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Effect of the tumor microenvironment on vincristine response in triple-negative and triple-positive primary canine mammary tumor cells: A 2D and 3D culture study

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Abstract

Canine mammary tumors (CMTs) are among the most common neoplasms in female dogs, with over half exhibiting malignant and aggressive behavior. Although vincristine is routinely used in veterinary oncology, its cytotoxic potential in relation to hormone receptor and HER2 status remains unclear. This study evaluated vincristine responsiveness in triple-positive (ER⁺/PR⁺/HER2⁺) and triple-negative (ER⁻/PR⁻/HER2⁻) primary cultured CMT cells grown under two-dimensional (2D) and three-dimensional (3D) culture systems, the latter better mimicking the tumor microenvironment. Tumor tissues excised from clinical cases were histopathologically confirmed as malignant, and receptor status was determined by RT-PCR. Primary CMT cells (CMT1-CMT5) and normal mammary epithelial cells were treated with graded concentrations of vincristine (0-100 μ M), and IC₅₀ values were calculated. Vincristine induced dose-dependent cytotoxicity in all CMT lines, with CMT2 and CMT3 showing the highest sensitivity, whereas CMT5 exhibited partial resistance. Normal mammary cells displayed significantly lower mortality ($p < 0.001$). No correlation was observed between cytotoxicity and receptor status. In 3D spheroids, vincristine reduced spheroid size, disrupted integrity, and induced apoptosis, though lower doses were less effective than in 2D cultures, indicating higher drug tolerance in 3D conditions. Flow cytometry confirmed apoptosis as the primary mode of cell death, with over 90% apoptotic cells after 48 h at 100 μ M vincristine. In conclusion, vincristine exerts selective cytotoxicity against malignant CMT cells independent of receptor status, and 3D spheroids serve as a more stringent *in vitro* model to study drug efficacy, resistance, and therapeutic optimization.

Keywords: Vincristine, IC₅₀, 3D tumor spheroid, Hormone receptor status

Introduction

Mammary tumors are the most common neoplasms in female dogs, occurring predominantly in intact animals, and are associated with mortality rates at least three times higher than those reported in human breast cancer (Egenvall *et al.*, 2005) [5]. The global incidence of canine mammary tumors (CMTs) is approximately 200 cases per 100,000 dogs annually, though rates are markedly lower in regions with routine early spaying practices (Elis *et al.*, 2023) [6]. Histologically, CMTs display a wide morphological spectrum ranging from benign adenomas to highly malignant carcinomas and sarcomas, with mixed tumors containing both epithelial and mesenchymal components being especially frequent in dogs (Goldschmidt *et al.*, 2016) [10]. This biological diversity complicates treatment responses and mirrors, to some extent, the molecular heterogeneity observed in human breast cancer.

Among chemotherapeutic agents, vincristine, a vinca alkaloid derived from *Catharanthus roseus*, is widely used in veterinary oncology. Vincristine binds to β -tubulin at the interface between tubulin heterodimers, disrupting microtubule assembly and inducing mitotic arrest (Boussios *et al.*, 2012; Field *et al.*, 2014) [2, 7]. Despite its broad use, vincristine efficacy is often limited by tumor heterogeneity, drug resistance mechanisms, and microenvironmental influences. Notably, receptor expression profiles, including estrogen receptor (ER), progesterone receptor (PR), and HER2, are increasingly recognized as important

determinants of biological behavior and treatment outcome in both human and canine mammary cancers. Triple-negative tumors (ER-/PR-/HER2-) are particularly aggressive, with higher metastatic potential and poor therapeutic responsiveness compared with hormone receptor-positive or HER2-positive tumors. Conversely, triple-positive tumors may display enhanced chemosensitivity.

To study drug-tumor interactions, primary cultures derived from freshly excised canine mammary tumors provide an excellent platform, as they retain key molecular and morphological features of the original tumor. Conventional two-dimensional (2D) monolayers are widely used for cytotoxicity screening but fail to fully replicate *in vivo* tumor complexity. In contrast, three-dimensional (3D) spheroid models mimic native tumor microenvironments, incorporating gradients of oxygen and nutrients, extracellular matrix deposition, and barriers to drug penetration (Mitrakas *et al.*, 2023) [15]. These features make 3D cultures more predictive of *in vivo* drug responsiveness, particularly in evaluating chemoresistance.

The present study aimed to evaluate the cytotoxic activity of

vincristine in primary cultured canine mammary tumor cells classified as triple-positive or triple-negative. In addition, we compared vincristine responsiveness between 2D monolayer cultures and 3D spheroid models to assess the influence of tumor receptor status and microenvironmental context on drug sensitivity.

Materials and Methods

Collection of tissue samples

Tissue samples were collected from 5 clinical cases of canine mammary gland tumors (CMTs) presented for surgery at the Referral Veterinary Polyclinic, ICAR-IVRI-Izatnagar, Bareilly. The normal/control mammary gland tissue was obtained from a dog post-mortem case. The tumor tissues were confirmed by histopathological analysis. The control sample was also confirmed by histology as normal mammary gland tissue. Patient data (age, breed, weight, tumor size, recurrence, growth type, and clinical stage) were prospectively recorded with informed owner consent. Tumor dimensions were measured using Vernier calipers. The details of clinical cases from which tumor tissues were collected are presented in Table 1.

Table 1: Clinical characteristics of Canine Mammary Tumor (CMT) cases. Data includes breed, age, tumor size, duration, tumor location on the mammary gland, and estrogen receptor (ER), progesterone receptor (PgR), and HER2 status for each case.

