in all tubes (1, 3, 6, or 10 beads) eluted concentrations of platinum greater than or equal to this peak plasma concentration for $\geq$ 12 hours, indicating that these beads should be at least as effective locally against local tumor cells as a single dose of carboplatin IV.

Concerning strategies for local chemotherapy, there have been 2 studies evaluating the effect of carboplatin directly on canine tumor cell lines and the IC_{50} of the drug *in vitro*.[7,38] Determination of IC_{50} values for dose-effect testing of drugs has been used to assess efficacy of chemotherapeutic agents in human and veterinary medicine.[7,39-41] In the *in vitro* studies,[7,38] with canine tumor cell lines, the IC_{50}s for mammary carcinoma, melanoma, and transitional cell carcinoma at 72 hours were found to be between 2.2 and 11.3 mg carboplatin/L (1.2 to 5.9 mg platinum/L). In our study, platinum concentrations were greater than these values for the entire 72 hour period in all tubes with carboplatin impregnated beads except those containing only 1 bead, suggesting that implantation of ≥ 3 beads in a tumor bed may effectively inhibit growth of these tumors. However, the duration for which platinum concentrations must be sustained above a minimal concentration to achieve long-term tumor control is not currently known. The IC_{50}s appeared to be time-dependent in the study of mammary carcinoma cells, decreasing by > 50% between 24 and 72 hours for carboplatin, indicating that tumor cell susceptibility may increase over time.[7] Additional studies are needed to determine the time period for which such a concentration must be maintained.

In the present study, platinum release increased rapidly during the first 12 hours and declined thereafter. Burst release, a phenomenon of initial rapid release of a compound from a substrate, is characteristic of elution of various compounds from CSH and polymethylmethacrylate beads, with 50 to 90% of total elution often occurring in the first 24 hours.[28-30,42,43] Burst release has been attributed to diffusion of the impregnated, hydrophilic compound from the exposed surface of the beads into the surrounding eluent.[12,17,31] Subsequent release occurs as a result of diffusion of the compound along the concentration gradient between the center and periphery of the

bead.[32,33,44] Complete release of the compound occurs upon dissolution of biodegradable substrates such as CSH, and release may continue until complete dissolution has occurred.[12,31,32,45-47] However, it is not known whether burst release of a chemotherapeutic agent is desirable for tumor control, and the ideal rate, pattern and duration of elution of platinum from any substrate have yet to be determined. The burst effect was diminished and release of compound more sustained when CSH beads were wrapped with either porcine small intestinal submucosa or biodegradable poly lactide-co-glycolide.[31,48] Additionally, carboplatin poly (L-lactide) microspheres have been dispersed in a thermosensitive biodegradable gel to prevent burst release and to prolong total release.[12] It has been suggested that wrapping the beads or dispersing them in gel diminishes the increase in porosity that occurs upon dissolution, thus protecting the CSH construct from further dissolution by the eluent.[12,31,48]

When evaluating over all time points, there was a significant difference in the percentage of total platinum eluted into tubes containing different numbers of beads. The percentage of total incorporated platinum eluted was significantly higher in tubes that contained 1 or 3 beads than those that contained 6 or 10 beads when all time points were compared. This difference was most notable early in the study; 89 and 86%, respectively, of incorporated platinum was eluted into the tubes with 1 and 3 beads during the first 12 hours, as compared to only 79 and 67%, respectively, in the tubes with 6 and 10 beads. This difference in burst release among bead groups may have been attributable to the physical presence of more beads in the tubes with 6 and 10 beads. The beads were placed in 5 mL of PBS within a conical test tube, and beads typically clustered and settled at the base of the tube. As the number of beads per tube increased, less of the total surface area of the beads would be exposed to the surrounding eluent owing to clustering of the beads.

As the initial release of compound from a bead is thought to occur via diffusion of the compound from the exposed surface of the bead, a decrease in the exposed surface area of the beads in the tubes with 6 and 10 beads may have resulted in slower initial release of platinum. It is possible that platinum was released more rapidly from beads in these groups once dissolution of the beads began, equalizing release among all bead groups over the entire study period. Consequently, over the 72-hour period evaluated, the mean ± SD concentration of platinum released per bead was 445.34 ± 31.5 mg platinum/L, regardless of the number of beads in the tube. This information may have added benefit once concentrations of carboplatin necessary for local tumor control and diffusion characteristics in living tissues are known, and could be used to help determine how many beads should be implanted into a tumor bed in future clinical research.

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Footnotes:

[a] Hess T, Miller J, Fettig A, et al. "Treatment of Canine Subcutaneous Soft Tissue Sarcomas With Surgical Excision and Intraoperative Placement of Platinum-Containing Biodegradable Beads", Veterinary Cancer Society Annual Conference (Las Vegas, NV). October 18-21, 2012.

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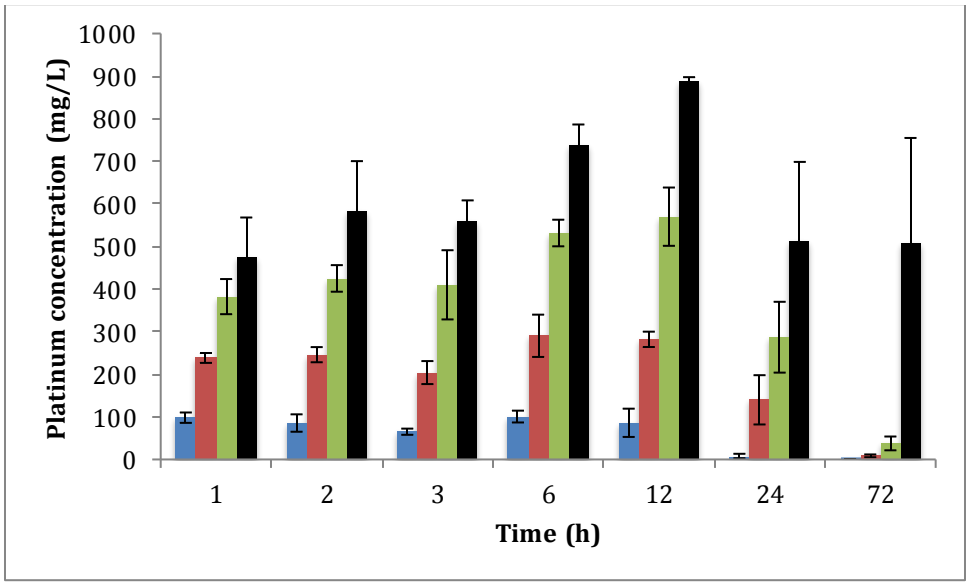
[d] Matrix III, US Patent 6391336, Royer Biomedical Inc, Frederick, MD

