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PEG-liposomal doxorubicin as a potential agent for canine metastatic osteosarcoma – *in vitro* and *ex ovo* studies

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Abstract

Introduction: Appendicular osteosarcoma (OSA) is a highly aggressive and metastatic primary bone tumour in dogs. Standard therapy is amputation and adjuvant chemotherapy (*e.g.* with doxorubicin). Liposomal drug delivery may augment therapeutic efficacy and reduce negative side effects. Polyethylene glycol (PEG)-liposomal doxorubicin treats human metastatic cancers effectively. The study aimed was to evaluate PEG-liposomal doxorubicin's inhibitory effect on canine metastatic proliferation and migration *in vitro*. It also aimed to appraise the drug's extravasation inhibition *in vivo* using the human medicine–proven chick embryo chorioallantoic membrane *ex ovo* model. **Material and Methods:** The canine D-17 OSA cell line was cultured and inoculated with decreasing concentrations of PEG-liposomal doxorubicin and conventional doxorubicin in a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) test of cell viability, proliferation and cytotoxicity. Flow cytometry with Annexin V and Draq 7 staining confirmed the MTT test results, indicating dead, early and late apoptotic, and live cells. The inhibitory effect of the two preparations on cancer cell migration was investigated with a wound-healing assay. Culture plates seeded with cells were prepared. The cell monolayer was scratched and images of cells migrating to the scratch were captured at 0 h, 12 h and 24 h. Also, embryos were removed from three-day-incubated fertilised chicken eggs. On the 12th day, labelled D-17 cells were injected into each embryo. Embryos in one group received 100 µL of phosphate-buffered saline as controls, those in another group 30 µg/mL of PEG-liposomal doxorubicin, and those in the last group 6 µg/mL of conventional doxorubicin. The effectiveness of the intravascular administration of the D-17 cells was confirmed under a microscope. **Results:** PEG-liposomal doxorubicin inhibited the migration of canine OSA cells more effectively than conventional doxorubicin ($P \leq 0.05$). The *ex ovo* model showed that both drugs had similar impacts on canine metastatic OSA. **Conclusion:** The liposomal form of the drug may be considered a potentially effective compound in canine metastatic OSA; nevertheless, further *in vivo* studies are essential to confirm this hypothesis.

Keywords: caelyx, chick chorioallantoic membrane, cancer, extravasation, migration.

Introduction

Appendicular osteosarcoma (OSA) is a highly malignant primary bone cancer and is both locally aggressive and highly metastatic (22). The treatment of choice, which consists of an amputation followed by adjuvant chemotherapy (carboplatin, cisplatin and doxorubicin), results in a median survival time range of almost 300 to almost 500 days (21). Unfortunately around 90% of patients will ultimately succumb to metastatic disease. Metastases disseminate to regional lymph nodes, the lungs or parenchymal organs such as the liver or spleen (20). Doxorubicin is one of the drugs in the anti-cancer protocol for canine OSA; however, its use is limited by life-threatening side effects such as cardiotoxicity (heart failure or dysrhythmia) (23).

In human medicine, nanoparticles and liposomes have been extensively investigated as effective drug delivery systems for anticancer compounds. Liposomal structures are characterised by their high ability to

target neoplastic cells either actively or passively; also the side effects specific to free cytostatic drugs are milder in therapy regimes with this delivery system (32). Liposomes are designed to be multifunctional, with different components providing control over such properties as biodistribution, targeting specificity, permeability and circulation half-lives (2). Doxil (polyethylene glycol (PEG)) was the first nanoparticle anticancer drug that received marketing approval, becoming available in 1995 (1). Currently, 16 clinically approved liposomal drugs are in use, examples of which are DepoCyt (cytarabine), Doxil/Caelyx/Myocet (doxorubicin), DaunoXome (daunorubicin), DepoDur (morphine) and Visudyne (verteporfin) (17). Doxil or Caelyx is a polyethylene glycol–coated (PEGylated) liposomal doxorubicin approved in the USA and Europe, while Myocet, which is a non-PEGylated liposomal doxorubicin, is approved only in Europe. Different loading methods, lipid compositions or sizes of Doxil/Caelyx and Myocet result in different circulation times and toxicities (32). PEG-liposomal doxorubicin is used as a treatment for ovarian cancer in women for whom the first choice treatment has failed and as a treatment for AIDS-related Kaposi's sarcoma (8). Furthermore, the first-phase clinical trial of PEG-liposomal doxorubicin in people with metastatic OSA focusing mainly on maximal tolerated dose assessment has yielded promising initial results for efficacy against metastatic disease (33). Nevertheless, there is still a lack of studies on the efficacy and safety of PEG-liposomal doxorubicin in OSA (33).