CMT case no	Breed	Age (Years)	Tumor size (cm)	Duration of tumor	Location on mammary gland	ER, PgR and HER2 status
CMT1	Labrador	5	7.0 x 5.5	5 months	2nd	ER-/PR-/HER2-
CMT2	Local Breed	4	3.0 x 2.5	3 months	1st	ER-/PR-/HER2-
CMT3	Spitz	6	8.0 x 6.0	5 months	1st	ER+/PR+/HER2+
CMT4	Local Breed	9.5	5.0 x 7.0	1 yr	1 st and 2 nd	ER-/PR-/HER2-
CMT5	Labrador	8	5.0 x 7.2	10 months	1 st and 2 nd	ER+/PR+/HER2+
N1	GSD	6	Nil	Nil	Nil	Nil

Culture of CMT cells

Primary cultures were established from surgically excised tissues obtained from clinical cases of canine mammary tumour and normal mammary gland. Tumor tissues were aseptically collected following surgical resection from clinical cases presented to the Division of Surgery and Radiology, ICAR-IVRI, Izatnagar. Tissues were washed thoroughly in sterile phosphate-buffered saline (PBS) containing antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) to minimize microbial contamination. Tumor cells were isolated by enzymatic digestion and mechanical dissociation, followed by filtration to obtain a single-cell suspension. Cells were seeded and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and antibiotics. Sub-culturing was performed using standard trypsinization, and cells were incubated in a humidified atmosphere at 37 °C with 5% CO₂ until reaching confluence [7, 8]. Five primary canine mammary tumor (CMT) cell types, designated CMT1-CMT5, and cells from a normal mammary gland tissue (N1) were used in this study.

Histology

Fresh tumor tissues were fixed in 10% neutral buffered formalin (NBF) for 24 hr, processed, dehydrated, and embedded in paraffin. Sections (4 µm) were stained with Hematoxylin and Eosin (H&E) and prepared for histological analyses. H&E-stained slides were evaluated for malignant cancer characteristics and classified into different cancer types as per Goldschmidt *et al.* (2011) [9].

RT PCR for determination of ER, PR, and HER2 Status

Total RNA was isolated from CMT and normal tissues using Trizol method as described previously [10] and reverse-transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) as per manufacturer's instructions. Qualitative PCR was performed to amplify ER (ESR1), PR (PGR), and HER2 (ERBB2) genes, with GAPDH as the internal control. Reactions (20 µL) contained 2× PCR Master Mix, 0.5 µM of each primer, and 0.5 µL cDNA template. Cycling conditions were: 95 °C for 3 min; 35 cycles of 95 °C for 30 s, 60 °C for 30 s annealing, 72 °C for 45 s; and a final extension at 72 °C for 5 min. Products were resolved on 2% agarose gel stained with ethidium bromide and visualized under UV light.

Cytotoxicity assay

Five CMT cell types, along with normal canine mammary cells were cultured from surgically excised tissues, were used for cytotoxicity testing. Cells were seeded at a density of 5×10^3 cells/well in 96-well microtiter plates containing DMEM supplemented with 10% fetal bovine serum (FBS) and incubated overnight to allow cell attachment. Vincristine was added to the wells in serial three-fold dilutions, with each concentration tested in triplicate. Plates were incubated for 48 h at 37 °C in a humidified atmosphere containing 5% CO₂. Following treatment, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; 5 mg/mL; HiMedia Pvt. Ltd.) was added to each well and incubated for 4 h. Thereafter, the medium containing MTT was aspirated, and the insoluble formazan crystals were dissolved in DMSO. Absorbance was measured at 570

nm with a reference wavelength of 690 nm using a 96-well microplate reader.

IC₅₀ determination

The half-maximal inhibitory concentration (IC₅₀) was determined from the dose-response curve. Cell viability was measured across a range of drug concentrations in triplicate, and the IC₅₀ value was calculated by regression analysis using the PRISM software.

Development of a 3D tumor spheroid model

Primary cells were isolated by enzymatic dissociation using 0.25% trypsin-EDTA solution for 10-15 min at 37 °C, followed by neutralization with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The resulting cell suspension was filtered through a 70 µm cell strainer to remove debris and centrifuged at 1200 rpm for 5 min. Cell viability was assessed by the trypan blue exclusion method, and cells were seeded at a density of 1×10^4 cells/well to generate spheroids. Three-dimensional (3D) CMT spheroids were developed using the 1.5% agarose-coated plate method (Raval *et al.*, 2018) [18]. Briefly, 96-well flat-bottom microtiter plates were coated with sterile 1.5% agarose to prevent cell adhesion, and freshly isolated CMT cells were cultured in DMEM supplemented with 10% FBS and antibiotics. The plates were incubated at 37 °C in a humidified atmosphere containing 5% CO₂, and spheroid formation was monitored daily under an inverted phase-contrast microscope until compact and uniform 3D spheroids were formed.

Vincristine cytotoxicity evaluation in 3D tumor model

Five different 3D canine mammary tumor (CMT) spheroid types (CMT1-CMT5) and one (1) normal canine mammary spheroid control (N1) were used to evaluate the cytotoxic effect of vincristine. Spheroids (~400-500 µm in diameter) were selected after 5-7 days of culture and transferred to 96-well agarose-coated plates. Vincristine (Sigma-Aldrich, USA) was freshly prepared in sterile PBS and prepared 1mg/ml stock filtered with 0.2-micron syringe filter and divided into final concentrations of 0 µM, 10 µM, 50 µM, and 100 µM, with each concentration tested in triplicate. Spheroids were incubated for 48 h at 37 °C in a humidified atmosphere containing 5% CO₂. Following treatment, spheroid morphology and size were monitored using an inverted phase-contrast microscope, and serial images were captured at defined intervals (0 h, 24 h, 48 h, and 72h). The diameter and volume of spheroids were measured using ImageJ software (NIH, Bethesda, MD, USA), and percentage reduction in spheroid size was calculated relative to untreated controls (0 µM vincristine).

