

Preclinical Evaluation of Concurrent Medicinal Mushroom–Based Immune-Enhancement Supplementation in Dogs Undergoing Chemotherapy for Various Cancers

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ABSTRACT: Chemotherapy is one of the most common treatments for canine cancer, although it is frequently quite toxic and can result in disruptions of the dog’s normal biochemical and physiological cycles. Chemotherapy is known to often drive the dog’s white blood cell count down (neutropenia), causing the immune system to weaken and raising susceptibility to opportunistic infections. This often causes chemotherapy schedules to be interrupted, resulting in less-effective treatment. Neutropenic patients are commonly symptomatic, exhibiting such side effects as anorexia, diarrhea, vomiting, fever, and death. Historically, neutropenia is one of the main factors considered in directing chemotherapy cycles. This study was conducted to determine the effectiveness of a popular mushroom-based veterinary immune-enhancement supplement, K-9 Immunity™, and its adjunct K-9 Transfer Factor™, when used concurrently with chemotherapy. The objectives of this study were several: to determine if immune supplementation could reduce neutropenia when partnered with chemotherapy, to determine if the incidence of side effects would be reduced, and to determine the overall effect on quality of life in the face of the chemotherapy. In addition to their regularly prescribed cancer treatments and medicines, dogs in this study received a daily dose of nonspecific immune-enhancement supplements: one 500 mg capsule per 4.5 kg body weight per day of K-9 Immunity™ and one 3000 mg wafer per day (for dogs greater than 11 kg) or 1/2 wafer per day (for dogs under 11 kg) of K-9 Transfer Factor™. Four different cancer types were tracked and evaluated in this study: lymphosarcoma (LSA) (N = 21), osteosarcoma (OSA) (N = 20), mast cell tumor (MCT) (N = 19), and hemangiosarcoma (HSA) (N = 4). Various chemotherapy or palliative therapy protocols were used according to tumor type and patient suitability. Standard chemotherapy protocols were followed throughout the study, so long as biological parameters were found to be within the normal ranges. Blood parameters monitored included CBC, chemistry panel, and urinalysis, as well as radiographs and other tests per individual protocol. All patients remained in their homes with clients who were asked to make daily assessments of appetite, attitude, vomiting, diarrhea, diet, medications, and other supplements, by way of a questionnaire. Patients were brought to the clinic routinely for assessment by the veterinary staff and received treatment as prescribed. The results of this study indicate that as an adjunct to chemotherapy, these supplements do reduce the common side effects for dogs who become neutropenic. The study also showed that this type of immune-modulation therapy relieves many of the symptoms usually associated with chemotherapy and allowed the dogs to maintain a quality of life more closely related to their norm. These results indicate that further research into nonspecific immune-modulation therapy as an adjunct to chemotherapy is warranted, with the goal of improving the quality of care and quality of life that we can deliver to our canine cancer patients.

KEY WORDS: immune supplements, K-9 Immunity, K-9 Transfer Factor, dog chemotherapy, canine lymphosarcoma, mast cell tumor, canine osteosarcoma, canine hemangiosarcoma, medicinal mushrooms

ABBREVIATIONS

CBC: complete blood cell count; **CCNU (Lomustine):** 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; **CHEM:** full blood chemistry panel; **CHOP:** chemo protocol using a specific regime of drugs; **DFI:** disease-free interval; **DIC:** disseminated intravascular coagulation; **GI:** gastrointestinal; **HSA:** hemangiosarcoma; **IgA:** Immunoglobulin A; **IgG:** Immunoglobulin G; **IgY:** Immunoglobulin Y; **i.l.:** intralesional; **i.m.:** intramuscular; **i.v.:** intravenous; **LSA:** lymphosarcoma; **LTFU:** lost to follow-up; **MCT:** mast cell tumor (mastocytoma); **N = 21:** number of cases is 21; **NED:** no evidence of disease; **NSAID:** nonsteroidal anti-inflammatory drug; **NT:** new tumor type appeared; **OSA:** osteosarcoma; **PD:** progressive disease; **SD:** stable disease; **s.q.:** subcutaneous; **TLR:** Toll-like receptors; **u:** units; **UA:** urine analysis; **VCCC:** Veterinary Cancer Care Center; **VCOG:** Veterinary Co-Operative Oncology Group; **VSS:** Veterinary Surgical Specialists; **WBC:** white blood cell; **WHO:** World Health Organization; **5FU:** 5-fluorouracil; **>=50%:** greater than or equal to 50%; **<=50%:** less than or equal to 50%.

I. INTRODUCTION

Cancer is the body's failure to recognize and destroy cells that do not replicate normally. A healthy immune system searches out and destroys these irregular cells before overgrowth can occur. When the immune system is not functioning properly, this cell overgrowth is free to continue to tumor formation. The key to successful cancer treatment is in triggering the immune system's recognition and response to these cells before uncontrolled overgrowth occurs. The body's own immune system thereby is able to fight off the cancer.¹⁻⁵

The currently accepted theory of solid-tumor formation is that three criteria must take place for cancer to occur:

1. Cells must have errors in the replication process. These replication errors can be influenced by toxins (environmental or otherwise), viral infection, radiation, age, and other unknown factors.
2. Once these errors in replication occur, the immune system fails to recognize them as aberrant, allowing further replication of these errors.
3. The immune system remains in an unresponsive state for a long enough period of time, allowing the aberrant growth to become self-sustaining, resulting in tumor formation.

When considered in this context, cancer is viewed primarily as an immune-dysfunction disease. Anything that can be done to enhance or correct the immune recognition and destruction of aberrant

cells shifts the balance more toward the destruction of these cells than their replication. Modern cancer treatment protocols are focused only on destruction of the aberrant cells. Although this is vital in any successful cancer treatment, it can only be viewed as one arm of a two-pronged approach, if we hope to increase long-term survival rates. The real issue to be addressed in long-term survival is that when the chemotherapy, radiation, and surgery are all finished, and all the cancer cells have been destroyed, the underlying immune dysfunction that allowed the proliferation of the tumor in the first place still exists. This is why it is so important to try to enhance immune response during cancer treatments, and why immune supplementation is thought to positively affect the outcome of conventional cancer-treatment methods.^{6,7}

Cancer is the leading cause of death in senior dogs (>5 years old) in the United States, accounting for approximately 50% of deaths in this group of animals.^{8,9} Because the mechanisms of action in most cancers are similar or even identical between humans and canines,^{10,11} it has become common practice in the veterinary field to apply the same palliative protocols to dogs as would be used in treatments for similar human diseases. As the use of mushroom-derived polysaccharides has been shown to positively affect the outcome of chemotherapy and reduce the chemotherapy-associated side effects in a number of human studies, their use as an adjunct with cancer treatment over the last 20 years has become quite common. There is a large quantity of research indicating the effectiveness of this type of adjunct treatment.^{3,4,12-18}

This report is on a 1-year-long study of dogs receiving immune supplementation concurrently with chemotherapy. It was initiated in June of 2007 at the Veterinary Cancer Care Center (VCCC) in Santa Fe, New Mexico (USA). The main purpose of this study was to assess whether the same positive effects would be seen in canines undergoing similar chemotherapy regimens as has been seen in human trials.