There are few clinical and preclinical studies investigating nanoparticles in veterinary medicine (32). PEG-liposomal doxorubicin (Doxil/Caelyx) has been tested in a canine model to evaluate its bio distribution, pharmacokinetics and safety (5). Vail *et al.* (27) demonstrated a 25.5% complete response to PEG-liposomal doxorubicin treatment in dogs with tumours of various histologic sites and types. Using the preclinical mouse model, Lou *et al.* (14) concluded that PEG-liposomal doxorubicin successfully inhibited primary pulmonary tumour growth and arrested metastatic cells in the mouse peritoneal cavity, while conventional doxorubicin was only able to reduce the growth of the primary tumour without preventing metastasis.

Metastasis appears when cancer cells spread from the primary tumour to surrounding tissues and to distant organs. It is the main cause of mortality in cancer patients. Most cancer cells are recognised as foreign and rapidly destroyed in circulation by the host immune system; however, some of them form metastatic foci (10). The ability of cells to extravasate into the surrounding tissue has long been considered a major rate-limiting step in the metastatic cascade (15). Preventing metastatic extravasation seems an appropriate way to reduce metastatic dissemination and potentially may prolong a patient's life. Although metastatic disease kills almost 90% of cancer sufferers whose cause of death is cancer, most oncological research does not concern *in vivo* metastatic studies (3).

Chick embryo chorioallantoic membrane (CAM), which is highly vascularised, offers a simple, cheap and highly accessible preclinical oncological model and in these aspects compares favourably to other animal models (12). The CAM *ex ovo* model has been successfully used in human medicine to analyse the anti-extravasation efficacy of anticancer compounds *in vivo* (12), and this validated the model's selection for the present experiment. The research aim was to analyse the inhibitory effect of PEG-liposomal doxorubicin on the canine D-17 metastatic OSA cell line's proliferation, migration and extravasation cascade stages, both *in vitro* and *in vivo*.

Material and Methods

The canine OSA cell line D-17 from American Type Culture Collection (ATCC, Manassas, VA, USA) was cultivated under standard conditions (5% CO_2, 95% humidity, and 37°C) in Eagle's minimum essential medium (ATCC) with additions of heat-inactivated foetal bovine serum, penicillin–streptomycin (50 IU/mL), and amphotericin B (2.5 mg/mL). Cells with 90% confluence in a logarithmic growth phase were harvested with trypsin (0.025%) (Sigma Aldrich, St. Louis, MO, USA). The numbers of living and dead cells were counted with a Countess II automatic cell counter (Thermo Fisher, Waltham, MA, USA).

Two drugs were used for the study: PEG-liposomal doxorubicin as the commercial Caelyx PEG-liposomal product containing 2 mg hydrochloride doxorubicin (Janssen-Cilag International, Beerse, Belgium), and conventional doxorubicin as Adriblastina PFS 2 mg/mL, with its active substance doxorubicin hydrochloride (Pfizer, New York, NY, USA).

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) test was used to measure cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity. The test also was used to establish the half maximal inhibitory concentration (IC_{50}) and the 20% cell death inhibitory concentration (IC_{20}) (24). Cells of the D-17 line were reseeded into 96-well cell culture plates (Sigma Aldrich) and incubated for 24 h. When the confluency was approximately 90%, the PEG-liposomal doxorubicin and conventional doxorubicin were added in decreasing concentrations of 250, 100, 50, 25, 10, 5, 2.5, 1 and 0.5 µg/mL. After 24 h, 10 µL of the MTT labelling reagent was added to each well. Four hours later, each well was filled with 100 µL dimethyl sulfoxide (Sigma Aldrich) prior to recording absorbance readings. Supernatant optical densities were read at 555 nm using an Infinite M 1000 spectrophotometer (Tecan, Männedorf, Switzerland). Untreated control cells were used to normalise absorbance. The test was performed three times for result reliability.