Flow Cytometric Analysis of Vincristine-Induced Apoptosis in 3D spheroids

To investigate the mechanisms of vincristine-induced cytotoxicity, tumor spheroids derived from two CMT cell lines were treated with vincristine at concentrations of 0, 50, and 100 µM for 48 hours. Following treatment, cells were harvested by trypsinization and subjected to apoptosis analysis using dual staining with Annexin V-FITC (excitation: 488 nm; emission: 530 nm) and propidium iodide (PI; excitation: 535 nm; emission: 617 nm). Flow cytometry was then used to distinguish between different

cell populations: viable cells (Annexin V⁻/PI⁻), early apoptotic cells (Annexin V⁺/PI⁻), late apoptotic cells (Annexin V⁺/PI⁺), and necrotic cells (Annexin V⁻/PI⁺).

Statistical Analysis: Quantitative data are expressed as mean ± standard deviation (SD). Statistical comparisons between two groups were performed using unpaired Student's t-test, whereas multi-group comparisons were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. When assumptions of normality or homogeneity of variance were violated, appropriate nonparametric or mixed-effects models were applied. Statistical significance was defined as $p < 0.05$. All analyses and graphical representations were conducted using GraphPad Prism 7.0 (GraphPad Software, CA, USA).

Results

Clinical Presentation and Tumor Characteristics

Tumor tissues were collected and cultured from a total of five clinical cases of canine mammary tumors (CMTs) and one control mammary gland tissue from dog post-mortem case. The affected dogs included two Labradors, two local breeds, and one Spitz, with ages ranging from 4 to 9.5 years (Mean ± SD: 6.5 years).

Tumors size ranged from 3.0 × 2.5 cm to 8.0 × 6.0 cm, with an average dimension of ± 5.6 × 5.64 cm. The duration of tumor development before presentation ranged from 3 months to 1 year. Tumors were predominantly located in the 1st and 2nd mammary glands, with three cases affecting both glands concurrently (CMT4 and CMT5) or individually (CMT1, CMT2, CMT3). Grossly, the tumors appeared nodular and firm, with some showing ulceration and hemorrhagic surfaces, as shown in Figure 1. Histopathological evaluation confirmed all tumor tissues as malignant subtypes. Detailed characteristics of the tumor tissues are provided in Figure 2.

Hormone Receptor Expression

RT-PCR analysis revealed that 2 out of 5 cases (40%) were positive for estrogen receptor (ER), progesterone receptor (PR), and HER2 (ER+/PR+/HER2+). Representative agarose gel electrophoresis images of RT-PCR amplicons are shown in Figure 3. The positive cases included CMT3 (Spitz, 6 years) and CMT5 (Labrador, 8 years). The remaining three tumors (CMT1, CMT2, and CMT4) were negative for all three markers (ER-/PR-/HER2-). The control sample (N1) exhibited neither pathological changes nor receptor.

CMT 2D cell and 3D spheroid culture characteristics

At the early stage of culture (Day 1-2; Figure 4A), a limited number of spindle-shaped and polygonal fibroblastic cells were observed migrating outward from the tumor explants and adhering to the culture substrate. By Day 3-4 (Figure 4B), a progressive outgrowth of fibroblast-cells was evident, with the formation of discrete cellular clusters surrounding the explants. Continued proliferation during Day 5-6 (Figure 4C) led to the development of a denser monolayer characterized by morphological heterogeneity. By Day 7-8 (Figure 4D), cultures demonstrated increased confluency, with fibroblast cells becoming predominant and polygonal fibroblastic cells distributed intermittently within the monolayer. At Day 9-10 (Figure 4E), nearly confluent cultures were achieved, indicating successful adaptation of

tumor-derived cells to *in vitro* conditions. In contrast, primary cultures established from normal mammary tissues (Figure 4F) exhibited more uniform fibroblast morphology and a comparatively slower proliferation rate relative to tumor derived cells. The mean time to reach 70-80% confluency was 2 days for CMT-derived cultures versus 5 days for normal mammary cells.

Cells within the spheroids displayed tight intercellular adhesion, with a proliferative outer layer and a progressively compact or quiescent core, closely resembling the *in vivo* tumor microenvironment. The sizes of the spheroids ranged from 400-500 μm , depending on the seeding density, and they maintained structural integrity throughout the culture period.