Dogs undergoing chemotherapy may experience several side effects due to the toxic drugs administered, which include (but are not limited to) pain, diarrhea, constipation, hair loss, nausea, vomiting, fever, and death. In addition, blood-related side effects, such as a decrease in the number of infection-fighting white blood cells (*neutropenia*), low red blood cell count (*anemia*), and low platelet count (*thrombocytopenia*) have also been noted.^{18, 19, 20}

Of these various side effects, one of the most critical is neutropenia. This is a blood disorder characterized by an abnormally low number of neutrophils, a type of white blood cell that usually comprises 50%–70% of the circulating white blood cells. Neutrophils provide the initial major defense against infections by destroying bacteria and viruses in the blood. Neutropenia is often seen with chemotherapy and can impair the patient's ability to fight off opportunistic infections, leaving the patient more susceptible to illness. Without prompt medical attention, the condition may become life-threatening (febrile neutropenia or neutropenic sepsis).^{20, 21}

One purpose of this study was to explore the possibility of dietary immune supplementation helping to boost white blood cell (WBC) levels in dogs undergoing chemotherapy treatments for various types of cancer. Other considerations addressed in this study were disease-free intervals and various biologic parameters, such as blood chemistry, urinalysis (UA), radiographs, and overall patient quality of life, as measured by symptomatic episodes. The basic questions posed at the onset of this study were as follows: Can K-9 Immunity™ prevents neutropenia, thereby avoiding missed chemotherapy treatments? Will K-9 Immunity™ supplementation present a more favorable treatment outcome? Is it possible that K-9 Immunity™ can improve quality of life for those patients undergoing chemotherapy or palliative treatments?

II. MATERIALS AND METHODS

A. Study Entry Criteria and Explanation of Cancer Types

Subjects were chosen based on four of the most common canine tumor types: lymphosarcoma (LSA) (n = 21); osteosarcoma (OSA) (n = 20); mast cell tumor (MCT) (n = 19); and hemangiosarcoma (HSA), both splenic (n = 2) and cutaneous (n = 2). There were no control patients for this study; rather, all comparisons were taken from historical data. The standard measurements used for assessing the severity and disease progression for these and other cancer types are referred to as stages and grades. According to the National Cancer Institute, tumor grade is a system used to classify cancer cells in terms of how abnormal they look under a microscope and how quickly the tumor is likely to grow and spread. Staging describes the extent or severity of an individual's cancer based on the original (primary) tumor and the spread of that cancer in the body.²²

Dogs involved in this study received either chemotherapy per prescribed protocols based on Veterinary Co-Operative Oncology Group (VCOG) accepted practices and dosages, or other approved therapies such as surgery with no chemotherapy, as determined on an individual basis by the attending veterinarian. Chemotherapy dosages were adjusted on a case-by-case basis according to the dog's response, to ensure humane and safe administration and outcome. Subjects were evaluated at the Veterinary Cancer Care Center (VCCC) for protocol procedures associated with the individual tumor type diagnosed. Clinical parameters noted were weight, tumor size (where applicable), biological data, side effects, tumor status, immune-supplement disbursement, and retrieval count, as well as treatment type and/or rest weeks. Miscellaneous medications, supplements, or procedures were also noted.

All patients in this study were housed in their own home environment, and the immune supplements were administered by the individual dog owners on a daily basis. Assessments of appetite, attitude, and activity levels were also made by the owners on a daily basis using a "monthly calendar" that was supplied to them, with parameters for the assessment of the pet's daily routine, including not

only appetite and attitude, but diet, vomiting, and diarrhea, ease of administration of supplements, and usage of other medications or supplements. Calendars and supplement containers were returned to the clinic at each visit for evaluation and monitoring to assure compliance. This information was then transferred to a master spread sheet for each patient. The master sheet for each subject also contained biologic information such as complete blood cell count (CBC), chemistry, urinalysis, radiographs, weight, and patient status.

B. Entry Criteria for All Tumor Types

The entry criteria for each tumor type are very similar among the four cancers monitored. All patients were required to have a CBC and a full blood chemistry panel (CHEM) (with or without UA), as appropriate, a surgical report or baseline abdominal ultrasound for MCT and HSA, a 2-view abdominal radiograph for LSA, and bone-lesion radiographs for OSA. For MCT and LSA, a 2-view chest radiograph may or may not have been required according to patient presentation, and 3-view chest radiographs were required for all OSA and HSA patients. A fine needle aspirate of the lymph nodes, with or without liver or spleen aspirate (if abnormal), was required for MCT, and an aspirate of the bone marrow was needed for LSA if the CBC was abnormal. MCT dogs had their masses and lymph nodes measured, and all cancer types had a cytological (LSA), histopathologic (HSA, MCT, OSA), or radiographic (OSA) diagnosis.

C. Parameters Recorded

The parameters recorded for the tumor types included the side effects of chemotherapy; VCOG grades of neutropenia, if present; the number of unscheduled rest weeks; GI toxicity; disease-free intervals (DFI); review of monthly calendars; tumor or lymph node size for OSA and LSA; and survival time and/or remission status. The remission status recorded was as follows:

1. Complete remission (100% resolution of gross disease)

2. Partial remission ($\geq 50\%$ reduction of gross disease)
3. Progressive disease ($\leq 25\%$ reduction of gross disease or new lesions)
4. Stable disease ($< 50\%$ decrease or $< 25\%$ increase in gross disease)

D. Monitoring of Chemotherapy Patients

Patients undergoing chemotherapy had a CBC performed at each chemotherapy visit and nadir; OSA patients had additional serum chemistry at the halfway point and at the end of therapy. Each of the monthly calendars was also reviewed and recorded. All tumor types underwent restaging as required; HSA and LSA patients were restaged after 4 cycles of chemotherapy and then every 3 months thereafter until 1 year. MCT patients were restaged at the last chemotherapy session, then every 3 months for 1 year. OSA patients were restaged every 3 months for 1 year. Any dog undergoing multiple chemotherapy protocols had full chemistry workup prior to each doxorubicin treatment.

