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Evaluation and Comparison of Different Surgico-chemotherapeutic Regimens for the Treatment of Malignant Canine Mammary Tumours

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ABSTRACT

Background: Cancer has been regarded as the most dreaded disease for both human and animals because of its incurability. Canine mammary tumours are common and major problem encountered by academicians and practicing veterinarians. As a result, the current study was conducted to examine the effectiveness and overall impact of different surgico-chemotherapeutic approaches in treating malignant mammary tumors in dogs.

Methods: The investigation involved 18 clinical instances of mammary tumors in different breeds, regardless of age or sex and were divided into three groups of six animals each. In Group S, only surgical excision of tumour was performed while animals of Group SD and Group SV were treated with surgical excision followed by administration of Doxorubicin (30 mg/m^2) BSA and Vincristine sulphate (0.025 mg/kg) intravenously alongwith DNS at 7th and 14th post-operative days respectively. Different physiological and haemato-bichemical parameters *i.e.* Hb, PCV, TLC, TPC, DLC, Serum glucose, TSP, SUN, SC, ALT, AST and ALP were recorded preoperatively, postoperatively and following chemotherapy at 10th, 30th and 60th day intervals.

Result: The present study showed transient change in the physiological, haematological and biochemical profiles following surgery and chemotherapy treatment. The histopathological analysis showed more cases of adenocarinoma which were followed by mixed carcinoma. Surgery combined with chemotherapy (doxorubicin and vincristine) resulted in minimal to no reoccurrence with few adverse reactions including inappetance, vomition, anaemia and alopecia. It was concluded that the most effective treatment for malignant mammary tumors in dogs is surgical excision followed by sequential vincristine therapy, which efficiently regresses the tumor without causing relapse.

Key words: Canine, Doxorubicin, Malignant, Mammary tumour, Regimen, Surgico-chemotherapy, Vincristine sulphate.

INTRODUCTION

Canine mammary tumors are the second most prevalent type of tumor after skin tumors (Moulton 1990), making up about 50% of all tumors in female dogs, of which 40-50% are malignant (Sorenmo, 2003) and most common type of tumors in female dogs which are not spayed. However, male dogs are also affected but the prevalence is only 1% (Rutterman *et al*., 2000). The most frequently affected sets of glands are the fourth and fifth pairs and can be a single or multiple tumours affecting one or more glands. If lump in the mammary gland is detected, it should be examined as it is the first alarming indication for tumour. The various types of mammary tumour include fibroadenoma, mixed adenocarcinoma and intraacinar carcinoma. Female sex hormones progesterone and estrogen are important for appropriate mammary gland development; early exposure to these hormones is strongly linked to an increased risk of developing mammary gland tumors (Petrov *et al*., 2014). The risk of developing mammary tumors in bitches spayed before their first estrous cycle is approximately 0.5%, whereas the risk is approximately 8% in bitches spayed between their first and second estrous cycles and the risk is 26% after the second estrous cycle (Brodey *et al*., 1983). According to Kishor *et al*. (2016), the age range where mammary tumors occurred most commonly was 8-10 years, then 10-12 years.

Now a days, a number of chemotherapeutic drugs like Cyclophosphamide, Chlorambucil, Vincristine, Vinblastine, Carboplatin, Cisplatin, Methotrexate, 5-Fluorouracil and

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Doxorubicin etc are being used for the treatment of canine mammary tumours. Majority of scientific community with maximum research grant are engaged for development of appropriate cancer therapeutics. No single chemotherapy regimen that has been shown to be completely effective. In veterinary practice, surgery still remains the treatment of choice for the majority of canine tumors but their recurrence following surgery is common in dogs due to the growth of the residual tumour which carry high risk of metastasis. In such cases, radical surgery is advocated followed by chemotherapeutic measures to arrest the recurrence resulting from remnants. Therefore, adjunct chemotherapy *i.e.* surgical excision along with chemotherapy is a newer approach for the treatment of tumours. Doxorubicin is an anthracycline glycoside antibiotic that was initially produced by *Streptomyces peucetius var. caesius* and exerts its cytotoxic effect as a DNA-intercalating agent to inhibit further DNA and RNA biosynthesis (Lori *et al*., 2010). Vincristine sulfate is an antimicrotubule drug obtained from the plant *Vinca rosea* that causes mitotic arrest by interfering with the polymerization or depolymerization of microtubules, which plays a critical role in cell function and division (Gustafson and Page, 2013). Since there are few studies on the use of vincristine sulphate and doxorubicin as adjuvant chemotherapy for the treatment of canine malignant mammary tumors in clinical cases, therefore, the primary goal of present study was to evaluate the effectiveness and systemic impact of consecutive vincristine sulphate and doxorubicin chemotherapy in conjunction with surgical removal of malignant mammary tumor in dogs.