[e] Midwest Laboratories, Inc., Omaha, NE

[f] SPSS version 23.0, SPSS Inc., Armonk, NY

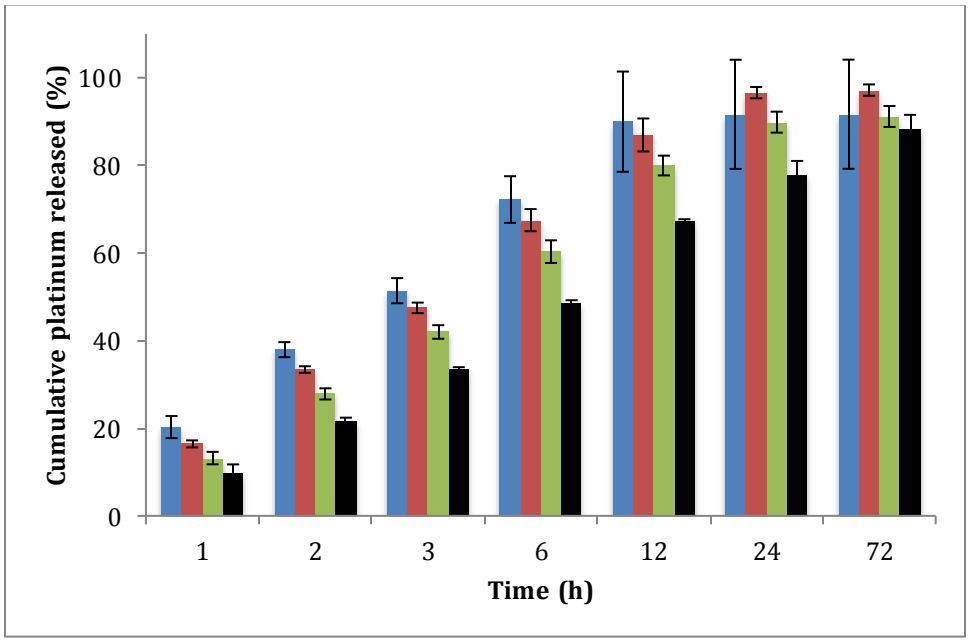
Figures and tables:

Figure 2.1:



Cumulative platinum concentration (mean ± SD of triplicate experiments) in the eluent of carboplatin-impregnated CSH beads placed in groups of 1 (blue bars), 3 (red bars), 6 (green bars), or 10 (black bars) into tubes of PBS (pH, 7.4) at 37°C. Each bead contained 4.6 mg of carboplatin (2.4 mg of platinum) and 18.4 mg of CSH with dextran. Initial placement of the beads into solution was considered time 0; all eluent was removed for analysis and replaced with 5 mL of fresh PBS at each time point.

Figure 2.2:



Cumulative percentage (mean ± SD of triplicate experiments) of the total incorporated platinum eluted from carboplatin-impregnated CSH beads into PBS at predetermined time points (same sample as in Figure 1). ***See*** Figure 1 for remainder of key.

Table 2.1:

Time	**Mean (%)**	**SD (%)**	**Range (%)**
1 hour	14.93	4.31	8.54-22.11
2 hours	30.26	6.37	21.27-39.92
3 hours	43.56	7.20	33.04-54.36
6 hours	62.18	9.65	48.18-78.33
12 hours	80.99	10.58	66.59-102.50
24 hours	88.95	9.17	74.02-105.14
72 hours	92.06	6.46	80.62-105.19

Mean percentage of the total incorporated platinum eluted from carboplatin-impregnated CSH beads into PBS at predetermined time points, irrespective of group. Concentrations at all times differed significantly from each other (all $P<0.0001$; except 24 h vs 72 h, $p=0.054$)

Table 2.2:

Group	Mean ± SEM (%)	95% CI (%)
1 bead	65.0 ± 1.78 *†	60.9–69.1
3 beads	63.6 ± 1.78 ‡§	59.5–67.7
6 beads	57.7 ± 1.78 *‡‖	53.6–61.8
10 beads	49.5 ±1.78 †§ ‖	45.4–53.6

Cumulative percentage of total platinum content eluted from carboplatin-impregnated CSH beads into PBS across all time points. Beads were placed in 5 mL of PBS (pH 7.4) at 37°C in groups of 1, 3, 6, or 10 for 72 hours.

*$P = 0.02$. †$P < 0.001$. ‡$P = 0.048$. §$P = 0.001$, ‖$P = 0.012$

CI = Confidence interval.

CHAPTER 3

CHARACTERIZATION OF LONG-TERM ELUTION OF PLATINUM FROM CARBOPLATIN-IMPREGNATED CALCIUM SULFATE HEMIHYDRATE BEADS IN VITRO BY TWO DISTINCT SAMPLING METHODS

Introduction:

Local, sustained- release chemotherapeutic strategies have been developed to decrease the incidence of systemic toxicity while optimizing regional control of non-resectable, incompletely or marginally resected tumors.[1-10] However, many of these delivery systems are not commercially available, have resulted in unacceptable regional complications, or have demonstrated minimal improvement in clinical outcome.[5,8,10,11]

Carboplatin-impregnated calcium sulfate hemihydrate beads are a commercially available[a] delivery system for sustained release of the platinum-containing agent, carboplatin. Calcium sulfate hemihydrate (CSH) is a proven biodegradable carrier for drug release and causes minimal reaction in tissues.[12-18] Published studies[19] and anecdotal reports[a] have shown promising efficacy of cisplatin-and carboplatin-impregnated biodegradable beads against various tumors in horses and soft tissue sarcomas in dogs, with negligible local side effects compared to other carriers. We described the short-term elution characteristics of carboplatin-impregnated CSH beads based on findings of an *in vitro* pilot study.[20]

Elution is the practice of extracting one material from another by washing it with a solvent. This process has been used historically as an *in vitro* method to predict the theoretical efficacy of antibiotic- and chemotherapeutic-impregnated carriers for sustained release of these compounds

in chronically infected wounds or tumor beds, respectively.[15,18,21] Studies evaluating elution characteristics have most commonly employed a sampling method in which the entirety of the eluent volume is exchanged at each sampling time.[12,15,18,21-34]

In humans, the skin and skeletal muscle contain approximately 2/3 of the extracellular fluid volume, combined. Available evidence suggests that interstitial fluid in normal skin and skeletal muscle is completely exchanged every 24-48 hours.[35] However, if the tissues are significantly disrupted by inflammation, neoplasia, fibrosis, or surgical intervention, the normal exchange of fluid is altered.[36,37] It is likely that the most commonly used sampling method does not adequately mimic the fluid dynamics of these altered *in vivo* conditions. To date, no elution studies have taken into account the differences in dynamics of fluid exchange between normal and disrupted tissues.

The purpose of this study was to characterize the long-term elution of platinum from carboplatin-impregnated CSH beads by comparing two distinct methods of sampling: one that mimics an environment with rapid and complete fluid exchange, and one that mimics an environment where no fluid exchange occurs. We hypothesized that sampling of eluent by the two distinct methods would result in significant differences in platinum concentrations in the measured eluent samples. An additional objective was to use the platinum concentration measured in this study to define the *minimum* and *maximum* concentrations of platinum that could be expected to elute from carboplatin-impregnated CSH beads in any *in vivo* condition.

Materials and methods:

All carboplatin-impregnated beads evaluated in this study were created at an accredited compounding pharmacy.[b] Briefly, a forged metal bead mold with a synthetic polytetrafluoroethylele-based coating[c] was used to create chains of uniform, 3 mm diameter beads[d] containing either 4.6 mg carboplatin (2.4 mg of platinum) with 18.4 mg CSH or 23.0 mg of CSH (used as a control); both formulations included dextran (at a final concentration of 0.67 mg/bead; added to slow release of the agent).