E. Monitoring for Surgery Only or Palliative Patients

The monitoring for patients who received surgery only or palliative treatments included a monthly physical exam and repeat blood work every 3 months (CBC, CHEM, with or without UA, as appropriate). Also reviewed and recorded were the monthly calendar, measurement of the masses, and lymph nodes. The patients were also restaged every 3 months for the full year according to the tumor type. In addition, OSA patients had remission status recorded; a lameness score; graded I–IV; 3-view chest radiographs; bone-lesion radiographs; and a full blood workup every 3 months.

F. Explanation of Individual Tumor Types

1. Lymphosarcoma (LSA)

LSA is one of the most common cancer types seen in dogs and begins in cells of the immune system.

It is characterized by an uncontrolled growth of lymphocytes (cells that normally function as part of the immune system). It affects dogs of any breed and age, although most dogs are middle-aged or older at the time of diagnosis.²¹ There are several stages used in assessing LSA according to standards of the WHO clinical staging system²²:

Stage I: Single lymph node enlarged

Stage II: Multiple nodes enlarged on either side of the diaphragm (the body cavity separating the chest and abdominal regions)

Stage III: Multiple nodes enlarged on both side of the diaphragm

Stage IV: Involvement of the liver and/or spleen

Stage V: Bone marrow involvement or involvement of other organs (e.g., gastrointestinal, skin, or nervous system)

Each numbered stage is further divided into substages (A and B). Patients substaged A feel well, whereas patients with substage B are ill. Survival times for most LSA dogs treated with combination chemotherapy protocols are in the range of approximately 1 year.^{21,22} In this study, all LSA patients were quite far along in disease progression. Of the twenty-one LSA dogs on this study, three were staged as IIIA and one was staged as IIIB and was also found to have hypercalcemia. Seven of the twenty-one were staged as IVA, one of those having T-cell lymphoma, one having hypercalcemia, and two having both hypercalcemia and T-cell lymphoma concurrently. Three of the twenty-one dogs were staged as IVB, and three of the twenty-one were staged as VB, one having pulmonary carcinoma.

2. Osteosarcoma (OSA)

OSA is the most common primary bone cancer in dogs and is estimated to account for roughly 85% of tumors in the canine skeleton. This type of cancer most often occurs in the front limbs, with tumors near the “wrist” (in the radius or ulna), but it can actually occur in any bone in the body. Middle-

aged to older large and giant breed dogs are most often affected. Dogs with this type of cancer often undergo surgery to remove the limb where the tumor is located, or occasionally limb-sparing surgery is performed. Following surgery, it is common practice to follow with chemotherapy treatment. Approximately one half of the dogs with OSA treated in this manner will be living 1 year after diagnosis. Approximately 25% of the dogs treated in this fashion are still living 2 years after diagnosis. Dogs treated with either amputation or palliative radiation therapy (without chemotherapy) live, on average, approximately 6 months before complications of the local tumor or metastasis cause death or prompt owners to consider euthanasia.^{19,21}

Staging for OSA is based on human values and consists of grade, anatomic location of the primary tumor, and the presence of regional or distant metastasis. Substages are A (intracompartmental tumor) and B (extracompartmental tumor).

Stage I: Low-grade tumor without metastasis

Stage II: High-grade tumor without metastasis

Stage III: Presence of regional or distant metastasis

On the basis of this grading system, most dogs with OSA present in stage IIB. In fact, nineteen of the twenty OSA dogs involved in this study were staged as IIB, and four of the nineteen had distant metastasis. One of the twenty dogs was staged as IIIB.

3. Mast Cell Tumor (MCT)

MCT is an overgrowth or mass composed of mast cells. MCTs can involve the skin, subcutaneous tissue, and muscle tissue. MCTs in dogs are very common, accounting for approximately 20% of all skin tumors.^{19,21} These tumors can arise from any skin site on the body and can have a variety of appearances. It is recommended that before any skin mass is removed, the cells from it be collected for examination to rule out mast cell or other malignant or benign tumor.²¹ Mast cells are easily identified on aspiration (withdrawing of cells from the mass via needle).

MCTs are staged and graded according to the WHO clinical staging system²³:

Stage I: One tumor confined to the skin, with no regional lymph node involvement

Stage II: One tumor confined to the skin but with regional lymph node involvement

Stage III: Many tumors that are often large, deeply infiltrating tumors, with or without lymph node involvement

Stage IV: Any tumor with distant spread evident. This stage is further divided into substage A, the dog is apparently well with no clinical signs of illness, and B, with clinical signs of illness. To determine the tumor stage, evaluation of other lymphoid organs must be performed.

In determining the prognosis of MCT of the skin, grade must also be considered. Grade is determined by the biopsy results (histopathology).

Grade I: Well-differentiated, mature cells with a low potential for metastasis

Grade II: Intermediately differentiated cells with potential for local invasion and moderate metastatic behavior

Grade III: Undifferentiated, immature cells with a high potential for metastasis

The prognosis for completely excised Grades I and II tumors is excellent. The prognosis for incompletely removed Grades I and II tumors treated with radiation therapy after surgery is also excellent, with approximately 90%–95% of dogs having no recurrence of the tumor within 3 years of receiving radiation therapy. The prognosis for dogs with Grade III tumors is considered guarded because local recurrence and/or spread is likely in most dogs.

Of the nineteen dogs involved with this study, two were classified as Grade I, both having clean margins, meaning that all the aberrant cells were removed during surgical excision, and they both had a low mitotic index, meaning that the remaining cells were likely slow replicating. Fourteen of the nineteen MCT dogs in this study were classified as Grade II, and six of the fourteen had dirty margins, meaning that upon examination of the

excised mass after surgery it was found that not all the cancer cells were removed. Three of these fourteen dogs also showed a high mitotic index, whereas six of them had a low mitotic index. Two of the nineteen MCT dogs were classified as Grade III, one with dirty margins and high mitotic index and the other with dirty margins and low mitotic index. One subject had gross disease, and stage and grade were unknown, whereas six of the nineteen dogs had an unknown mitotic index.

4. Hemangiosarcoma (HSA)

HSA most often appears on the spleen, right heart base, or liver, although varieties can also appear on the skin or in other locations. HSA of the spleen or liver is the most common tumor to cause hemorrhage in the abdomen. HSA of the skin usually appears as a small red or bluish-black mass. This type of cancer can also occur under the skin, it and is suspected that in the skin, it is caused by sun exposure. Occasionally, HSA of the skin can be a metastasis from visceral HSA. Other sites of the tumor may include bone, kidney, bladder, muscle, mouth, and the central nervous system. HSA can cause anemia, thrombocytopenia (low platelet count), and disseminated intravascular coagulation (DIC). Clinical signs of visceral HSA include loss of appetite, arrhythmia, weight loss, weakness, lethargy, collapse, pale mucous membranes, and/or sudden death. An enlarged abdomen is often seen, due to hemorrhage. Metastasis is most common to the liver, omentum, lungs, or brain. The usual estimate of the average time from discovery of the tumor until death occurs in affected dogs is 6 to 8 weeks, but death occurs more rapidly than this in a number of cases, usually due to hemorrhage.