Flow cytometry with Annexin V and Draq 7 staining was performed to confirm the results obtained from the MTT test. The Annexin V and Draq 7 test allows the quantitative analysis of dead, early and late apoptotic, and live cells. In the study Draq 7 (BioLegend, San Diego, CA, USA) replaced propidium iodide (PI) or 7-AAD, as Draq 7 has no emission overlap with doxorubicin (31). Cells were cultivated in serum-free medium (Sigma Aldrich). After 24 h, the cells were treated with IC_{50} doses of PEG-liposomal and conventional doxorubicin. On the next day, media with the studied drugs were transferred to cytometry probes (Abcam, Cambridge, UK) with binding buffer. Study samples were divided into four groups: unstained, with addition of Annexin V 450 (Abcam), with addition of Draq 7 (Abcam), and with addition of Annexin V and Draq 7. The percentages of apoptotic, necrotic and viable cells were analysed with an emission wavelength in the 450–750 nm range with a FACS Aria II flow cytometer (BD Biosciences, San Jose, CA, USA). The FACS Diva software application version 6.1.3 (BD Biosciences) was used to analyse the results. The experiment was repeated three times.

The wound-healing assay as an *in vitro* test was carried out to assess the inhibitory effect of conventional and PEG-liposomal doxorubicin on cancer cell migration. Jonkman *et al.* (11) showed that it is an effective tool to define the impact of particular substances on cell migration. In the experiment, cells were seeded on six-well cell culture plates (Sigma Aldrich), after 24 h the medium was replaced by Mitomycin C (Abcam) and the plates were left for 3 h. Mitomycin C is a proliferation inhibitor applied experimentally to eliminate the contribution of cell division to wound closure (28). In the next step, serum-free medium containing an IC_{20} dose of liposomal doxorubicin, serum-free medium containing an IC_{20} dose of conventional doxorubicin, and serum-free medium alone as a control were added. The cell monolayer was wounded with a sterile, plastic 100 µL pipette tip (Eppendorf, Hamburg, Germany). Images of cells migrating to the scratch were captured at three time points (t1 = 0 h, t2 = 12 h and t3 = 24 h) with a Primovert inverted microscope (Zeiss, Jena, Germany). Pictures were analysed with the Zen Pro 2012 programme (Zeiss). The rate of cell migration was analysed by calculating the distance between the edges of the wound. One hundred measurements of the wound were performed at each time point for each sample.

The *ex ovo* model is an effective preclinical model which allows the extravasation of cancer cells to be determined (13). Fertilised Ross 308 eggs (Koko-eko hatchery, Marylka, Poland) were incubated in standard conditions with temperature in the 36–39°C range and high 65–70% humidity. On the third day of embryo development, eggshells were opened with a drill and chick embryos were transferred into black polystyrene weighing boats of 100 mL capacity sized 85 × 85 mm (Global Scientific, Northallerton, UK) (Fig. 1B). The embryos were transported in batches using plastic boxes with water on the bottom to provide proper humidity (Fig. 1B).

On the 12th day of the experiment, D-17 cells in a concentration of 1.0×10^5/mL were labelled with green 5-chloromethylfluorescein diacetate cell tracking dye (Life Technologies, Paisley, UK). On the same day, the labelled cancer cells were injected into each embryo. Intravenous injections were performed under a microscope (Keyence, Neu-Isenburg, Germany) with 50-µm-diameter glass microneedles. Chick embryos were divided into three groups of 7: a PEG-liposomal doxorubicin group (PEG-liposomal doxorubicin at IC_{50}), a non-liposomal conventional doxorubicin group (doxorubicin at IC_{50}) and a control group. Embryos were injected with 100 µL of phosphate-buffered saline, 30 µg/mL of PEG-liposomal doxorubicin or 6 µg/mL of conventional doxorubicin, according to their group. After injection, labelled D-17 cells were evaluated under a Zeiss Axio Examiner Z1 microscope (Zeiss) to confirm that they were located within the CAM blood vessels, which were labelled with tomato lectin DyLight 649 (Thermo Fisher). The effectiveness of the intravascular administration of the D-17 cells was confirmed under the microscope with a 40× objective (Fig. 2).