All beads were formed and evaluated in triplicate (groups A, B, C) for Sampling Methods 1 and 2. For Method 1, three carboplatin-impregnated CSH beads were placed in a tube containing 5 mL of PBS and maintained at 37° Celsius and pH 7.4 with constant agitation (Figure 3.1). The PBS was sampled from the beads by evacuation of all 5 mL of the eluent fluid at 1, 2, 3, 6, 9, and 12 hours, and 1, 2, 3, 6, 9, 12, 15, 18, 22, 26, and 30 days. The fluid was then replaced with 5 mL of fresh PBS at each time point (Figure 3.2). Control beads without carboplatin were also evaluated using this sampling method.

For Method 2, tubes corresponding to each sampling time point (1, 2, 3, 6, 9, and 12 hours, and 1, 2, 3, 6, 9, 12, 15, 18, 22, 26, and 30 days) were established at time zero, with each tube containing 3 carboplatin-impregnated CSH beads and 5 mL PBS (Figure 3.3). Ambient conditions were the same as described for Method 1. The PBS was sampled from only the assigned tubes at each time point by evacuation of all 5 mL of the eluent fluid (Figure 3.4). Control beads without carboplatin were also evaluated using this sampling method.

Samples from Method 1 were compared with samples from Method 2. Also, the first 72 hours of samples from Method 1 were compared with samples from a previously conducted 72-hour pilot study that also utilized Method 1. All eluent samples from Method 1 and Method 2 and the pilot study were analyzed for platinum concentration by inductively coupled plasma-mass spectrometry (ICP-MS; limit of detection, 0.1 parts per million).[e]

Statistical methods:

The distribution of the data was evaluated by means of a Shapiro-Wilk test, skewness, kurtosis, and q-q plots. Data that were normally distributed were reported as mean, standard deviation (SD), and minimum-maximum (range) values, while non-normally distributed data were reported by median, 25^{th} to 50^{th} percentiles, and range. Data that were not normally distributed were log transformed for parametric testing. A general linear model for repeated measures was used to determine if there was a difference in platinum concentrations over time (1 h, 2 h, 3 h, 6 h, 12 h, 1 d, 3 d) and by group (present study vs. pilot study). This same statistical test was used to compare Methods 1 and 2 over time (1 h, 2 h, 3 h, 6 h, 9 h, 12 h, 1 d, 2 d, 3 d, 6 d, 9 d, 12 d, 15 d, 18 d, 22 d, 26 d, 30 d). Mauchly's test was used to assess sphericity. Because sphericity was not found, the Greenhouse-Geisser test was used to determine within subjects effects. A commercial statistical software program[f] was used to analyze the data. A $p < 0.05$ was used to determine statistical significance.

Results:

There was a significant difference in platinum concentrations over time when evaluating the first 72 hours of samples evaluated by Method 1 ($F = 34.3$, $P = 0.0001$), but no difference was found between samples from the pilot study and the first 72 hours of Method 1 samples from the present study ($F = 0.105$, $P = 0.762$).

Regarding Methods 1 and 2 of the present study, there were significant differences in platinum concentrations released over time ($F = 58.77$, $P = 0.0001$) and between methods ($F = 264576.20$, $P = 0.0001$) (Table 3.1). Platinum concentrations (mg/L) measured by Method 2 were significantly higher (all $p < 0.01$) than concentrations measured by Method 1 for all times except 1 hour ($p = 0.495$) (Figure 3.5).

There were significant differences in the percent mg of incorporated platinum released over time ($F = 78.13$, $P = 0.0001$) and between methods ($F = 12290.48$, $P = 0.0001$) (Table 3.2). The percent mg of total incorporated platinum that was released over time was significantly higher for samples measured by Method 2 (all $p < 0.01$) than those measured by Method 1 for all times except hour ($p = 0.487$).

Discussion:

Calcium sulfate hemihydrate has been extensively studied as a drug delivery substrate and is a proven depot for drug release, or elution.[12-17] Drug elution studies should evaluate species-specific *in vivo* pharmacokinetics and pharmacodynamics, but inherent in such studies are risks to the species involved and significant costs.[38] Review of the literature, therefore, reveals an emphasis on *in vitro* evaluation of drug elution and variability in study design.[12-18,21-34] One consistency among *in vitro* studies is the use of a sampling method that involves complete exchange of eluent medium such as PBS or canine serum.[12,15,18,21-34] Although some investigators removed only an aliquot of eluent for drug measurement and replenished the same volume using fresh medium,[2,4,39] only one investigative team attempted to mimic *in vivo* conditions by exchanging a diminishing volume of eluent over the course of the study.[13] However, this component of the study design was based solely on the authors' clinical impression that wound effusion diminishes over time, a supposition that does not apply to all wounds.[13]

As the most commonly used sampling method could promote more rapid elution by creating a new concentration gradient at each sampling time, the authors of the present study used two distinct sampling methods to evaluate platinum release from carboplatin-impregnated CSH beads. Comparing results of the two sampling methods allowed the authors to 1) characterize the long-term elution of platinum from commercially available carboplatin-impregnated CSH beads and 2) define a range of platinum concentrations that could be expected to elute in different *in vivo* conditions.

Delivery systems allowing sustained, local release of a chemotherapeutic agent for the treatment of neoplasia offer the advantage of achieving high concentrations at a tumor site with minimal risk of systemic toxicity.[2-11,18,21] Elution of substrate-bound drugs in tissue is largely dependent on the distribution of the drug, or concentration gradient, between the substrate and the biological tissue[40]. The shape, size, and porosity of the substrate carrier are also important[12-18,21-34]. Distribution, absorption, and elimination of the drug are then mediated by the drug's extracellular concentration and the vascularity, lymphatic density, cellular microenvironment, and fluid dynamics of the wound bed[36,37,40-42]. Inflammatory or neoplastic infiltrations or surgical intervention could sufficiently alter the tissue environment to result in variation in drug distribution, absorption, and elimination among patients.[36,37] In patients with a normal tissue microenvironment and interstitial fluid dynamics, complete exchange of extracellular fluid might be expected to occur quite frequently, often within hours.[36] However, in patients with significant inflammatory, neoplastic, or fibrous tissue infiltration, little to no exchange of extracellular fluid may occur, or exchange of fluid might occur very slowly.[36,37]

In the present *in vitro* study, sampling Method 1 involved complete exchange of eluent at every time point to more closely mimic a wound environment where rapid and complete extracellular fluid exchange would be expected. Results of Sampling Method 1, therefore, represent the *minimum* concentration of platinum that would be expected to occur *in vivo*. Sampling Method 2 involved no exchange of eluent, and mimicked a wound environment where fluid exchange would be expected to be negligible. Therefore, results of Sampling Method 2 represent the cumulative release of platinum into the surrounding medium over time and the *maximum* concentration of platinum that would be expected to occur *in vivo*. Because wounds or tumor

beds will vary among dogs and cats in size, vascularity, severity of inflammation, and rate and proportion of wound fluid exchange, the actual concentration of platinum eluted *in vivo* from carboplatin-impregnated CSH beads should fall within the range of these minimum and maximum values.