Staging for HSA is based on WHO criteria²³:

Stage I: Primary tumor confined to spleen, without evidence of rupture, and measuring less than 5 cm, with no evidence of metastasis

Stage II: Primary tumor less than 5 cm or evidence of rupture, with or without regional metastasis

Stage III: Invasive tumor greater than 5 cm with evidence of regional metastasis

Of the four HSA dogs involved in this study, two had subcutaneous tumors, one of which had distant metastasis. The other two HSA dogs had splenic HSA, one of which had a “burst” or hemorrhagic spleen.

G. Chemotherapy Drugs Used in Study

Carboplatine (dosage of 300 mg/m² i.v. every 3 weeks.) for use with LSA and OSA. *Lomustine* (CCNU) (dosage of 60–90 mg/m² by mouth every 3 weeks) for use with LSA and MCT. *5-Fluorouracil* (5FU) (dosage of 150 mg/m² i.l. weekly) use in MCT. *Doxorubicin* (adriamycin) (dose of 25–30 mg/m² every 3 weeks) for use with LSA and OSA. *Oncovin* (Vincristine) (dosage of 0.5–0.7 mg/m² i.v. weekly) for use with LSA and HSA. *Cyclophosphamide* (Cytosan) (dosage of 200–300 mg/m² by mouth over 2–3 days; may also be dosed at 250 mg/m² i.v.) for use with LSA, MCT, and HSA. *Chlorambucil* (Leukeran) (dosage of 2–6 mg/m² by mouth daily, every other day) for use with LSA and MCT. *Vinblastine* (dosage of 2.0 mg/m² i.v. every week) for use with LSA and MCT. *Bleomycin* (dose of 10–20 mg/m² s.q.) for use with MCT and LSA. *Cisplatin* (dose of 10 mg/m² i.l.) for use with OSA. *Prednisone* (dosage of 1–2 mg/kg by mouth for 3 weeks, then taper) for use with LSA, OSA, and MCT. *Dacarbazine* (dosage of 800–1000 mg/m² i.v. every 3 weeks) for use in LSA rescue therapy after coming out of remission. *Asparaginase* (dosage of 10,000 µ/m² i.m. or s.q.) for use with LSA.

Metronomic Therapy (low-dose continuous chemotherapy) is a combination of chemotherapy, pain relief, antibiotic, and antiangiogenesis therapies. Drugs used are *Cytosin* 10–25 mg/m² by mouth every other day; *Piroxicam* (feldene) or other NSAID therapy based on clinician preference, typically 0.3 mg/kg by mouth daily; and *Doxycycline* 10 mg/kg orally, daily.

H. Treatment Protocols

There are several protocols used for each cancer type, and they are used according to both the tumor type and the tolerance of the individual animal. In

addition to this, LSA treatment comprises a multitude of different protocols because it is a highly variable cancer that may enter into and exit out of remission several times during the lifetime of the patient. Once a patient comes out of remission, a different protocol is used because the aberrant cells become resistant to previously administered treatment drugs.

1. LSA Chemotherapy Protocols

Chemotherapy was administered for 16–24 weeks, according to one or more of the following protocols:

1. *CHOP protocol*. Vincristine is administered in the 1st week, cytosin in the 2nd week, vincristine again for the 3rd week, and adriamycin for the 4th week, repeated for 4–6 cycles.
2. *Doxorubicin/dacarbazine protocol*. Doxorubicin is administered for 1 week, dacarbazine for the 2nd week, and then the weekly cycles are repeated.
3. *Lomustine protocol*. This single agent is administered repeatedly once or twice a week, repeated every 3–6 weeks, depending on the tolerance of the animal.
4. *Vincristine and cyclophosphamide protocol*. Vincristine and cyclophosphamide are administered during week 1, vincristine is administered during weeks 2 and 3, and both vincristine and cyclophosphamide are administered again during week 4 and routinely, every 2–3 weeks, for maintenance over a 6-month period.
5. *L-asparaginase/lomustine protocol*. L-asparaginase is administered for the 1st week, then lomustine is administered the 2nd week. A CBC is taken for baseline chemistry in weeks 3 and 4 and before any dosage of lomustine, or after every 3 doses have been administered. This treatment cycle is repeated for a period of 4–6 months.
6. *Vincristine/lomustine protocol* is administered weekly for 16–24 weeks. The 1st week vincristine is given, with lomustine administered in the 2nd week and only a CBC taken for weeks 3 and 4. Weeks 1–4 are repeated, and

- prednisone is given initially at 5 mg every 12–24 hours then tapered.
7. *Vincristine/chlorambucil protocol* was administered weekly for 16–24 weeks. The 1st week both vincristine (vinblastine may be substituted) and chlorambucil were administered. Chlorambucil alone was administered for weeks 2 and 3 and then the drug cycle was repeated.
 8. *Chlorambucil* was used as a single agent; administration was repeated once or twice a week, and this once or twice weekly administration was repeated every 3–6 weeks.
 9. *Lomustine* was used as a single agent; administration was repeated every 3–6 weeks.
 10. *Doxorubicin* was used as a single agent; administration was repeated every 3–6 weeks for a maximum of 5 doses or 180–240 mg/m² total.
2. *Lomustine* was used as a single agent every 3–4 weeks for a total of 6–8 treatments. Both CBC and blood chemistry were monitored. Prednisone was administered daily for 2 weeks, then tapered.
 3. *Vinblastine* was used as a single agent weekly for a total of 6–8 treatments. CBC was taken and monitored. *Prednisone* was administered daily for 2 weeks, then tapered.
 4. *Vinblastine/lomustine* protocol administered vinblastine during week 1, required CBC only for week 2, lomustine was administered for week 3, and the CBC was taken only for week 4. This protocol was continued for 4 cycles with the CBC being monitored weekly as well as chemistry for lomustine treatments. Prednisone was given daily for 2 weeks, then tapered.
 5. Intralesional chemotherapy with the single agent 5-FU or bleomycin was administered weekly.