MATERIALS AND METHODS

The current investigation was carried out on 18 clinically suspected cases of canine mammary tumors (17 female and 1 male) at the Teaching Veterinary Clinical Complex (TVCC) and Department of Veterinary Surgery and Radiology, College of Veterinary Science and A.H., Anjora, Durg (C.G.) between July 2017 and July 2018. They were aged 5-12 years and weighed between 10-20 kg. The breed wise distribution of dogs was as follows: 4 Labrador, 4 German shepherd, 3 Non-descript, 3 Pomeranian, 1 Golden Retriever, 2 Lasa Apso, 1 Cross breed. Owner's consent was also taken into consideration before grouping of these animals for surgico-chemotherapy. The screening of the affected animal was done on the basis of physical examination of tumour mass, affected gland, sex and reproductive status. Pre-surgical evaluation of all the animals was done before start of regimens in terms of physiological and haematological profile to ensure the general health status of animals. The physiological profile was within normal range but haematological profile showed some variation. There was increase in total leucocyte count and Hb and PCV values were less than normal range, so all the animals were stabilized by antibiotic and supportive (oral haematinics) therapy before surgery. Fine needle aspiration cytology of the growth or mass was done in which tissue was taken from the growth using a tiny needle to detect whether the growth present in the gland are tumorous or not.

Regimens

The 18 animals were divided into three groups, consisting of six animals each: Group S, Group SD and Group SV. Mammectomy was performed under general anaesthesia (atropine sulphate @ 0.02 mg I/M, xylazine hydrochloride @ 1 mg I/M and ketamine @ 5 mg I/V) in all the animals. Group S underwent simply surgical removal of the tumor while Group SD and Group SV had surgico-chemotherapeutic regimens and sequential chemotherapy after seven days following operation. On the 7^{th} and 14^{th} post-operative days, Vincristine sulphate (0.025 mg/kg) was given intravenously coupled with DNS in group SV, whereas Doxorubicin (30 mg/m^2) BSA was given intravenously along with DNS in group SD. Dogs in Group SD and SV received supportive therapy using Tribivet @ 0.05-0.2 mg/kg b.wt. I/M for five days followed by Polybion syrup once tsf bid and Tab Liv-52® daily throughout their chemotheraptic regimens therapy.

Physiological profiles

Rectal temperature (ºF), heart rate (beats/minute) and respiratory rate (breaths/minute) were measured preoperative, during surgery at 10, 30, 60, 90 and 120 minutes, as well as on the 10^{th}, 30^{th} and 60^{th}day post surgery following the chemotherapeutic regimen.

Haemato-biochemical profiles

About 1 ml blood sample was taken from dog's cephalic or saphenous vein in vials containing ethylene diamine tetra acetic acid (EDTA) before and on the 10^{th} , 30^{th} and 60^{th} day after surgery and following the chemotherapeutic regimen. The automated haematology blood cell counter (B. C. 2800 Vet. Mindray) was used to estimate hemoglobin (gm/dl), packed cell volume (%), total erythrocyte count (millions/ mm^3), total leukocyte count (thousand / mm^3) and total platelet count (lac / mm^3). Blood smears were prepared from the obtained blood in order to manually perform the Differential Leukocyte Count (DLC). The smear was fixed for one minute in methanol and then stained for thirty minutes using Geimsa stain. A percentage was used to express the counts. In order to measure biochemical parameters, 3 ml of peripheral venous blood was drawn from the dog in a sterile, clean vial before and at 10^{th}, 30^{th} and 60^{th} day following surgery and the chemotherapy regimen. Serum was separated for the estimation of serum glucose (mg/dl), total serum proteins (gm/dl), serum urea nitrogen (mg/dl), serum creatinine (mg/dl), aspartate aminotransferase (U/L), alanine aminotransferase (U/L) and alkaline phosphatase (U/L) using a semi-automated biochemistry analyzer (Diasil-100 Systronics make).