Recommended dosages for carboplatin are currently dictated by the systemic maximally tolerated dosage, not by targeted plasma or tissue concentration.[1] Currently, carboplatin chemotherapy for dogs involves a carboplatin dosage of 300 mg/m^2 of body surface area administered IV every 21 days. A single IV dose achieves a peak plasma concentration (PPC) of approximately 80 mg of carboplatin (42.1 mg platinum)/L 4-6 hours following IV administration.[1,43] In the present study, platinum concentrations measured by Methods 1 and 2 were maintained over 1.3 times the PPC for up to 24 hours and from 1.3-15 times the PPC for up to 30 days for Methods 1 and 2, respectively.

Only two studies that have investigated targeted tissue concentrations by evaluating the effect of carboplatin directly on canine tumor cells.[44,45] These studies determined the 50% inhibitory concentration (IC_{50}), or the concentration of carboplatin necessary to achieve 50% inhibition of replication of tumor cells *in vitro*. Determination of IC_{50} values for dose-response testing of drugs has been used to assess efficacy of carboplatin in human and veterinary studies.[44-48] In these studies, the IC_{50}s for mammary carcinoma, melanoma, and transitional cell carcinoma at 72 hours were between 2.2-11.3 mg carboplatin/L (1.2-5.9 mg platinum/L). Although platinum concentrations measured by Method 2 were maintained 13-90 times above IC_{50} values from 1 hour to 30 days, platinum concentrations measured by Method 1 fell below IC_{50} values between

48 and 72 hours. This discrepancy highlights the importance of accurate modeling in *in vitro* studies. IC_{50} concentrations appear to be time-dependent, and the duration for which platinum concentrations must be sustained above some minimal concentration to achieve tumor control is not known.[44-48] Further studies are needed to evaluate the interval and longer-term IC_{50}s of carboplatin for various susceptible tumor types and the ideal rate, pattern, and duration of platinum elution from carboplatin-impregnated CSH beads.

The minimum and maximum platinum concentrations that could be expected to elute from individual carboplatin-impregnated CSH beads from 1 hour to 30 days are listed in Table 3.3. The actual concentration eluted will depend on the physical environment of the wound or tumor bed. These values are median values of experiments performed in triplicate, and are derived from measurement of platinum elution by sampling Method 1 (minimum concentration) and sampling Method 2 (maximum concentration). These measurements may prove clinically useful once IC_{50}s for local tumor control are known. It should be noted that these concentrations are derived from an *in vitro* study, and are therefore only estimates of concentrations that will be achieved *in vivo*. The accuracy of these estimations depends on the accuracy with which our sampling methods model the two most extreme wound or tumor bed conditions. Further studies are needed comparing the findings of our *in vitro* study with tissue concentrations of platinum measured *in vivo*.

The sampling Method 1 used in this study was also used in a preliminary study conducted by the authors.[20] The pilot study evaluated platinum elution from carboplatin-impregnated CSH beads over 72 hours from the same manufacturer[b] over 72 hours. When platinum elution measured

during the pilot study was compared with the first 72 hours of elution measured by sampling Method 1 in the present study, there was a significant difference in platinum concentrations over time, but not between the pilot study and the present study. This finding indicates precision in sampling as well as consistency in total bead platinum concentration among beads with different manufacturer lot numbers.

Significant differences were found when comparing concentrations of platinum measured by Methods 1 and 2 for nearly all time points (Figure 5). The mg/L of platinum and the percent mg of platinum incorporated into each bead that were released by each time point were significantly different from nearly every other time point, indicating that neither a constant amount nor a constant proportion of platinum were released by each sampling time (Table 3.1 and Table 3.2). Platinum release did increase sharply in the first 24 hours of sampling for Method 1, and declined thereafter, a phenomenon known as "burst release" in elution studies.[12-18,21-34] Interestingly, a burst of release was not noted for sampling Method 2, as substantial proportions of the total incorporated mg platinum were released until the 12th study day (Table 3.2). This finding raises the question whether the phenomenon of burst release is actually an artifact of sampling Method 1 because at each sampling point with this method, a new gradient is created between the bead and freshly added PBS containing no drug at all.

Sampling method significantly affected the concentrations of platinum released from carboplatin-impregnated CSH beads at nearly all time points. A sampling method that involves frequent and complete exchange of eluent fluid may mimic a wound bed environment with normal vascularity, cellularity, and fluid dynamics but may not accurately apply to clinical

situations. Elution profiles of local drug delivery systems measured by this sampling method may not be reliably applied to patients. Further studies are needed comparing *in vitro* elution profiles of drugs with pharmacokinetic and pharmacodynamic profiles obtained from *in vivo* studies.

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Footnotes:

[a] Hess T, Miller J, Fettig A, et al. "Treatment of Canine Subcutaneous Soft Tissue Sarcomas With Surgical Excision and Intraoperative Placement of Platinum-Containing Biodegradable Beads", Veterinary Cancer Society Annual Conference (Las Vegas, NV). October 18-21, 2012.

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[c] University of Vermont Instrumentation and Modeling Facility, Burlington, VT

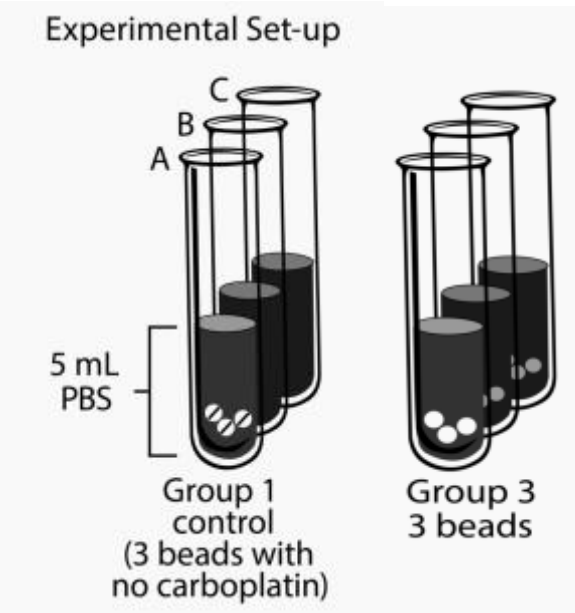
[d] Matrix III, US Patent 6391336, Royer Biomedical Inc, Frederick, MD