Vincristine Cytotoxicity in 2D Cultures

The cytotoxic effects of vincristine on primary canine mammary tumor (CMT1-CMT5) cells and normal mammary fibroblastic cells (N1) were evaluated over 48 h using both morphological assessment and quantitative viability analysis. At baseline (0 μM), all cells exhibited normal spindle-shaped or polygonal morphology with intact adherence and confluent monolayers (Figure 5A). Exposure to 10 μM vincristine induced early cytotoxic effects in tumor cells, including partial rounding, shrinkage, and reduced density (Figure 5B). Mortality varied across tumor lines, ranging from 6.50% in CMT5 to 52.57% in CMT2, whereas N1 cells remained largely unaffected, confirming tumor-specific cytotoxicity. At 50 μM , tumor cells showed extensive rounding, detachment, and fragmentation, with mortality ranging from 43.32% (CMT5) to 69.14% (CMT2). In contrast, N1 cells showed only 19.37% mortality, highlighting their relative resistance. At the highest concentration (100 μM), near-complete tumor cell death was observed, with CMT2 (92.00%) being most sensitive, followed by CMT3 (84.11%), CMT4 (78.22%), CMT1 (66.66%), and CMT5 (57.40%). Although significantly lower than tumor cells, normal N1 cells also showed significant mortality (40.31%) at this dose. Notably, CMT5 displayed mortality levels closer to N1 cells, indicating partial resistance. Morphological observations confirmed these quantitative findings, showing progressive cell rounding, detachment, and fragmentation corresponding with increasing vincristine concentration (Figure 5).

Vincristine responsiveness and receptor status

A key finding was that vincristine cytotoxicity did not correlate with hormone receptor expression. Both triple-positive ($\text{ER}^+/\text{PR}^+/\text{HER2}^+$) and triple-negative ($\text{ER}^-/\text{PR}^-/\text{HER2}^-$) tumors exhibited similar dose-response patterns. Interestingly, CMT5, a triple-positive tumor, showed the lowest sensitivity, while CMT3, also triple-positive, demonstrated substantial cytotoxicity. Likewise, the triple-negative tumors (CMT1, CMT2, and CMT4) displayed variable responsiveness, further underscoring that receptor status is not predictive of vincristine sensitivity. Additionally, heterogeneity in response among tumor cells, particularly the partial resistance observed in CMT5,

highlights intrinsic variability in drug susceptibility across individual tumors. Collectively, these results demonstrate that vincristine exerts selective, dose-dependent cytotoxicity in CMT cells, is independent of triple-positive or triple-negative receptor status, and exhibits variable efficacy among different tumor subtypes, reflecting inherent resistance in some populations.

Effect of vincristine on 3D spheroid growth of canine mammary tumor (CMT) cells

To assess vincristine cytotoxicity under 3D culture conditions, multicellular spheroids (400-500 μm) were generated and exposed to increasing concentrations of vincristine (0, 10, 50, and 100 μM). Untreated control spheroids maintained compact, well-organized structures, exhibiting a mean surface area of 4.715 mm^2 (Figure 7A). Treatment with 10 μM vincristine moderately reduced spheroid area to 4.148 mm^2 ($p < 0.05$), with peripheral cell loosening while the core remained intact (Figure 7B). Compared with 2D cultures, lower doses of vincristine showed less pronounced cytotoxicity in 3D spheroids, likely due to limited drug penetration and micro environmental protection. At 50 μM , spheroid area decreased further to 3.558 mm^2 ($p < 0.01$ vs. control; $p < 0.05$ vs. 10 μM), with central densification indicative of core cell death (Figure 7C). Exposure to 100 μM caused extensive fragmentation, loss of spherical integrity, and dispersion of cells into the surrounding matrix, with spheroid area collapsing to near-negligible levels ($p < 0.001$ vs. control and lower doses) (Figure 7D). Quantitative analysis confirmed a strong dose-dependent inhibition of 3D tumor growth (Figure 7E). These findings indicate that while vincristine effectively suppresses CMT spheroid growth at higher doses, lower concentrations are less effective in 3D models than in 2D monolayers, highlighting the influence of tumor microenvironment on drug sensitivity.

Vincristine-Induced Apoptosis in Canine Mammary Tumor Cells Assessed by Annexin V-FITC/PI Flow Cytometry

To investigate vincristine-induced apoptosis in canine mammary tumor (CMT) cells, Annexin V-FITC/PI dual staining was performed, and the populations of viable, early apoptotic, late apoptotic, and necrotic cells were quantified by flow cytometry. In the untreated control group (N1), the majority of cells (99.02%) were viable, with negligible proportions of early apoptotic (0.98%), late apoptotic (0.00%), or necrotic (0.00%) cells, confirming minimal spontaneous cell death under basal conditions (Fig. 8A). Vincristine treatment (representative data shown at 100 μM for 48 h) induced a marked apoptotic response in CMT cells after 48 hrs of treatment with more than 90% cells in either early or late apoptosis. The viable cell fraction was reduced to 3.50%, with a significant increase in early apoptotic cells (15.86%) and, more prominently, late apoptotic cells (79.12%). A minor proportion of necrotic cells (1.53%) was also detected (Fig. 8B).