Histopathological analysis

Following surgery, the tissue samples were collected and rinsed with normal saline to remove extraneous blood and other debris.The specimens were cut into 2 mm cubes and fixed in 10% neutral buffered formalin in accordance with standard protocol and were embedded in paraffin wax. These sections of the paraffin embedded tissues were cut at 3-5 µm thickness and stained using Haematoxylin and Eosin (H and E) according to the standard protocol (Luna, 1968) for microscopic analysis under a light microscope to obtain details of the histopathology.

Evaluation and comparison of surgico-chemotherapeutic regimens

The effectiveness of different regimens were assessed based on the evaluation of the wound and the tumor's recurrence following two weeks of chemotherapy doses. The wound was examined on days 0, 3rd and 10th day following surgery to determine the nature of wound (normal or oedematous) and the type of discharge (bloody, serosanguinous, foetid, or pussy).Any negative consequences of the regimens were also noted.

Statistical analysis

The collected data was subjected to analysis of variance (ANONA) and Duncan's multiple range test (DMRT) in order to compare the mean value within each group at various time intervals as well as between groups at different time intervals. The statistical software package SPSS v17.0 was used to analyze all the data, which were displayed as mean±Standard Error and the level of significance was set at 5%.

RESULTS AND DISCUSSION

Examination of animals before regimens

All 18 cases of mammary tumour affected dogs presented to Department of Veterinary Surgery and Radiology were unspayed and uncastrated. On clinical examination, the shape of mammary tumours varied from ovoid, elongated, rounded to irregularly nodular and ulcerative (Fig 1). Most of the tumours were solitary and pedunculated. Grossly, tumours were soft to firm in consistency. The physical examination of tumour mass revealed ulcerative tumour in 6 cases (33.33%) followed by and non-ulcerative in 6 cases (33.33%), 2 cases were smooth (11.11%) and 4 cases were intact and subcutaneous (22.22%). The occurrence of mammary tumour was more in 5th gland (6 cases) (33.33%) followed by 4th to 5th gland (4 cases) (22.22%), 4th gland (4 cases) (22.22%) and 3rd to 4th gland (4 cases) (22.22%). The present study, revealed that mammary tumours were most by affecting the 5th gland followed by 4th to 5th gland, 4th gland and 3rd to 4th gland probably because of their greater size containing more mammary tissue and thereby subjected to a greater range of physiological changes, predisposing them to neoplasms. Dileepkumar *et al.* (2014) and Lather *et al.* (2017) have also reported maximum occurrence in 5th inguinal mammary gland. The incidence of mammary tumour was observed more in female (94.44%) as compared to male (5.55%). In the present study, out of 18 cases of mammary tumour, only one male dog was affected with mammary tumour and rest were female dogs. Similar findings were recorded by Rutterman *et al*. (2000) and Dhami *et al*. (2010) who reported that higher incidence of mammary tumours in female dogs as compared to male dog. Palta (2000); Bala (2005) and Gupta *et al*. (2012) reported mammary tumour in one male dog. In fact, canine mammary tumours are specific tumour in females and rare in males and are often associated with hormonal abnormalities (Moulton *et al*., 1970). This could be due to action of ovarian hormones (estrogens and progesterone) on mammary gland tissue during different stages of development which could be a risk factor associated with the development of mammary tumours (Petrov *et al*., 2014). In the present study, the incidence of mammary tumour was more in intact/ unsprayed dogs. Thus, it could be inferred that unspayed bitches have greater risk for occurrence of mammary tumours as compared to spayed ones. The reason could be hormone dependency of proliferating neoplastic cells. This is further supported by the observation of temporary regression of already existing mammary tumours after spaying. Alenza *et al.* (2000) also reported that intact females had a 3 to 7-fold greater risk of developing

a. Mammary tumour involving both 4th and 5th mammary gland.

b. Ulcerative mammary tumour with maggots in female dog.

c. Intact mammary tumour in female dog.

d. Ulcerative mammary tumour with maggots in male dog.

Fig 1: Location of canine mammary tumour involving mammary glands.

mammary tumours than neutered females. Fine needle aspiration cytology of the growth or cutaneous mass smear showed cellular anisocytosis, increased pleomorpism with change in shape of identical type cells which were in clusters. Cells were large in size with giant nucleus and devoid of its architectural details. Few areas in the slide of smear revealed minimum variation in shape and size of cells, well adherent with each other as well as nuclear detail was remained unaltered. The mammary gland structure remained uniform and epithelial cells have formed bigger clusters. These cytological findings are suggestive of tumorous growth. Similar findings have also been reported by Chavan *et al.* (2016).