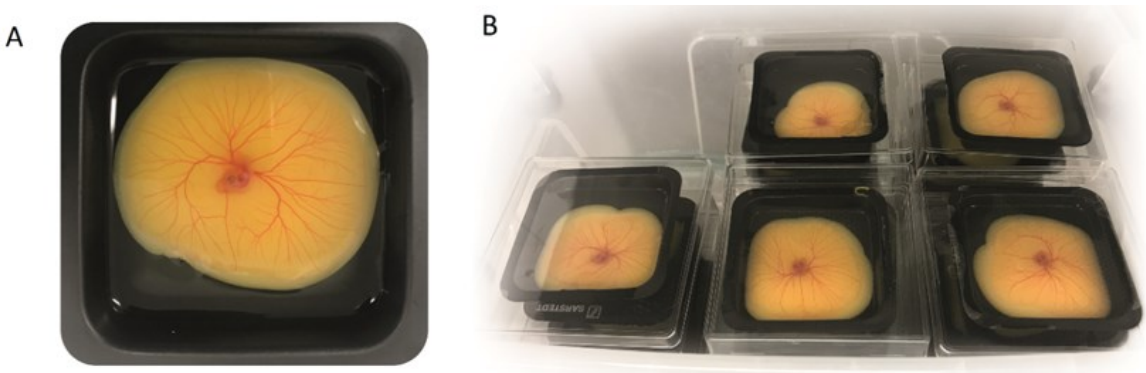

Fig. 1. Embryos prepared for the *ex ovo* experiment: A – the chorioallantoic membrane and cancer cell injection site; B – embryos in weighing boats covered with plastic Petri dishes

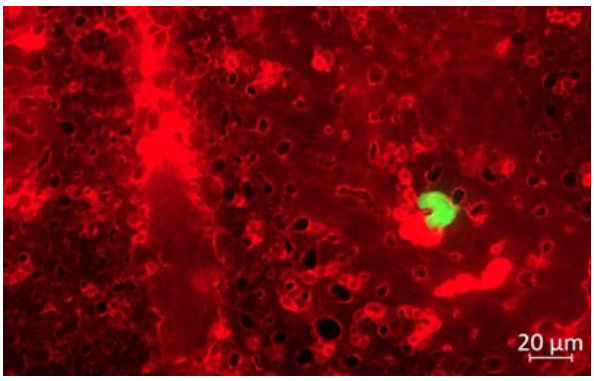

Fig. 2. Stained D-17 cell located intravascularly in the lumen of the CAM blood vessel. Scale bar 20 µm

Sterile silicon rings (Zegir, Warsaw, Poland) were used to define the evaluation area. The proportion of cancer cells that were initially arrested in the CAM vessels and the proportion of cancer cells that crossed the vessel lumen were determined. To analyse the efficacy of extravasation, all cells within each silicon ring were counted at t1 and t2 using the Zeiss Axio Examiner Z1 with a 4× objective. Extravasation efficacy in each group was calculated using the formula

$$\text{Extravasation rate} = \frac{\text{number of extravasated cells (t2)}}{\text{number of intravascular cells (t1)}} \times 100\%$$

After the experiment all embryos were euthanised. The experiment was repeated in three biological replicates.

Statistical analysis. One-way analysis of variance and *post-hoc* Tukey tests (Prism 5.0; GraphPad, San Diego, CA, USA) were used for statistical analysis of the wound-healing assay and *ex ovo* experiment. Each data point was represented as mean ± standard error of the mean (for the scratch assay and *ex ovo* assay) or mean ± standard deviation (for the Annexin V and Draq 7 tests). The value of P of ≤ 0.05 was established as significant, while P ≤ 0.01and P ≤ 0.001 were established as highly significant.

Results

Potent cytotoxic effects of both assessed drugs were observed on the D-17 OSA cell line after 24 h of incubation (Fig. 3); however, the IC_{50} dose of conventional doxorubicin was more than fourfold lower than that of PEG-liposomal doxorubicin (Table 1).