2. OSA Chemotherapy Protocols

1. *Carboplatine* was used as a single agent protocol every 3 weeks for 4–6 treatments.
2. *Doxorubicin* was used as a single agent every 2–3 weeks for 4–6 treatments.
3. *Doxorubicin/carboplatine* or *cisplatin* protocols were administered for 3 cycles, with the CBC taken and doxorubicin administered in week 1, only CBC taken in week 2, CBC taken and carboplatine administered during week 3, only CBC is taken during weeks 4 and 5, and both CBC taken and doxorubicin administered during week 6. This cycle was repeated for a total of 3 doxorubicin treatments and 3 carboplatine treatments.
4. Intralesional chemotherapy was administered every other week, with cisplatin, until a pain response was noted.

3. MCT Chemotherapy Protocols

1. *Chlorambucil* was used as a single agent daily or weekly for 4–6 months. CBC must be done every 2–3 weeks. Prednisone was administered daily for 2 weeks, then tapered.

4. HSA Chemotherapy Protocol

1. *Doxorubicin/metronomic* protocol required doxorubicin every 2 weeks × 5 treatments along with metronomic chemotherapy (piroxicam, cytoxan, doxycycline) daily to every other day.
2. Maintenance chemotherapy protocol required vincristine every 2–3 weeks along with metronomic chemotherapy daily to every other day.

5. Immune-Enhancement Supplements Used in Conjunction with Chemotherapy

K9 Immunity™ is an orally administered medicine (class “Dietary Supplement” in the United States) intended for daily use in adult dogs undergoing conventional therapy. It assists in optimizing immune-system function and consists of a heterogeneous mixture of multiple heteropolysaccharide moieties derived from six species of medicinal mushrooms (*Agaricus brasiliensis* (= *A. blazei* ss. Heinem.), hybrid *Cordyceps sinensis*, *Lentinus edodes*, *Grifola frondosa*, *Ganoderma lucidum*, and *Trametes versicolor*). It is supplied in red- and

white-colored capsules containing 500 mg active ingredients each.

K9 Transfer Factor™ is a specially formulated canine supplement containing several types of antibodies, proline-rich polypeptides (aka Transfer Factors), and other immunoproteins for use adjunctively with K-9 Immunity™. These immune protein molecules enhance the absorption and bioavailability of the polysaccharide immune modulators in K-9 Immunity™ by bonding to the polysaccharides and acting as a bridge between the immune-cell surface receptors and the polysaccharides. Both K-9 Immunity™ and K-9 Transfer Factor™ are manufactured by Aloha Medicinals Inc. of Carson City, NV (USA), and were obtained directly from the manufacturer for this trial.

6. Supplement Dosage Adjuncts to Chemotherapy

Standard canine chemotherapy protocols were used according to cancer type, along with daily supplementation with the veterinary supplements. Each dog received a dose of one 500 mg capsule of K9 Immunity™ per 4.5 kg body weight per day. Each capsule contains 500 mg of a formula comprising over 200 different structural types of immune-modulators of the complex polysaccharide class, enzymatically derived from six species of medicinal mushroom. To ensure proper uptake and bioavailability of the K-9 Immunity™ supplement, all dogs also received K9 Transfer Factor™ daily, at a dose of one 3000 mg wafer per day for dogs greater than 11 kg and 1/2 wafer per day for dogs 11 kg and under. Each K9 Transfer Factor™ wafer contains 3000 mg of the immune proteins IgA, IgG, and IgY, along with proline-rich polypeptides, also known as Transfer Factors. These immunoproteins are derived from bovine colostrums, bovine serum, and avian sources (chicken egg yolk).

7. Laboratory and Chemistry Panels

All lab work was performed either in house at VCCC, at the referring veterinarians' offices, or at Antech Diagnostic Laboratories, Phoenix, AZ (USA).

Samples were taken aseptically from the jugular vein when possible via sterile procedure with needle and syringe. Urine for UA was normally obtained via free catch, unless there was an indication for cystocentesis (i.e., possibility of urinary tract infection), which then was performed by sterile procedure with needle and syringe. Cytologies, chemistries, and UA were performed both in house for speed of diagnosis, with confirmation at Antech Labs. Cytology samples were obtained by fine needle aspirate using a sterile procedure. Biopsy samples were interpreted at Antech Labs, as well as at the Colorado State University School of Veterinary Medicine laboratories, Colorado State University (USA). Most samples were obtained by the referring veterinarian prior to the subject's initial visit to VCCC. Those requiring biopsy or surgery were referred to Veterinary Surgical Specialists of New Mexico. Ultrasound, when required, was performed at VCCC and also at Veterinary Surgical Specialists of New Mexico, 4000 Montgomery Blvd., NE Albuquerque, NM 87109 (VSS). All radiographs were obtained either at the referring veterinarian or at VSS. Where applicable, measurements of tumors were taken with standard metric caliper. Other testing and laboratory procedures performed were at the discretion of the attending veterinarian and in keeping with the best and most humane treatment for the patient. All testing was performed per standard protocol.

III. RESULTS

A. Incidence of Neutropenia for All Tumor Types

The severity of neutropenia is divided into four grades according to neutrophil count as determined by the Veterinary Co-Operative Oncology Group (VCOG).²⁴

Grade I - Neutrophil count of 1500–2500/μL. This is fairly common in patients undergoing chemotherapy. These patients should be closely monitored, with delayed treatment until cells reach normal levels (above 3000/μL).

Grade II - Neutrophil count of 1000–1500/μL. Broad-spectrum antibiotics should be adminis-

tered and regular blood counts taken to monitor the bone marrow.

Grade III - Neutrophil count of 500–1000/ μ L.

Grade IV - Neutrophil count of 1–500/ μ L.

Grades III and IV require immediate action because there is a very high risk for infection.

B. Neutropenia in LSA Patients

Of the twenty-one dogs diagnosed with LSA, eleven (52.38%) developed Grade I neutropenia, but only one (4.76%) was symptomatic. Four (19.04%) of these 21 subjects developed Grade II neutropenia, but only two (9.52%) were symptomatic. One (4.76%) dog developed Grade III neutropenia and was symptomatic, whereas two (9.52%) dogs developed Grade IV neutropenia, but only one (4.76%) of them became symptomatic. According to the VCOG neutropenia scores, four (19.04%) of the twenty-one dogs attained both Grade I and II neutropenia over the course of the study, whereas one (4.76%) of the twenty-one dogs attained both Grades I and IV neutropenia. Five subjects had to postpone 1 treatment, two subjects had to postpone 2 treatments, one subject had to postpone 3 treatments, and one subject had to postpone 4 treatments.

C. Neutropenia in OSA Patients

Of the twenty dogs diagnosed with OSA, four (20%) developed Grade I neutropenia, none of which showed any symptoms. Three (15%) dogs developed Grade II neutropenia, and no dogs developed Grades III or IV neutropenia. None of the neutropenic dogs with OSA showed any symptoms of their condition. However, one non-neutropenic subject became symptomatic and had to postpone one chemotherapy treatment. In addition, one subject was categorized as having both Grades I and II neutropenia over the course of the study.