Physiological profiles

In all the three groups, there was a non-significant drop in rectal temperature and respiration rate up to 60-minute interval, after which they increased non-significantly and approached normal value. On the other hand, heart rate increased significantly ($P<0.05$) in all the groups within the first 30 minutes and then steadily declined throughout the remaining 120 minutes of monitoring. However, the values were within physiological limits in all the three groups. Dropped rectal temperature and respiration rate following anaesthesia might be due to synergistic action of ketamine and xylazine. Hellebrekers *et al.* (1998) also reported similar results. Heart rate, breathing rate and rectal temperature did not significantly vary in any of the three groups during the surgico-chemotherapeutic regimens. Similar findings were noted by Todorova *et al*. (2005) in dogs with mammary tumors following surgery and during the first, second and third injections of chemotherapy.

Haemato-biochemical profiles

Haematological paramaters did not differ significantly in different groups at various time intervals before, post-surgery and after chemotherapeutic regimens. The reduction in haemoglobin levels following the administration of vincristine sulphate and doxorubicin may have been the consequence of chemotherapy-induced impairment to the bone marrow's ability to produce red blood cells. Anaemia caused by bone marrow depression results in a progressive reduction in haemoglobin concentration as also reported by Brar *et al.* (2002). In groups SD and SV, there was a non-significant drop in TEC, which could be due to chemotherapy induced erythrocytopenia resulting from myelosuppression. Brar *et al.* (2002) and Todorova *et al.* (2005) also made similar observations. There was a significant ($P<0.05$) decrease in TLC in groups SD and SV due to the cytotoxic drugs which inhibit the bone marrow's replicating precursor cells and thus results in reduced leucocyte production. A dose-dependent, reversible leukopenia was described by Todorova *et al.* (2005) as the main sign of doxorubicin bone marrow haematologic toxicity. Group SV had a non-significant increase in total platelet count (thrombocytosis) upto 30th day followed by a decline whereas, a non-significant decrease in TPC (thrombocytopenia) was observed in group SD (surgical excision plus doxorubicin therapy), where dogs with mammary tumors received two doses of treatment. The localization of doxorubicin hydrochloride occurs in the bone marrow of long bones and as chemotherapy treatment advances, the bone marrow's capacity to generate platelets is diminished and leads to immune-mediated thrombocytopenia. However, a non-significant rise in TPC (thrombocytosis) was found in group SV, where dogs with mammary tumors received both surgical excision and two doses of vincristine sulphate therapy. Consequently, since thrombocytosis was noted with very minor haematological changes, so it is possible to conclude that the chemotherapeutic agent vinca alkaloid vincristine sulphate is a safe drug. Significant ($P<0.05$) drop in the number of neutrophils was observed in groups SD and SV following chemotherapeutic drug (doxorubicin and vincristine sulphate) that normally localizes into the bone marrow of long bones and whose myelosuppressive activity reduces the neutrophil percentage, Consequently, it is possible to state that intravenous chemotherapy causes neutropenia. In contrast to this; there was negligible neutrophil decline in group S. Similar to this, Ravikumar *et al.* (1999) noted a steady decline in neutrophil count until vincristine therapy ended, which was the result of decreasing bone marrow production.The reversible leukopenia and neutropenia that were significantly ($P<0.05$) found in this study after chemotherapy (doxorubicin and vincristine) was most likely caused by the action of cytotoxic drugs, which might have reduced the bone marrow's replicating precursor cells and produced myeloid toxicity. The current study confirms the findings of Srivastava *et al*. (2009), who reported alterations in their haematological parameters after vincristine therapy due to depression of reticuloendothelial system. Similarly, Khan *et al*. (2017) also noted a significant ($P<0.05$) decrease in haematological parameters after surgical excision and doxorubicin treatment in dogs which could be due to complete suppression of bone marrow activity, accidental infection, or an allergic reaction.