Analysis of the cytometry test confirmed the results of the colorimetric test (MTT assay). The D-17 cells were incubated for 24 h with the studied drugs at IC_{50} doses. Both drugs exerted comparable apoptotic and necrotic effects (Fig. 4). It was indicated that doxorubicin acted mainly through cell apoptosis on the D-17 OSA cell line, as the number of apoptotic cells was notably higher (P ≤ 0.05) than that of necrotic cells (Fig. 5).

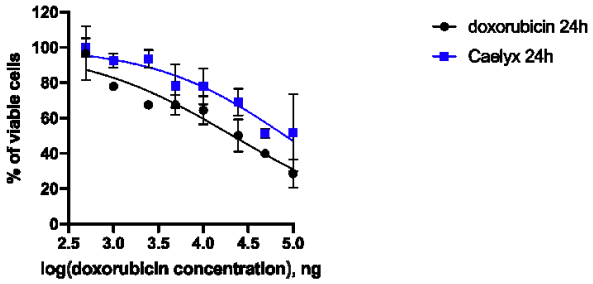

Fig. 3. Viability of canine D-17 cells (osteosarcoma cell line) after exposure to 100 µL of polyethylene glycol–liposomal doxorubicin (Caelyx) or conventional doxorubicin for 24 h. Curved lines represent the log (inhibitor) *vs* normalised response curve. Standard deviation is represented by the error bars

Table 1. Half maximal inhibitory (IC_{50}) doses of polyethylene glycol–liposomal and conventional doxorubicin after 24 h incubation for the canine D-17 osteosarcoma cell line

	IC_{50} after 24 h (µg/mL)
PEG-liposomal doxorubicin	28.862
Conventional doxorubicin	6.090

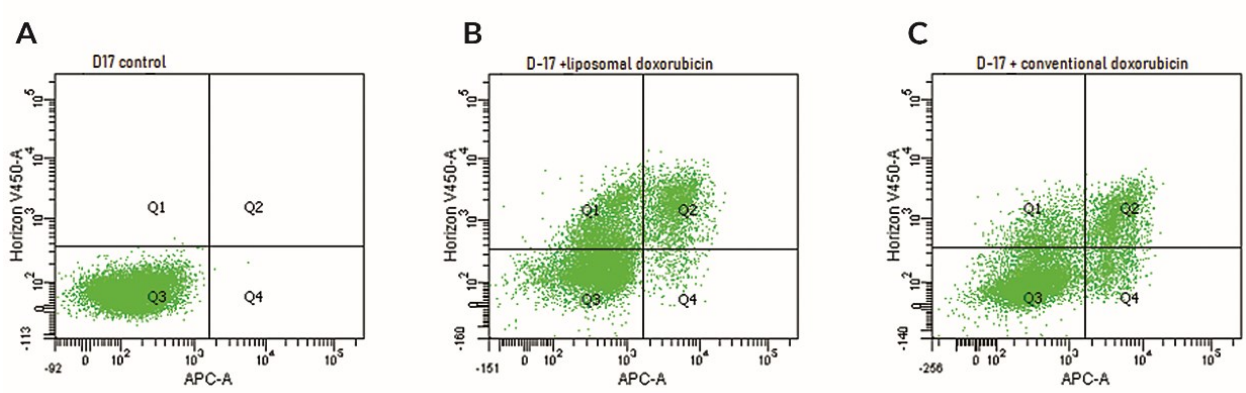

Fig. 4. Scatterplots showing D-17 subpopulations distinguishable as necrotic (Q1), late apoptotic (Q2), live (Q3) and early apoptotic (Q4) cells after incubation for 24 h with the tested drugs at IC_{50} doses. Scatterplots are for: A – control group cells (no treatment); B – polyethylene glycol–liposomal doxorubicin–treated cells; C – conventional doxorubicin–treated cells. Horizon V450-A refers to Annexin and APC-A to Draq 7 detection filters