[e] Midwest Laboratories, Inc., Omaha, NE

[f] SPSS version 23.0, SPSS Inc., Armonk, NY

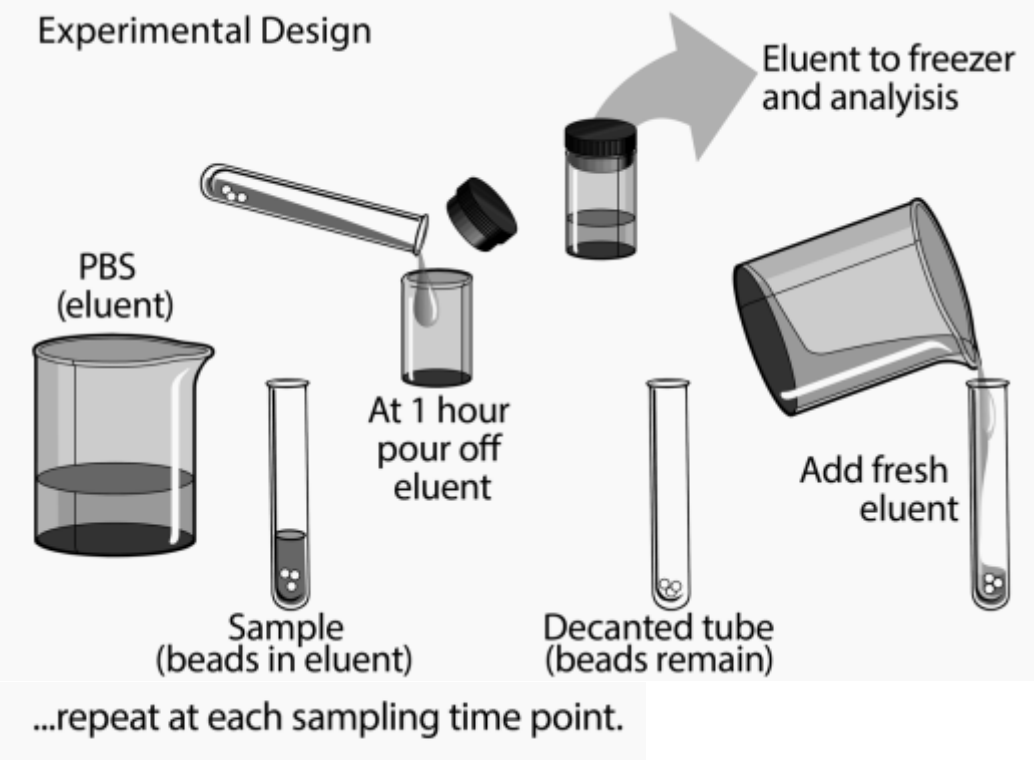
Figures and tables:

Figure 3.1:



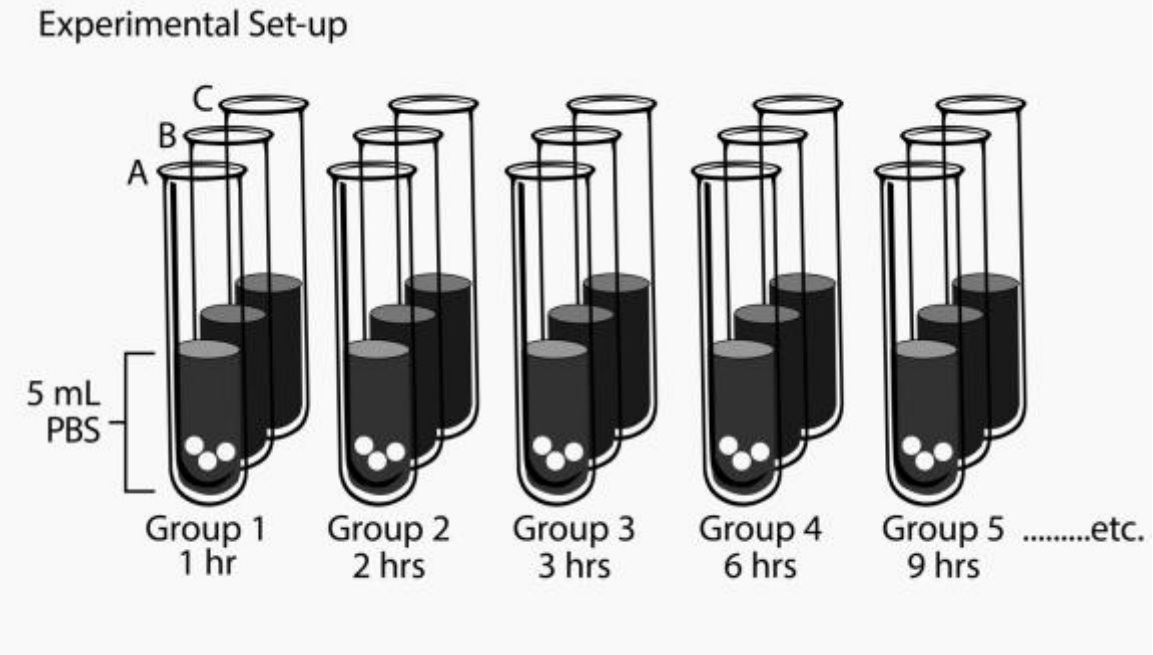
Experimental set-up for Method 1. Three carboplatin-impregnated CSH beads were placed in an eluent well containing 5 mL of PBS and maintained at 37° Celsius and pH 7.4 with constant agitation.

Figure 3.2:



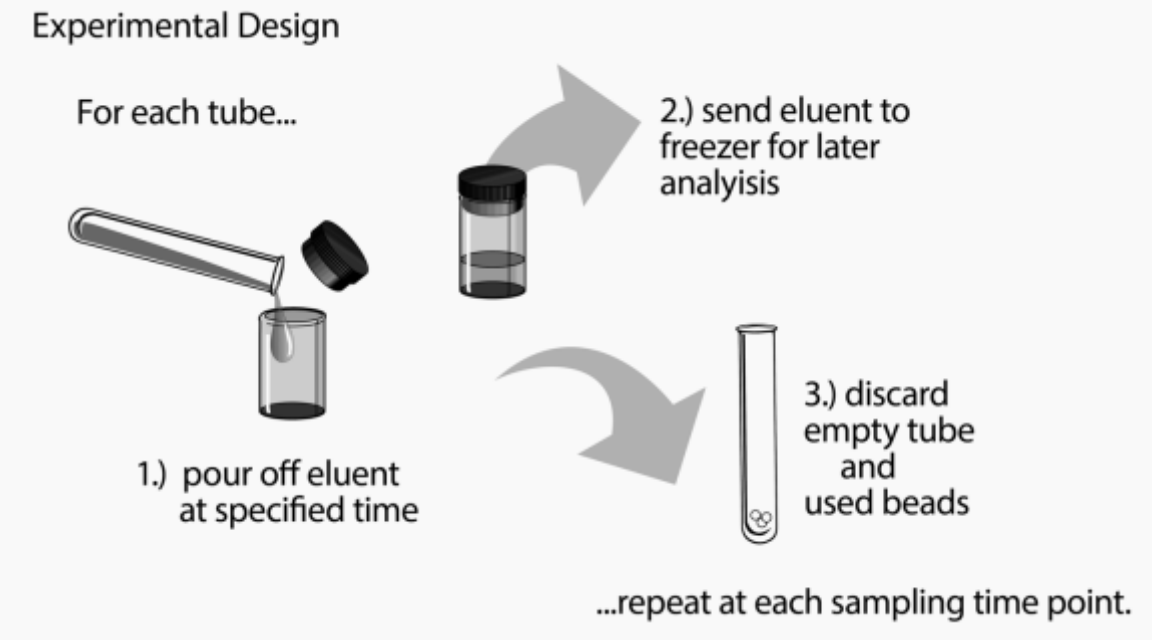
Experimental design for Method 1. The PBS was sampled from the beads by evacuation of all 5 mL of the eluent fluid at 1, 2, 3, 6, 9, and 12 hours, and 1, 2, 3, 6, 9, 12, 15, 18, 22, 26, and 30 days. The fluid was then replaced with 5 mL of fresh PBS at each time point.