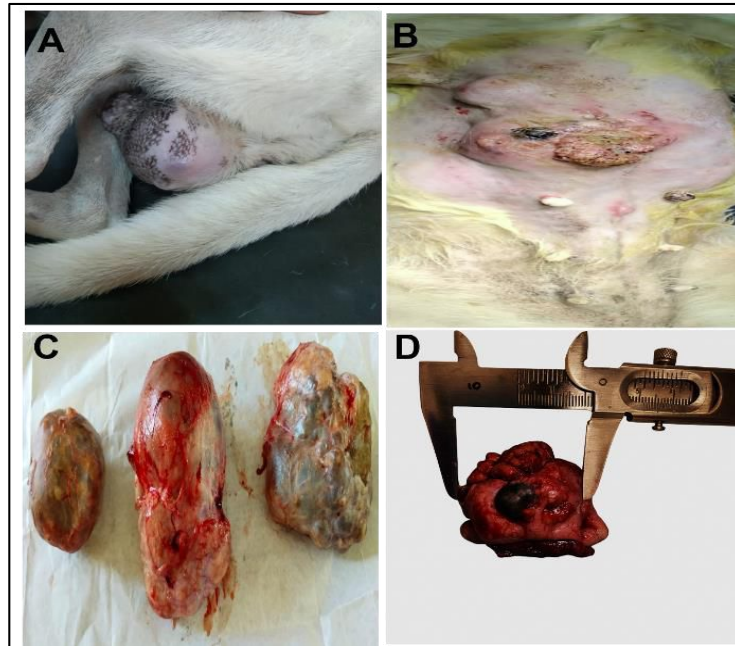


Fig 1: Gross morphology of canine mammary tumors (CMT). (A) Clinical presentation of a dog showing a firm, enlarged mammary mass. (B) Ventral view of the animal depicting multiple mammary tumors with ulceration and necrosis of overlying skin. (C) Excised mammary tumors of varying sizes and morphology following surgical resection. (D) Measurement of an excised tumor mass using a digital vernier calliper to determine tumor dimension.

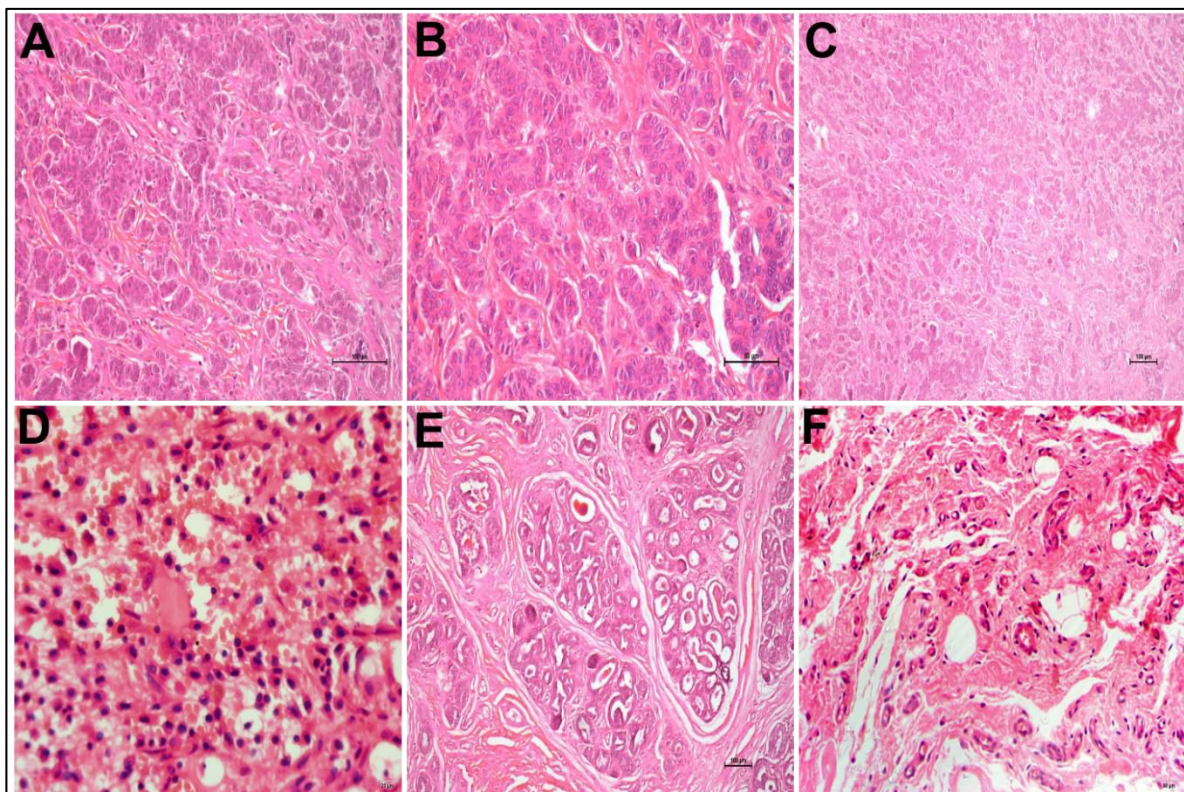


Fig 2: Histopathological classification of canine mammary tissues stained with hematoxylin and eosin (H&E). (A) Solid carcinoma showing densely packed sheets of epithelial tumor cells with minimal tubular differentiation, (B) Anaplastic carcinoma characterized by pleomorphic tumor cells with marked nuclear atypia and a high mitotic index, (C) Medullary carcinoma showing large, syncytial sheets of poorly differentiated epithelial cells with scant stromal support. (D) Simple tubular adenoma: Ducts and tubules are lined by single layer of cuboidal to columnar cells with moderate amount of eosinophilic cytoplasm. Tubules were supported by scant to moderate amount of fibrovascular stroma with or without amorphous amphophilic secretion. Minimal anisokaryosis and anisocytosis. Mitotic figures were absent. (E) Malignant mixed tumor showing comprising pleomorphic epithelial cells, spindle-shaped mesenchymal cells, and foci of cartilaginous differentiation. (F) Normal mammary gland tissues displaying well-organized glandular and ductal structures with intact epithelial and myoepithelial layers. (Scale bar = 50 µm).