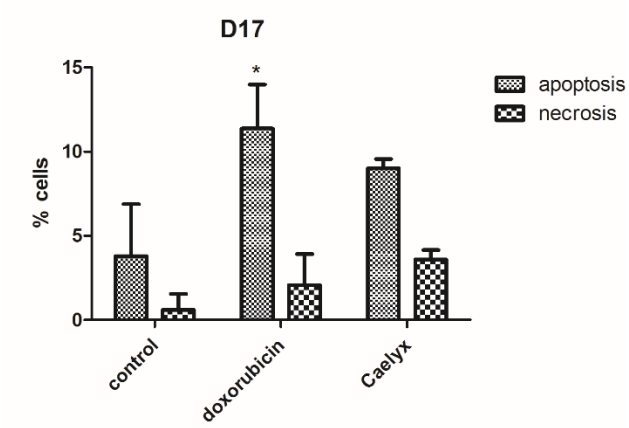

Fig. 5. The effects of polyethylene glycol–liposomal and conventional doxorubicin on apoptosis and necrosis of canine D-17 osteosarcoma cells. From left to right, the bars represent the percentage of apoptotic and necrotic untreated control cells, cells treated with conventional doxorubicin, and cells treated with PEG-liposomal doxorubicin (Caelyx) at IC_{50} doses after 24 h incubation. Error bars represent standard deviation. P-value ≤ 0.05 (*) was assigned significance

An *in vitro* wound-healing assay was used to analyse the ability of conventional and PEG-liposomal doxorubicin to inhibit D-17 cell migration. Both drugs significantly inhibited cancer cell migration ($P \leq 0.001$), with a statistically significantly ($P \leq 0.01$ after 12 h and $P \leq 0.05$ after 24 h) greater inhibition of cells treated with nanoparticle PEG-liposomal doxorubicin in comparison to those treated with conventional doxorubicin (Figs 6 and 7).

Directly after intravenous injection, cancer cells in each study group were spread evenly in the CAM vasculature (Fig. 8 A, C and E). After 24 h there was a notably high percentage of extravasated cancer cells in the control group and a substantially lower amount of extravasated cancer cells in embryos treated with the studied drugs ($P < 0.0001$). No statistical difference in the percentages of cells with extravasation between those treated with conventional doxorubicin and those treated with liposomal doxorubicin was observed (Figs 8 and 9). The rate of extravasation of D-17 cancer cells in the control group after 24 h was 48%.