Figure 3.3:



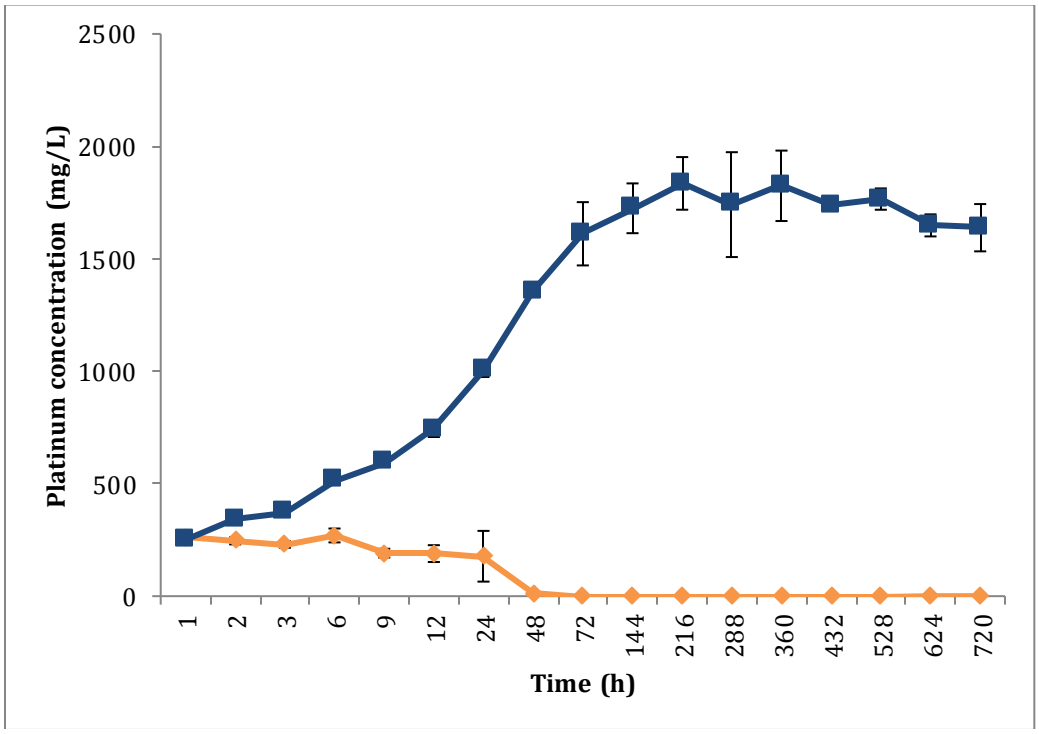
Experimental set-up for Method 2. Tubes corresponding to each sampling time point were established at time zero, with each tube (Time 1 hr, Time 2 hrs, Time 3 hrs, …) containing 3 carboplatin-impregnated CSH beads and 5 mL PBS.

Figure 3.4:



Experimental design for Method 2. The PBS was sampled from only the assigned tubes at each time point by evacuation of all 5 mL of the eluent fluid.

Figure 3.5:



Platinum concentrations (mean ± SD of triplicate experiments) eluted from carboplatin-impregnated CSH beads into PBS (pH 7.4, 37°C) at predetermined time points over time, as sampled by Methods 1 and 2. Each bead contained 4.6 mg of carboplatin (2.4 mg of platinum) and 18.4 mg of CSH with dextran. Initial placement of the beads into solution was considered time 0. Method 1 is depicted in orange with data points represented by diamonds, while Method 2 is depicted in dark blue with data points represented by squares.

Table 3.1: Comparison of platinum concentrations (mg/L) that were measured over time in eluent by sampling Methods 1 and 2.

Time	**Group**	**Median**	**25-50%**	**Min-Max**
1 hour[a]	1	255.0	243-255	243-284
	2	252.0	242-252	242-258
2 hour[b]	1	253.0	228-253	228-258
	2	341.0	328-341	328-348
3 hour[c]	1	237.0	213-237	213-240
	2	370.0	367-370	367-382
6 hour[d]	1	258.0	247-258	247-305
	2	521.0	492-521	492-524
9 hour[e]	1	196.0	170-196	170-208
	2	586.0	586-586	586-617
12 hour[f]	1	203.0	147-203	720-775
	2	722.0	720-722	720-775
1 day[g]	1	180.0	63.5-180	63.5-289
	2	1022.0	970-1022	970-1023
2 days[h]	1	17.2	3.10-17.2	3.10-20.7
	2	1350.0	1350-1350	1350-1364
3 days[i]	1	0.48	0.190-0.480	0.190-0.490
	2	1586.0	1485-1586	1485-1763
6 days[j]	1	0.26	0.250-0.260	0.250-0.270
	2	1671.0	1651-1671	1651-1852

Table 3.1 (continued):

9 days[k]	1	0.18	0.170-0.180	0.170-0.190
	2	1857.0	1709-1857	1709-1940
12 days[l]	1	0.14	0.110-0.140	0.110-0.190
	2	1794.0	486-1794	1486-1943
15 days[m]	1	0.10	0.0900-0.100	0.0900-0.150
	2	1792.0	1687-1792	1687-1995
18 days[n]	1	0.09	0.000-0.0900	0.000-0.120
	2	1741.0	1714-1741	1714-1756
22 days[o]	1	0.07	0.000-0.0700	0.000-0.0900
	2	1790.0	1711-1790	1711-1796
26 days[p]	2	1657.0	1596-1657	1596-1693
30 days[q]	2	1601.0	1557-1601	1557-1757

[a]1 hour significantly different ($p<0.05$) from all times, except day 1 ($p=0.111$)

[b]2 hour significantly different ($p<0.05$) from all times, except 3 hour ($p=0.712$) and day 1 ($p=0.228$)

[c]3 hour significantly different ($p<0.05$) from all times, except 3 hour ($p=0.712$) and day 1 ($p=0.293$)

[d] 6 hour significantly different ($p<0.05$) from all times, except 9 hour ($p=0.202$), 12 hour ($p=0.989$), and day 1 ($p=0.855$)

[e] 9 hour significantly different ($p<0.05$) from all times, except 6 hour ($p=0.202$), 12 hour ($p=0.330$), and day 1 ($p=0.597$)

Table 3.1 (continued):

[f] 12 hour significantly different ($p<0.05$) from all times, except 6 hour ($p=0.989$), 9 hour ($p=0.330$), and day 1 ($p=0.824$)

[g] 1 day significantly different ($p<0.05$) from all times, except 1 hour ($p=0.111$), 2 hour ($p=0.228$), 6 hour ($p=0.855$), 9 hour ($p=0.597$), and 12 hour ($p=0.824$)

[h] 2 day significantly different ($p<0.05$) from all times, except day 18 ($p=0.06$) and day 22 ($p=0.06$)