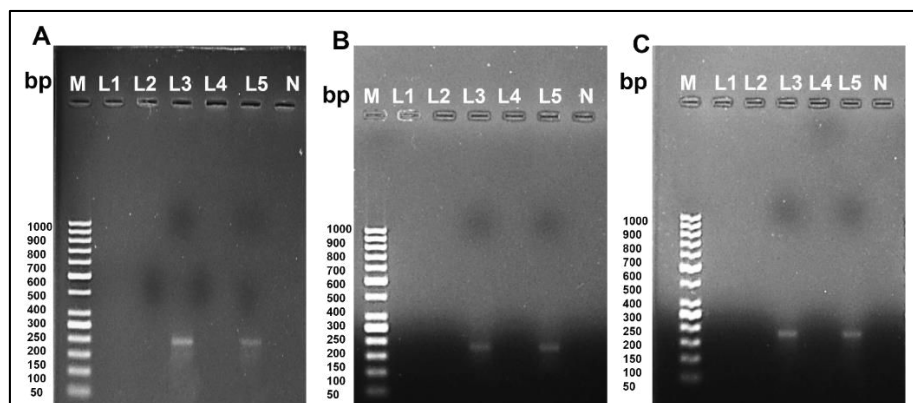


Fig 3: RT PCR representing amplification of ER, PR, and HER gene expression in 2 canine mammary tumor samples. Agarose gel electrophoresis showing amplicons for (A) estrogen receptor (ER), (B) progesterone receptor (PR), and (C) human epidermal growth factor receptor (HER). Lane M: 50 bp Puregene DNA ladder; L1-L5: representative tumor samples; N: negative control. Specific amplification bands corresponding to the expected product sizes were detected in positive samples, while no amplification was observed in negative controls.

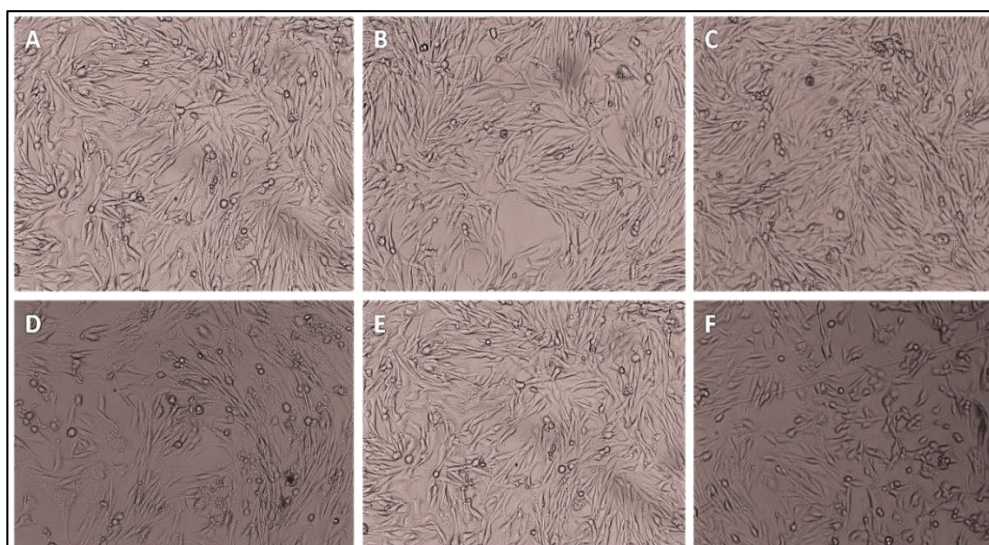


Fig 4: Primary culture of canine mammary tumor (CMT) cells (A-E) and normal mammary cells (F) established using the tissue explant method. (A-E) Phase-contrast micrographs showing adherent spindle-shaped fibroblast-like and fibroblastic tumor cells migrating out from tissue explants and proliferating to form monolayers. Cells were cultured in DMEM supplemented with 10% FBS and maintained at 37 °C in a humidified atmosphere with 5% CO₂.

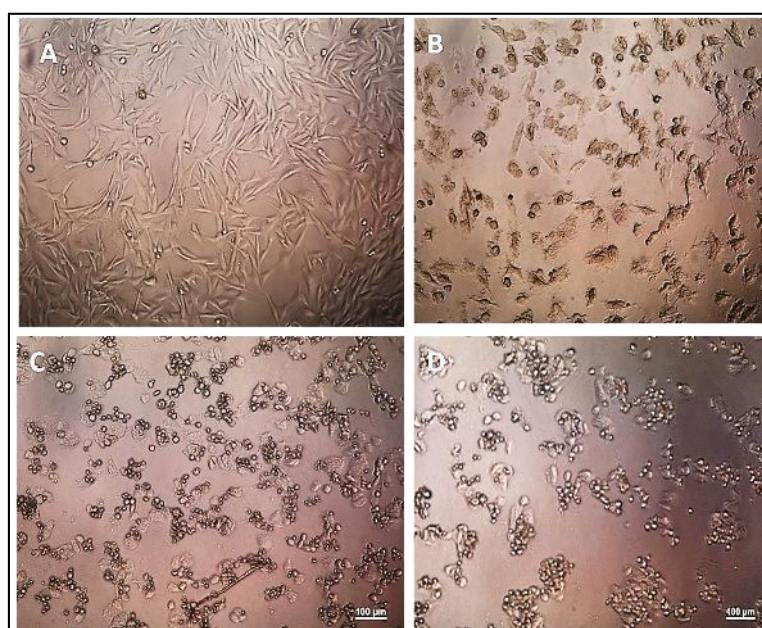


Fig 5: Dose-dependent cytotoxic effect of vincristine in MTT assay. Cells treated with (A) 0 μM, (B) 10 μM, (C) 50 μM, and (D) 100 μM vincristine show progressive loss of viability, morphological changes, and cell detachment.