Biochemical pofiles

Serum glucose levels in group SD exhibited a significant ($P<0.05$) increasing trend up to 60th days after doxorubicin therapy, while group SV showed an non-significant drop up to 60th days following vincristine therapy. The elevated glucose levels in group SD could be stress induced activity of doxorubicin drug which resulted in the production of glucocorticoids and mineralocorticoids as a result of the adrenal cortex being stimulated, as well as epinephrine and non-epinepherine from the medulla. The animals in the SD group had a significant ($P<0.05$) decrease in total serum protein value at 10th day which further increased significantly ($P<0.05$) upto 60th day. Despite this, there were marginal variations in the serum total protein values for group S and SV during the observation period. According to Sandhu and Rampal (2006), the marginal irregular pattern

of serum total proteins after chemotherapy could be due to gastro-intestinal disorder from cytotoxic drugs. Serum urea nitrogen and serum creatinine levels in group SD increased significantly ($P<0.05$) upto 30th day which further dramatically decreased ($P<0.05$) at 60th day whereas, group S and SV had irregular alterations during the observation period. Todorova *et al*. (2005) did not find any significant change in the blood urea nitrogen and serum creatinine level after 2nd dose of doxorubicin. Similarly, Srivastava *et al*. (2009) found that total protein, urea nitrogen, creatinine and glucose levels remained within normal range during the entire period of vincristine therapy with surgical excision. Khan *et al*. (2017) reported a significant ($P<0.05$) increase in BUN, serum creatinine, AST and ALT in dog treated with surgery alongwith doxorubicin therapy whereas, a non-significant change was observed in dogs treated surgically. The current investigation suggests that the elevation of AST and ALT levels in the SD and SV groups after chemotherapy may be the consequence of vincristine and doxorubicin hydrochloride detoxification in the liver, which is exclusively loaded for transaminase production (Todorova *et al*., 2005). In contrast to our study, Palta (2000) observed insignificant alterations in AST and ALT levels after neoadjuvant and adjuvant chemotherapy with vincristine in canine mammary neoplasm cases. However, Gupta *et al*. (2014) found a non-significant rise in ALT levels in the surgical excision group treated with vincristine following the second cycle of therapy, which might be due to an increase in the liver's metabolic activity for drug detoxification. The values of alkaline phosphatase (ALP) were towards the higher limit of normal range in all the three groups at the time of presentation. Similar finding were also observed by Bala (2005) at the time of presentation of canine mammary tumor. Alkaline phosphatase (ALP) levels in group S, SD and SV showed significant ($P<0.05$) decrease upto 30th day with a further increase upto the end of observation period. This suggests that increase in the alkaline phosphatase level could be attributed due to presence of malignancy and addition of chemotherapy (vincristine and doxorubicin) induced stress, which was intensely reflected by rise in alkaline phosphatase activity during the research period. Alkaline phosphatase activity rises in cases of cancinoma, according to Chauhan and Agarwal (2008) findings and values of serum ALP gradually increased following the first and second cycles of vincristine therapy (Gupta *et al*., 2014).

Histopathological analysis

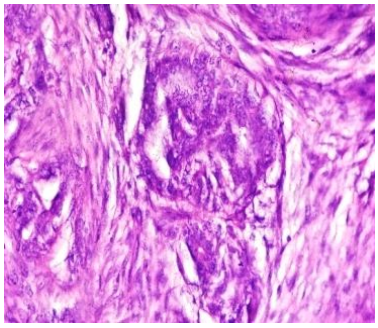
After histopathological confirmation regarding presence of malignancy, all eighteen cases were included in the study. Canine mammary mixed carcinoma (7) and canine mammay adenocarcinoma (11) were the two most prevalent tumors in the current investigation. Adenocarcinoma (61.11%) was most common, followed by mixed carcinoma (38.89%). This aligned with the research conducted by Nayyar (2002) and Ravikumar *et al*. (1999), which indicated that adenocarcinoma was the most prevalent kind of tumor, followed by mixed mammary tumors. On the other hand, according to reports from Moulton *et al*. (1970); Gill (1997) and Palta (2000) mixed mammary tumors were more prevalent followed by adenocarcinoma.