[i] 3 day significantly different ($p<0.05$) from all times, except day 6 ($p=0.507$), day 9 ($p=0.192$), day 18 ($p=0.730$) and day 22 ($p=0.655$)

[j] 6 day significantly different ($p<0.05$) from all times, except day 3 ($p=0.507$), day 18 ($p=0.846$) and day 22 ($p=0.722$)

[k] 9 day significantly different ($p<0.05$) from all times, except day 3 ($p=0.192$), day 12 ($p=0.257$), day 15 ($p=0.07$), day 18 ($p=0.846$) and day 22 ($p=0.990$)

[l] 12 day significantly different ($p<0.05$) from all times, except day 9 ($p=0.257$), day 15 ($p=0.216$), day 18 ($p=0.648$) and day 22 ($p=0.792$)

[m] 15 day significantly different ($p<0.05$) from all times, except day 9 ($p=0.07$), day 12 ($p=0.216$), day 18 ($p=0.469$) and day 22 ($p=0.620$)

[n] 18 day significantly different ($p<0.05$) from all times, except day 2 ($p=0.06$), day 3 ($p=0.730$), day 6 ($p=0.846$), day 9 ($p=0.846$), day 12 ($p=0.648$), day 15 ($p=0.469$), day 22 ($p=0.147$), day 26 ($p=0.128$), and day30 ($p=0.129$)

[o] 22 day significantly different ($p<0.05$) from all times, except day 2 ($p=0.06$), day 3 ($p=0.655$), day 6 ($p=0.722$), day 9 ($p=0.990$), day 12 ($p=0.792$), day 15 ($p=0.620$), day 18 ($p=0.147$), day 26 ($p=0.129$), and day30 ($p=0.130$)

Table 3.1 (continued):

[p] 26 day significantly different ($p<0.05$) from all times, except day 18 ($p=0.128$), day 22 ($p=0.129$), day 26 ($p=0.128$), and day 30 ($p=0.814$)

[q] 30 day significantly different ($p<0.05$) from all times, except day 18 ($p=0.128$), day 22 ($p=0.129$), day 26 ($p=0.130$), and day 30 ($p=0.814$)

Table 3.2: Comparison of percent of total platinum incorporated that was measured in eluent over time by sampling Methods 1 and 2.

Time	**Method**	**Median**	**25-50%**	**Min-Max**
1 hour[a]	1	17.6	16.8-17.6	16.6-19.6
	2	17.4	16.7-17.4	16.7-17.8
2 hour[b]	1	17.4	15.7-17.4	15.7-17.8
	2	23.5	22.6-23.5	22.6-24.0
3 hour[c]	1	16.3	14.7-16.3	14.7-16.6
	2	25.5	25.3-25.5	25.3-26.3
6 hour[d]	1	17.8	17.0-17.8	17.0-21.0
	2	35.9	33.9-35.9	33.9-36.1
9 hour[e]	1	13.5	11.7-13.5	11.7-14.3
	2	40.4	40.4-40.4	40.4-42.6
12 hour[f]	1	14.0	10.1-14.0	10.1-15.0
	2	49.8	49.7-49.8	49.7-53.4
1 day[g]	1	12.4	4.37-12.41	4.37-19.93
	2	70.5	66.9-70.5	66.9-70.6
2 days[h]	1	1.18	0.21-1.18	0.21-1.42
	2	93.1	93.1-93.1	93.1-94.1
3 days[i]	1	0.03	0.013-0.03	0.01-0.03
	2	109	102-109	102-122
6 days[j]	1	0.017	0.017-0.017	0.017-0.018
	2	115	114-115	114-128

Table 3.2 (continued):

9 days[k]	1	0.012	0.011-0.012	0.011-0.013
	2	128	118-128	118-134
12 days[l]	1	0.009	0.007-0.009	0.007-0.013
	2	124	102-124	102-134
15 days[m]	1	0.006	0.006-0.006	0.006-0.10
	2	124	116-124	116-138
18 days[n]	1	0.006	0.00-0.006	0.00-0.008
	2	120	118-120	118-121
22 days[o]	1	0.004	0.00-0.004	0.00-0.006
	2	123	118-123	118-124
26 days[p]	2	114	110-114	110-117
30 days[q]	2	110	107-110	107-121

[a]1 hour significantly different ($p<0.01$) from all times

[b]2 hour significantly different ($p<0.01$) from all times, except 3 hour ($p=0.357$)

[c]3 hour significantly different ($p<0.01$) from all times, except 2 hour ($p=0.357$)

[d] 6 hour significantly different ($p<0.01$) from all times, except 9 hour ($p=0.872$)

[e] 9 hour significantly different ($p<0.01$) from all times, except 6 hour ($p=0.872$)

[f] 12 hour significantly different ($p<0.01$) from all times

[g] 1 day significantly different ($p<0.05$) from all times

[h] 2 day significantly different ($p<0.05$) from all times

[i] 3 day significantly different ($p<0.05$) from all times, except day 9 ($p=0.183$), day 12 ($p=0.485$), day 18 ($p=0.146$), day 22 ($p=0.073$), day 26 ($p=0.753$), and day 30 ($p=0.846$)

Table 3.2 (continued):

[j] 6 day significantly different ($p<0.05$) from all times, except day 9 ($p=0.444$), day 12 ($p=0.908$), day 15 ($p=0.076$), day 18 ($p=0.839$), day 22 ($p=0.533$), day 26 ($p=0.445$), and day 30 ($p=0.424$)

[k] 9 day significantly different ($p<0.05$) from all times, except day 3 ($p=0.183$), day 9 ($p=444$), day 12 ($p=0.628$), day 15 ($p=0.946$), day 18 ($p=0.257$), day 22 ($p=0.406$), and day 30 ($p=0.102$)

[l] 12 day significantly different ($p<0.05$) from all times, except day 3 ($p=0.485$), day 6 ($p=0.908$), day 9 ($p=0.628$), day 15 ($p=0.653$), day 18 ($p=0.978$), day 22 ($p=0.883$), day 26 ($p=0.523$), and day 30 ($p=0.302$)

[m] 15 day significantly different ($p<0.05$) from all times, except day 6 ($p=0.076$), day 9 ($p=0.946$), day 12 ($p=0.653$), day 18 ($p=0.329$), day 22 ($p=0.466$), day 26 ($p=0.212$), and day 30 ($p=0.243$)

[n] 18 day significantly different ($p<0.05$) from all times, except day 3 ($p=0.146$), day 6 ($p=0.839$), day 9 ($p=0.257$), day 12 ($p=0.978$), day 15 ($p=0.329$), day 22 ($p=0.166$), day 26 ($p=0.093$), and day 30 ($p=0.239$)

[o] 22 day significantly different ($p<0.05$) from all times, except day 3 ($p=0.073$), day 6 ($p=0.533$), day 9 ($p=0.406$), day 12 ($p=0.883$), day 15 ($p=0.466$), day 18 ($p=0.166$), day 26 ($p=0.087$), and day 30 ($p=0.221$)

[p] 26 day significantly different ($p<0.05$) from all times, except day 3 ($p=0.753$), day 6 ($p=0.445$), day 12 ($p=0.523$), day 15 ($p=0.212$), day 18 ($p=0.093$), day 22 ($p=0.087$), and day 30 ($p=0.841$)

[q] 30 day significantly different ($p<0.05$) from all times, except day36 ($p=0.846$), day 6 ($p=0.424$), day 9 ($p=0.102$), day 12 ($p=0.302$), day 15 ($p=0.243$), day 18 ($p=0.239$), day 22 ($p=0.2221$), day 26 ($p=0.841$)

Table 3.3: The minimum and maximum platinum concentrations expected to elute from individual carboplatin-impregnated CSH beads *in vivo*.