D. Neutropenia in MCT Patients

Of the nineteen dogs diagnosed with MCT, two (10.53%) developed Grade I neutropenia, but only

one (5.26%) showed any symptoms. Another one (5.26%) of the nineteen dogs developed Grade II neutropenia but didn't show symptoms, none of the dogs developed Grade III neutropenia and one (5.26%) dog developed Grade IV neutropenia and showed symptoms. Both subjects who became symptomatic each had to postpone one chemotherapy treatment.

E. Neutropenia in HSA Patients

Of the four dogs diagnosed with HSA, none were neutropenic; however, two (50%) became ill with either vomiting or diarrhea but missed no chemotherapy treatments. Both of these symptomatic dogs received adriamycin.

F. Summary of Appetite and Attitude for All Tumor Types

Appetite and attitude were based on a median average of Grades 1–5, with 1 being anorexic with no appetite and having little or no interest in activity and 5 having normal or better appetite and or activity, as indicated by the pet owner on a monthly calendar provided by VCCC. These numbers were then assigned based on the subject having a consistent score for 3 consecutive days.

1. LSA

Of the twenty-one dogs diagnosed with LSA, none were recorded as having the anorexic Grade 1 appetite or attitude. Three (14.28%) dogs were recorded as having Grade 2 appetite, but none of the dogs were recorded as having Grade 2 attitude. Four (19.04%) dogs were recorded as having Grade 3 appetite, and 5 (23.80%) dogs were recorded as having a Grade 3 attitude. One (4.76%) dog was logged as having a Grade 4 appetite, and five (23.8%) dogs were logged as having a Grade 4 attitude. Eleven (52.38%) dogs maintained a Grade 5 appetite, and nine dogs (42%) maintained a Grade 5 attitude. There were

2 subjects whose data was unknown because their calendars were not received.

2. OSA

Of the twenty dogs diagnosed with OSA, none of them were recorded as having a Grade 1 or 2 appetite, whereas two (10%) of them were recorded as having a median Grade 3 appetite, 6 (30%) were recorded as having an almost normal Grade 4 appetite, and ten (50%) dogs were recorded as having normal or better Grade 5 appetite. One (5%) of the twenty dogs was recorded as having a Grade 1 attitude, 4 (20%) were recorded as having a Grade 2 attitude, four (20%) were recorded as having a Grade 3 attitude, six (30%) were recorded as having a Grade 4 attitude, and three (15%) were recorded as having a Grade 5 attitude. There were two subjects whose appetite and attitude data was unknown because their calendars were not received.

3. MCT

Of the nineteen dogs diagnosed with MCTs, none were recorded as having a Grade 1 or 2 appetite or attitude. There was one (5.26%) dog that was recorded as having a Grade 3 appetite and two (10.52%) dogs were recorded as having a Grade 3 attitude. There was one dog (5.26%) that was logged as having a Grade 4 appetite, and two (26%) dogs that were logged as having a Grade 4 attitude. There were fourteen (73%) dogs that maintained a Grade 5 appetite, and nine dogs (47.36%) that maintained a Grade 5 attitude. There were three subjects whose data was unknown because their calendars were not returned.

4. HSA

Of the four dogs diagnosed with HSA, one (25%) of the dogs was recorded as having a median Grade 3 appetite, and three (75%) maintained normal or better Grade 5 appetite habits. Two (50%) of these dogs were recorded as having a Grade 4

attitude, and two (50%) of these dogs maintained a Grade 5 attitude.

G. Symptomatic Gastrointestinal Episodes

Each value was assigned based on client observation of the subject in their home environment and in correspondence to the VCOG scoring for GI values. A grade of I was given to any subject who vomited or had diarrhea, or both, for 3 consecutive days. A grade of II was given to any subject who exhibited these side effects for 3 days or more but less than 5 days. There were no grades higher than II.

1. LSA

Of the twenty-one dogs diagnosed with LSA, sixteen (76.2%) dogs did not show any symptoms of GI toxicity, and three (23.8%) attained Grade 1 GI toxicity, and none attained Grade 2. One of the dogs that attained Grade 1 toxicity ate a large amount of green chili peppers and was sick for 3 days. This toxicity may or may not have been related to the chemotherapy treatments.

2. OSA

Of the twenty dogs diagnosed with OSA, seventeen (85%) did not show any GI toxicity at all, whereas three (15%) of them attained Grade 1 GI toxicity. None of the dogs attained Grade 2.

3. MCT

Of the nineteen dogs diagnosed with MCT, fourteen (73.7%) did not show any GI toxicity at all, four (21.05%) of them attained Grade 1 GI toxicity, and only one dog (5.26%) attained Grade 2 toxicity.

4. HSA

Of the four dogs diagnosed with HSA, two (50%) did not show any GI toxicity at all, whereas two

(50%) attained Grade 1 GI toxicity. None of them attained Grade 2 GI toxicity.

H. Summary of Survival Status for Each Tumor Type as of June 1, 2008

Status of the disease was evaluated and recorded by the attending veterinarian at each visit based on physical exam and/or lab findings. Some of these subjects did not undergo chemotherapy treatments. Surgery, amputation, and IL treatment modalities were also used. These factors may have had an influence on disease status. For survival and remission status, it is important to note that any individual dogs may have been classified as belonging to one or more of these status categories throughout the study. NED means No Evidence of Disease, which includes complete remission (100% resolution of gross disease) and partial remissions (greater than or equal to 50% reduction of gross disease). SD means Stable Disease (tumor stable or growth less than 20% after the start of therapy). PD means Progressive Disease (tumor increase of greater than 20% after start of therapy). Deceased means death via euthanasia (by euthasol dose 1 mL per 10 kg body weight) or home death. NT means that a New Tumor type appeared. LTFU means that the dog was Lost To Follow Up visits. In more than one case, the client chose early euthanasia over prolonged treatment if a subject came out of remission.

1. LSA

At the end of the year-long study, eight (38.09%) of the twenty-one dogs diagnosed with LSA showed no evidence of disease and were classified as experiencing complete remission, five (23.8%) of the twenty-one dogs experienced partial remissions for different periods of time on an individual basis, one (4.76%) of these dogs developed a new tumor type, fourteen (66.66%) were classified as having stable disease, twelve (57.14%) of the dogs were classified as having progressive disease, ten (47.62%) dogs in total with this cancer type died during the course of the study, and two (9.52%) were lost to follow-up. The dogs with this cancer

type stayed in the study anywhere from 2 to 12 months before the dog either died or the study was completed. It is noteworthy to mention that most subjects in this study, when first presented, already had advanced disease; one subject had pulmonary carcinoma, one subject had T-cell LSA, two subjects had hypercalcemia, and two subjects had T-cell LSA + hypercalcemia.