a) Canine mammary adenocarcinoma

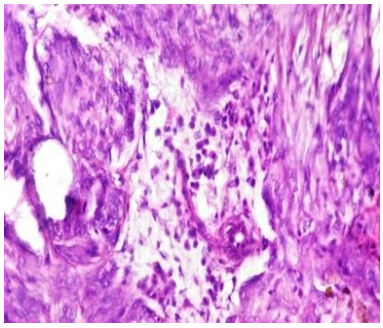
The histopathology of mammary tumour mass stained with Haematoxylin and Eosin showed papillary projections, distorted glandular epithelium, infiltration of lymphocytes with fibroblastic connective tissue and sheaths of neoplastic cells arranged in the form of islands with little stroma and many mitotic figures (Fig 2). These findings were in agreement with Jain *et al*. (2011) and Manjunatha *et al*. (2013).

b) Canine mammary mixed carcinoma

The histopathology of mammary tumour mass stained with Haematoxylin and Eosin revealed that growths were intermingled with the proliferating glandular epithelium of ducts, tubulopapillary and acini. The epithelial region showed many aberrant mitotic figures, big ovoid hyperchromatic nuclei with prominent nuclei and significant

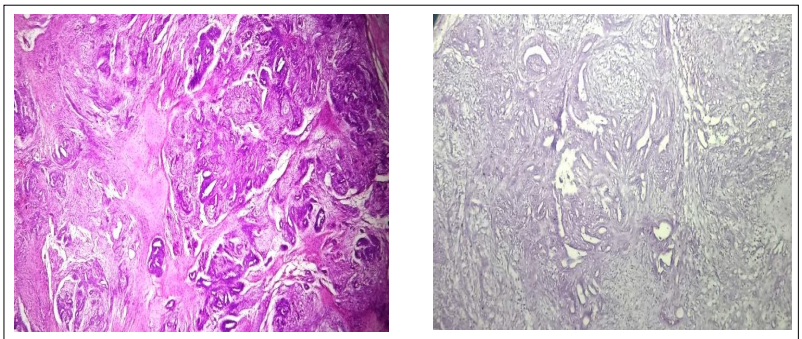


a. Adenocarcinoma showing distorted appearance of glandular epithelium (40×).



b. Adenocarinoma showing infiltrating lymphocytes with fibroblastic connective tissue and mitotic figures (20×).

Fig 2: Histopathology of canine mammary adenocarcinoma (H and E stain).



a. Mammary tumour composed of epithelial cells arranged in a tubulopapillary pattern with connective tissue components (20×).

b. Mixed mammary tumour depicting epithelial and connective tissue elements (10×).

Fig 3: Histopathology of canine mammary mixed carcinoma (H and E stain).

nuclear polymorphism of epithelial cells (Fig 3). Additionally, granulomatous masses showed round or polyhedral neoplastic cells with chondroid metaplasia. The observations of Dileepkumar *et al*. (2015) and Lather *et al*. (2017) were used to mimic these findings.

Evaluation and comparison of different surgico-chemotherapeutic regimens

In all three groups, the wound healing process proceeded normally without any complications or discharge. All the three groups showed complete wound healing in 8 to 10 days and were monitored for a minimum of three months following surgery and after completion of chemotherapy regimens (doxorubicin and vincristine sulphate). During the course of the chemotherapy, there was no recurrence in the group SV (surgical excision and vincristine therapy), in contrast to this, group SD (surgical excision and doxorubicin therapy) exhibited minimum recurrence (+) in one case while group S (surgical excision) showed mild to moderate recurrence (+, ++) in two cases. Inappetence vomiting, anemia and alopecia were the side effects associated with administration of chemotherapy drugs. In comparison to group SV, animals in group SD had a greater number of the aforementioned symptoms. But as palliative measure, the conditions of the animals were managed by supportive therapy alongwith administration of intravenous fluids, antaacids and liver tonics. These results corroborate the observations of Srivastava *et al*. (2009), who noted that surgery in conjunction with vincristine therapy resulted in complete regression of the tumor without recurrence. The overall response of treatment with surgical excision and chemotherapeutic drug administration (doxorubicin and vincristine) showed that vincristine sulfate was superior to doxorubicin due to its minimal side effects on haemato-biochemical profiles. Based on the aforementioned study, the most effective treatment for canine malignant mammary tumor is surgical excision followed by sequential vincristine therapy, since the tumor regressed completely without showing any signs of recurrence.

CONCLUSION

Surgical excision combined with sequential vincristine sulphate chemotherapy is a more effective treatment for canine malignant tumors than surgical excision combined with sequential doxorubicin chemotherapy because it effectively inhibits the growth of new tumor cells and metastasis.

Conflict of interest

All authors declare that they have no competing interests.

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