Table 2: Cytotoxic effect of vincristine on canine mammary tumor (CMT) cells and calculation of median effective dose (IC₅₀) from dose-response curves, indicating variable sensitivity among tumor cell types, whereas the normal cell line showed no measurable IC₅₀ within the tested concentration range.

CMT cell	% mortality (Average)		
	Vincristine Conc. (μM)		
	10.0 μM	50.0 μM	100.0 μM
CMT1	31.92	50.35	66.66
CMT2	52.57	69.14	92.00
CMT3	38.75	56.59	84.11
CMT4	27.06	49.83	78.22
CMT5	6.50	43.32	57.40
N1	0	19.37	40.31

IC ₅₀ Calculations	
CMT1	43.66
CMT2	9.96
CMT3	26.33
CMT4	42.79
CMT5	65.34
N1	--

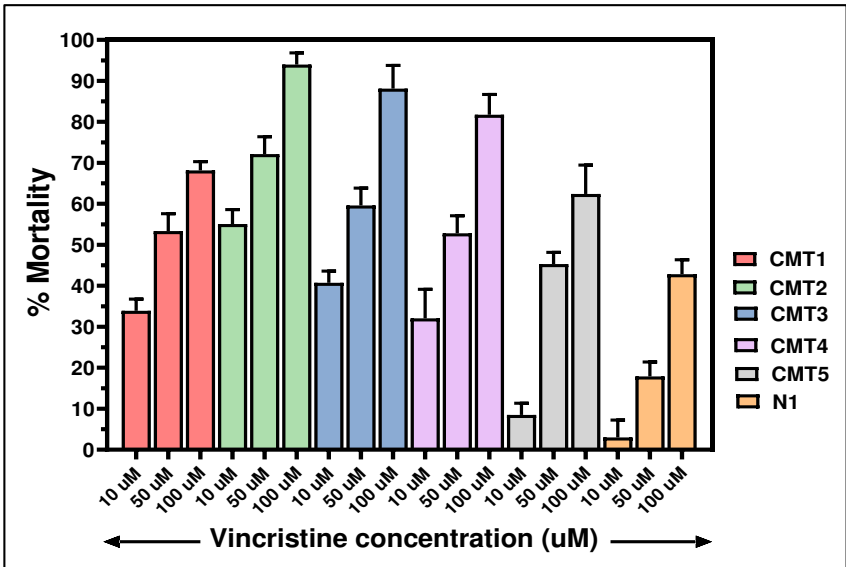


Fig 6: Percentage mortality of canine mammary tumor (CMT) cell lines treated with vincristine at different concentrations (0, 10, 50, and 100μM). Dose-dependent cytotoxicity was observed in CMT1-CMT5 cell types, with the highest mortality recorded at 50 μM. The normal canine cell line (N16) exhibited comparatively lower mortality across all concentrations, indicating selective cytotoxicity of vincristine towards tumor cells.

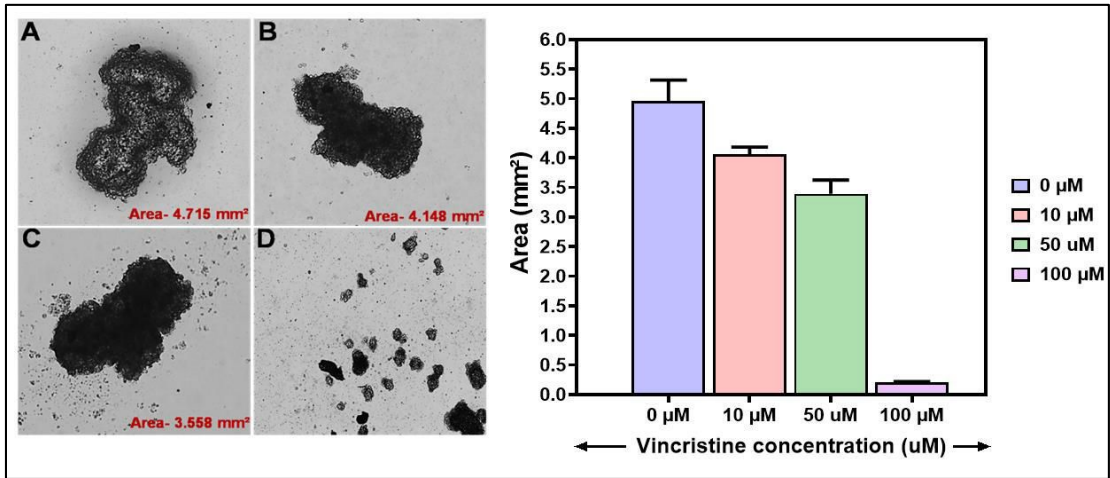


Fig 7: Effect of vincristine on 3D spheroid growth of canine mammary tumor (CMT) cells. Representative images of CMT 3D tumor spheroids treated with increasing concentrations of vincristine. (A) Untreated control spheroid (0 μM) showing compact morphology with an area of 4.715 mm². (B) 10 μM vincristine treatment reduced spheroid size to 4.148 mm²(C) 50 μM vincristine further decreased spheroid area to 3.558 mm², indicating growth inhibition. (D) 100 μM vincristine caused extensive disintegration of the spheroid structure, with marked fragmentation and loss of compactness.