2. OSA

Four (20%) of the twenty dogs diagnosed with OSA had NED at end of trial, four (20%) had PD, two (10%) had SD, nine (45%) dogs with this cancer type died over the course of the trial, and one (5%) was lost to follow-up. The dogs with this cancer type stayed in the study from 1 to 11 months before the dog either died or the study was completed.

3. MCT

Thirteen (68.42%) of the nineteen dogs diagnosed with MCT had NED at end of trial, three (15.80%) of the nineteen had PD, all of which died before the completion of the trial. One (5.26%) of the nineteen had an NT develop, one (5.26%) of the nineteen had SD, and one (5.26%) of the nineteen was lost to follow-up. Of the four dogs (21.05%) diagnosed with MCT that died by the end of this study, three were classified as having PD, and because of the aggressive cancer type, would normally have a low life expectancy. All dogs with this cancer type stayed in the study from 1 to 9 months before the dog either died or the study was completed.

4. HSA

One (25%) of the four dogs diagnosed with HSA showed NED at the end of the study, one of the four (25%) had PD, and two (50%) of the dogs were deceased. The dogs with this cancer type stayed in the study from 2 to 9 months before the dog either died or the study was completed. One of the HSA patients died acutely at home with no evidence of progressive disease.

IV. DISCUSSION

Studies to determine the efficacy of dietary supplements such as K-9 Immunity™ in the treatment of canine cancer are few. Many of these trials are simple studies with many variables. Because the average owners of cancer pets may already be instituting multiple supplement therapies on their own, it is difficult to get precise information and dosing procedures. The nature of the cancer process itself does not lend itself to ease of study because protocols are constantly changing, depending on the patient's disease and remission status, among other variables. Economics must also be considered because some cancer treatments are quite costly. This study was directed toward issues with neutropenia and the resulting side effects, but it became apparent during this trial that cancer treatment and its side effects are multifactorial, and other indicators need to be looked at as well. The statistical analysis needs to be evaluated, as well as a follow-up of patients and a confirmation of data comparisons to historical or other controls.

An easy and relatively inexpensive method of tracking the immune response to cancer treatment involves evaluation of the neutrophils in the bloodstream. Immunomodulators are used to boost the nonspecific immune components of the white blood cell line.²⁵ The neutrophil component of the complete blood count is associated with the short-term response of the immune system to an insult, such as chemotherapy. In tracking the response of the neutrophil line, this study noted that the K-9 Immunity™ supplement did defend the patient from the onslaught of opportunistic infections when affected by neutropenia, and that the quality of life the patient had previously experienced was maintained, in most cases. The main actions exhibited by the mushroom-derived polysaccharide

and proteoglycan components of K-9 Immunity™ and K-9 Transfer Factor™ appear to be activation and enhancement of various aspects of innate and specific immune response (host-mediated immune response). The number and activity of T lymphocytes (T cells), B lymphocytes (B cells), macrophages, and antigen-presenting cells (APC) were stimulated and/or modulated. The exact mechanism of action for these products is only partially understood, but it is thought to be mainly accomplished by binding beta-glucans, proteoglycans, and other heteropolysaccharides to specific cellular receptors on the surface of the various classes of immune cells, probably the CR3, Dectin-1, LacCer, TLR, and scavenger receptors.²⁵

As seen in Table 1 and Figure 1, it appears that the study subjects suffered fewer symptomatic episodes in conjunction with their neutropenia than could be expected based upon previous experience with similar patients. The number of patients who became neutropenic yet who were free of symptoms seemed (in the experience of these authors) to be better than historical averages, although definitive historical data on neutropenia and its associated side effects could not be found in a search of the scientific literature. Of the sixty-four dogs involved with this study, 56% showed no neutropenia, while only 44% acquired some grade of neutropenia. Of those that became neutropenic, only about 10% showed symptoms. This is less than 5% of the total subject becoming symptomatic. Only one of the study patients who was non-neutropenic became symptomatic. In addition, it is noteworthy that of the LSA patients, one subject had pulmonary carcinoma, one subject had T-cell LSA, two subjects had hypercalcemia, and two subjects had T-cell LSA + hypercalcemia. Having these additional factors added to the diagnosis indicates that the disease has progressed to a higher stage of illness. Hypercalce-

TABLE 1
Neutropenia and Symptomatic Episodes for All Tumor Types

	Grade I	Grade II	Grade III	Grade IV
Neutropenia	26.56%	12.55%	1.56%	4.68%
Symptomatic	3.12%	3.12%	1.56%	3.12%



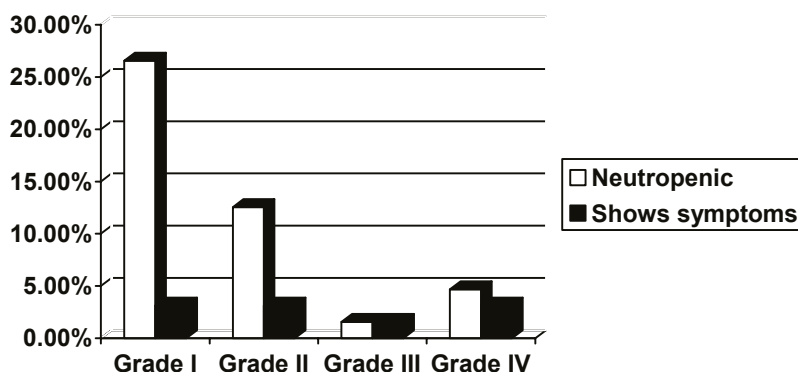


FIGURE 1. Number of dogs involved with this study that attained any grade of neutropenia and associated symptoms.

mia is not only a cause of morbidity on its own, but is usually associated with T-cell lymphoma, which historically has a shorter survival time than B-cell lymphoma. T-cell lymphoma affects the immune system directly and so may initiate both a higher incidence of neutropenia and other symptoms. In addition, there were a few subjects who were classified as having more than one grade of neutropenia over the course of the study. Symptomatic response was based on episodes of vomiting and/or diarrhea, as well as overall activity level as reported by owners on the monthly calendar. An overall average was taken, based on the subject being symptomatic for 3 consecutive days or more. One subject became symptomatic, even though neutrophil counts remained in the normal range.