Time	**Range of platinum (mg/L) eluted per bead**	
	Minimum	**Maximum**
1 hour	84.0	85.0
2 hour	84.3	114
3 hour	79.0	123
6 hour	86.0	174
9 hour	65.3	195
12 hour	67.7	241
1 day	60.0	341
2 days	5.73	450
3 days	0.160	529
6 days	0.0867	557
9 days	0.0600	619
12 days	0.0467	598
15 days	0.0334	597
18 days	0.0300	580
22 days	0.0234	597
26 days	0	552
30 days	0	534

CHAPTER 4

FUTURE DIRECTIONS

Carboplatin-impregnated CSH beads are a delivery system for sustained release of carboplatin and are intended for implantation at sites of gross tumor or of marginal tumor extirpation. Calcium sulfate hemihydrate is a proven depot for drug release, is biodegradable, biocompatible, inexpensive, and sterilizable.[1-3] Published studies and unpublished reports have shown promising efficacy of cisplatin-and carboplatin-impregnated CSH beads against various tumors in horses and soft tissue sarcomas in dogs without clinically significant local or systemic toxicity.[1,a] Moreover, carboplatin-impregnated CSH beads are commercially available and affordable, at a cost of only $90 per three beads. To date, no studies exist evaluating the safety and efficacy of carboplatin-impregnated CSH beads in cats.

Feline injection site-associated sarcoma (FISAS) is reported to develop at sites of vaccination, subcutaneous injection, or microchip implantation, with prevalence ranging from 1 to 1.3 per 1000 vaccinated cats.[4-11] It is estimated that at least 66 million felines comprise the household cat population in the United States, and over 40% of these cats receive at least 1 vaccine per year, creating a sizable population of cats at risk.[4] Despite changes in vaccines and vaccination protocols performed in an attempt to mitigate occurrence of this tumor, incidence of FISAS over the past 20 years has not changed, still representing 15% of feline cutaneous masses.[12] These tumors can be very challenging to treat, as they are characterized by rapid growth and non-selective penetration into surrounding tissues and organs.[4,13-15] Due to their infiltrative nature, recent studies suggest 5 cm lateral margins and 2 deep tissue planes are required for complete

surgical excision.[14] Local recurrence has been reported in 19-22% of sites with microscopic tumor-free margins compared to 58-69% recurrence for sites without tumor-free margins.[13,14] Complete surgical excision of the tumor affords the best prognosis, but often requires radical surgery including thoracic or abdominal wall excision, amputation or hemipelvectomy, or removal of the scapula or dorsal spinous processes.[5,14-16] Although adjuvant radiation therapy improves prognosis, many cats do not receive optimum treatment due to cost, invasiveness, or lack of access to a veterinary specialist.[14,16] The mean time to tumor recurrence for such cats has been reported to be only 66 days, and the time to recurrence is shorter with each successive surgery, suggesting that incomplete excision may select for survival of a more aggressive population of cancer cells.[16] Unfortunately, most tumors recur within 1-2 years of treatment and are uniformly fatal despite efforts to manage the recurrence.[16]

The studies outlined in the previous chapters document the characteristics of release of carboplatin from carboplatin-impregnated CSH beads *in vitro*. Our data reveal that carboplatin-impregnated CSH beads release carboplatin locally at levels greater than those achieved in plasma with IV administration of a single dose. Implantation of carboplatin-impregnated CSH beads at the time of radical surgery may introduce sufficiently high concentrations of carboplatin to the surgical site to result in cytotoxicity of cancer cells. This treatment may drastically improve survival outcomes by delivering high concentrations of carboplatin directly to the site of greatest need, the extirpated tumor bed. In one study, horses with cutaneous tumors such as soft tissue sarcoma and squamous cell carcinoma were treated with bead implantation alone, without removal of the mass if the mass was <1.5 cm diameter, or with marginal removal of larger masses and implantation of beads. Two years after bead implantation, 40/48 of horses showed no

signs or recurrence.[1] In another study, platinum-containing CSH beads were implanted in the wound following wide surgical excision of soft tissue sarcomas. Thirteen of 23 tumors did not recur, and overall median disease-free interval was not reached.[1] These findings suggest that for cats not undergoing radical surgery for FISAS, implantation of carboplatin-impregnated CSH beads may be performed alone or in combination with a more marginal, less invasive surgery. However, before carboplatin-impregnated CSH beads may be used in clinically-ill cats, studies are needed to determine I) the efficacy of carboplatin-impregnated CSH beads against FISAS cells *in vitro* (the IC_{50} of carboplatin for FISAS cells), II) the distribution of carboplatin in a three dimensional space after diffusion from the CSH bead and III) the *in vivo* pharmacokinetics and safety profile of carboplatin-impregnated CSH beads when implanted subcutaneously into cats.

Funding has been secured for phases I and II of this study through an American College of Veterinary Surgeons Foundation grant. In phase I, a controlled, experimental study will be performed to evaluate the *in vitro* cytotoxic effects of carboplatin-impregnated CSH beads against FISAS cell lines in culture. Five FISAS cell lines will be treated with concentrations of carboplatin ranging from 5-450 μM derived from carboplatin-impregnated CSH beads. Cell proliferation assays and apoptosis analyses will be performed to identify the carboplatin concentration needed to minimally result in 50% inhibition of cell growth or cytotoxicity of FISAS cells (IC_{50}). In phase II, an agarose gel tissue phantom will be used to approximate spatial distribution of carboplatin from carboplatin-impregnated CSH beads. In phase III of the study, carboplatin-impregnated CSH beads will be implanted subcutaneously in 4 healthy cats to determine the pharmacokinetic (PK) and safety profile of carboplatin-impregnated CSH beads in cats. Blood samples for platinum and PK evaluation will be taken at 1, 2, 3, 6, 12, and 24 hours and 2, 3, 7, 14, and 21 days. Small, 4-

mm subcutaneous tissue samples obtained at 3, 7, 14, and 21 days will be evaluated histopathologically for signs of local toxicity and for platinum content by inductively coupled plasma-mass spectrometry (ICP-MS).

Preliminary data from one FISAS cell line indicate that the IC_{50}s for FISAS cells and carboplatin are within the range of concentrations released by carboplatin-impregnated CSH beads *in vitro*. It is expected that carboplatin concentrations released from carboplatin-impregnated CSH beads will be sufficiently toxic to FISAS cell cultures to achieve at least 50% growth inhibition or cytotoxicity of cells. It is also expected that following implantation in cats, concentrations of carboplatin within tissues in proximity of beads will exceed the IC_{50}. Finally, it is expected that neither significant systemic concentrations of platinum nor local toxicity will be observed associated with bead implantation in cats.

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Footnotes:

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