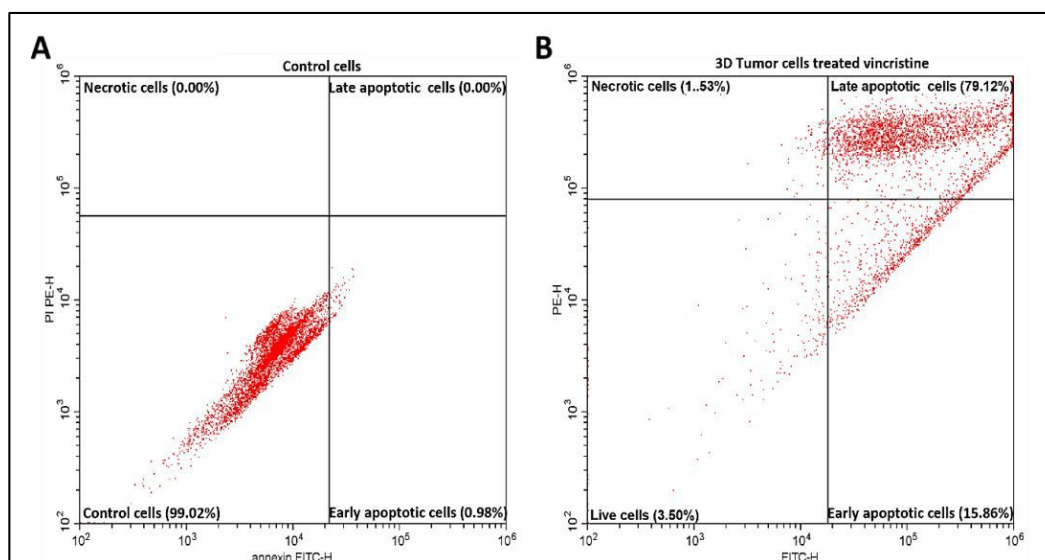


Fig 8: Flow cytometric analysis of Annexin V-FITC/PI-stained CMT cells. Control cells were mostly viable (99.02%) with minimal early apoptosis (0.98%) and no late apoptosis/necrosis. Vincristine (100 μ M, 48 h) reduced viability (3.50%) and induced apoptosis (early: 15.86%; late: 79.12%), with limited necrosis (1.53%)

Discussion

Canine mammary tumors (CMTs) represent a biologically diverse group of neoplasms, exhibiting variable morphology, receptor expression, and chemotherapeutic responsiveness (Misdorp *et al.*, 2002; Goldschmidt *et al.*, 2011) [14, 9]. In this study, we established primary cultures from five clinical CMT cases and a normal mammary fibroblastic gland tissue to evaluate vincristine cytotoxicity in both 2D monolayers and 3D spheroid models.

Our results demonstrate that vincristine induces a clear dose-dependent cytotoxic effect in all CMT cell types, with higher concentrations causing significant cell rounding, detachment, and death. Importantly, vincristine sensitivity did not correlate with hormone receptor status, as both triple-positive and triple-negative tumors showed comparable responses. This indicates that vincristine cytotoxicity in canine mammary tumors is receptor-independent, consistent with its mechanism of action as a tubulin-binding agent that disrupts microtubule dynamics rather than targeting hormone signalling pathways (Jordan *et al.*, 2004; Monks *et al.*, 2009) [12, 16].

Despite the overall cytotoxic trend, heterogeneity in drug response was evident. Certain tumor-derived cells, particularly CMT5, exhibited relative resistance even at higher vincristine concentrations, whereas others, such as CMT2, were highly sensitive. This variability highlights the intrinsic differences in chemoresistance among individual tumors, which may be influenced by factors such as genetic background, microtubule dynamics, and tumor-specific drug efflux mechanisms (Robey *et al.*, 2018) [19].

The 3D spheroid model further reinforced these observations. Compared with 2D monolayers, spheroids were generally more resistant to vincristine, showing slower shrinkage and delayed structural disruption at comparable doses. This is likely due to microenvironmental factors, including cell-cell and cell-matrix interactions, limited drug penetration, and oxygen/nutrient gradients, which are better recapitulated in 3D cultures (Friedrich *et al.*, 2009; Edmondson *et al.*, 2014; Thoma *et al.*, 2014) [8, 4, 20]. These findings emphasize the importance of using 3D models for preclinical evaluation of chemotherapeutic agents, as they

more accurately mimic *in vivo* tumor architecture and drug response.

Collectively, our study establishes that vincristine is effective against primary CMT cells *in vitro*, but responses vary across tumor types and culture systems. The lack of correlation with receptor status suggests that vincristine may be broadly applicable across CMT subtypes, while the observed heterogeneity underlines the need for individualized therapeutic strategies. Future studies integrating molecular profiling, drug resistance mechanisms, and combination therapy approaches could further improve the predictive value of *in vitro* models for clinical outcomes in canine mammary cancer (Uva *et al.*, 2009; Queiroga *et al.*, 2011) [21, 17].

Conclusion

Vincristine demonstrates potent, dose-dependent cytotoxic effects in primary canine mammary tumor cells while sparing normal mammary cells, highlighting its tumor-selective activity. The use of primary 2D cultures alongside 3D spheroid models, combined with histopathology and receptor profiling, provides a reliable framework to evaluate chemotherapeutic efficacy *in vitro*. Notably, vincristine sensitivity did not correlate with triple-positive or triple-negative receptor status, though variability among tumors revealed inherent differences in resistance. These findings emphasize the need for tailored therapeutic strategies and support further exploration of vincristine, including combination approaches, to address refractory or resistant tumor types.

Conflict of Interest

Authors declare no competing of interests.

Data Availability

Upon reasonable request, the datasets of this study can be available from the corresponding author

Ethics Approval

In the present experiment, no laboratory animals were sacrificed.

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