Appetite and attitude were both based on a median average of Grades 1–5, with 1 being poor (little or no appetite/activity on the subject's part), and 5 being normal or great, with the subject acting happy and exhibiting a normal routine. These values were assessed by pet owners daily and reported to the clinic at each visit. An overall average was taken, and a grade of 1–5 was given to each dog based on an appetite and attitude score recorded consistently for 3 consecutive days. Study findings seen in Tables 2–4 and Figures 2–4 reveal the general trend that appetite and attitude remained above average with low percentages of GI toxicity for all cancer types. Of the sixty-four total dogs in the study, 60.94% of them maintained a normal or better Grade 5 appetite, whereas 35.94% maintained a normal or better Grade 5 attitude. In

addition, totaling the successive results in Table 4 shows that 76.56% of all dogs in the study had no gastrointestinal toxicity whatsoever. This supports the finding that most dogs were able to continue normal routines with little or no problem.

The overall median survival for all subjects on trial appeared to be improved from historic averages, although this information is hard to tabulate on such a short open-label trial. The indications of Table 5 show that at least 50% of the total number of dogs taking K-9 Immunity™ and K-9 Transfer Factor™ adjunctively with chemotherapy in all cancer types reviewed were still living at the close of our year-long study. It is important to note that for Figure 5, any individual dog may have been classified as belonging to one or more of these status categories throughout the study. It can be clearly seen from these findings that K-9 Immunity™ does indeed improve the patient's quality of life when used adjunctively with chemotherapy, at least in the types of cancers evaluated in this study. For canine cancer families, this quality-of-life consideration is an important part of the decision-making process when considering treatment for their pets.

V. FUTURE STUDIES

The results of this study indicate that further research into nonspecific immune-modulation therapy as an adjunct to chemotherapy is warranted, with the goal of improving the quality of care and quality of life that we can deliver to our canine cancer

TABLE 2
Appetite

	Grade 1 (worst)	Grade 2	Grade 3	Grade 4	Grade 5 (best)
LSA	0%	14.28%	19.04%	4.76%	52.38%
OSA	0%	0%	10%	30%	50%
MCT	0%	0%	5.26%	5.26%	73.68%
HSA	0%	0%	25%	0%	75%

Note: LSA = lymphosarcoma; OSA = osteosarcoma; MCT = mast cell tumor; HSA = hemangiosarcoma.

TABLE 3
Attitude

	Grade 1 (worst)	Grade 2	Grade 3	Grade 4	Grade 5 (best)
LSA	0%	0%	23.8%	23.8%	42.86%
OSA	5%	20%	20%	30%	15%
MCT	0%	0%	10.53%	26.32%	47.4%
HSA	0%	0%	0%	50%	50%

Note: LSA = lymphosarcoma; OSA = osteosarcoma; MCT = mast cell tumor; HSA = hemangiosarcoma.

TABLE 4
Percentage of Dogs Showing No GI Toxicity

	LSA	OSA	MCT	HSA
Grade 1	76.20%	85%	78.95%	50%
Grade 2	100%	100%	94.74%	100%

Note: LSA = lymphosarcoma; OSA = osteosarcoma; MCT = mast cell tumor; HSA = hemangiosarcoma.

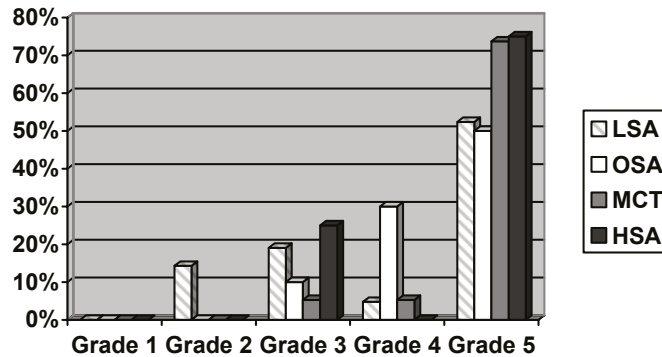


FIGURE 2. Appetites of all the dogs in the study by tumor type and grade. Grade 1 = no appetite; Grade 5 = normal or better appetite.

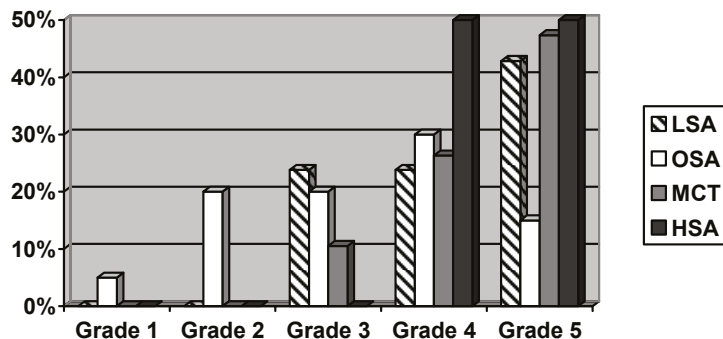


FIGURE 3. Grades of attitude for all tumor types for dogs in this study.

patients. There are many avenues to be explored in the study of immune modulators in cancer therapy in dogs. This particular study, in revealing the fact that neutropenia is not entirely controlled by immune supplementation, begs the question of how long those neutropenic periods were, or if the neutropenic episodes were any shorter due to the concurrent supplementation. Diets must also be explored in conjunction with supplements. Chemistry values can be further explored as well as other blood parameters such as effect on neutrophil functions. This preclinical trial evaluation shows the great promise held by combining some alternative medicine techniques with conventional medicine protocols. Further double-blind, placebo-controlled trials are planned in conjunction with a major veterinary university to further explore the potential of this method of treatment.

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FULL DISCLOSURE OF FINANCIAL TIES OF AUTHORS

J. Holliday and B. Gianotti are employed by Aloha Medicinals Inc., which manufactures the products

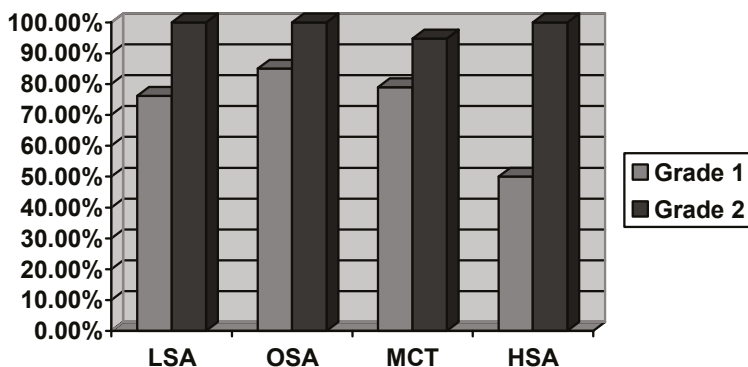


FIGURE 4. Percentage of dogs per tumor type without gastrointestinal side effects.