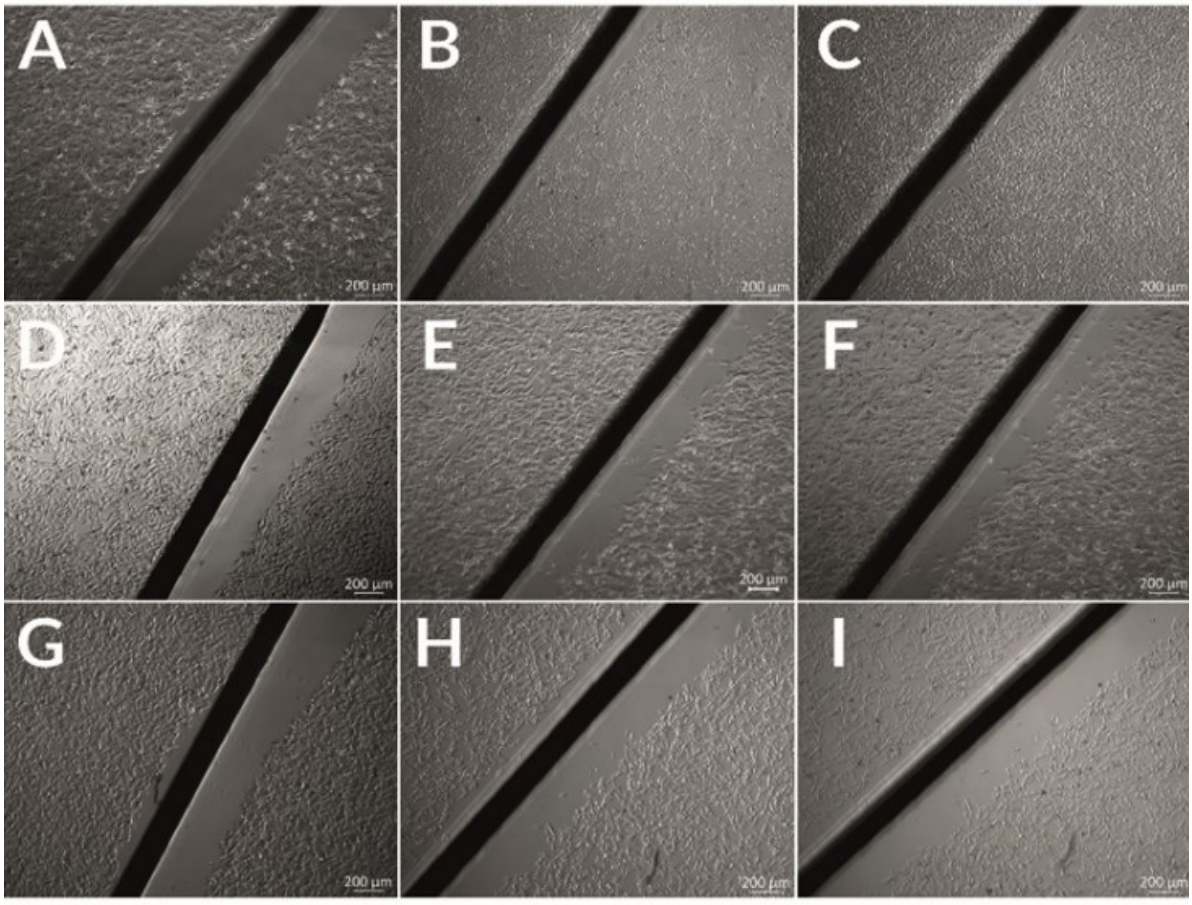

Fig. 6. Wound-healing assay microscopic images showing inhibition of canine D-17 osteosarcoma cell migration. A – untreated control cells at t1 (0 h); B – these cells at t2 (12 h); C – these cells at t3 (24 h); D – cells incubated with conventional doxorubicin at t1; E – these cells at t2; F – these cells at t3; G – cells incubated with polyethylene glycol–liposomal doxorubicin at t1; H – these cells at t2; I – these cells at t3

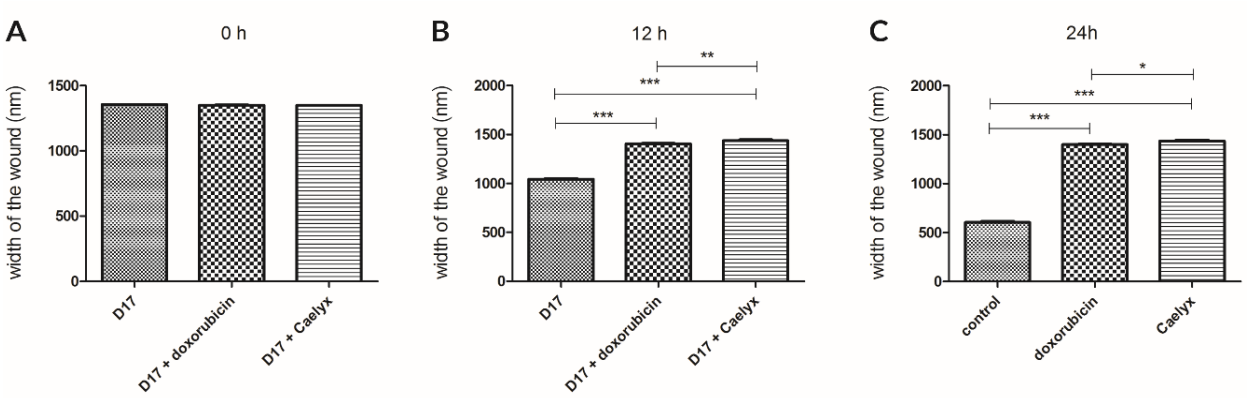

Fig. 7. Bar graphs showing the effect of the tested drugs inhibiting the migration of canine D-17 osteosarcoma cells at: A – t1 (0 h); B – t2 (12 h); and C – t3 (24 h). From left to right, the bars represent untreated and incubated control group cells, conventional doxorubicin–treated and incubated cells, and polyethylene glycol–liposomal doxorubicin–treated and incubated cells at each time point
*** – very high statistical significance at $P \leq 0.001$; ** – high statistical significance at $P \leq 0.01$; * – statistical significance at $P \leq 0.05$

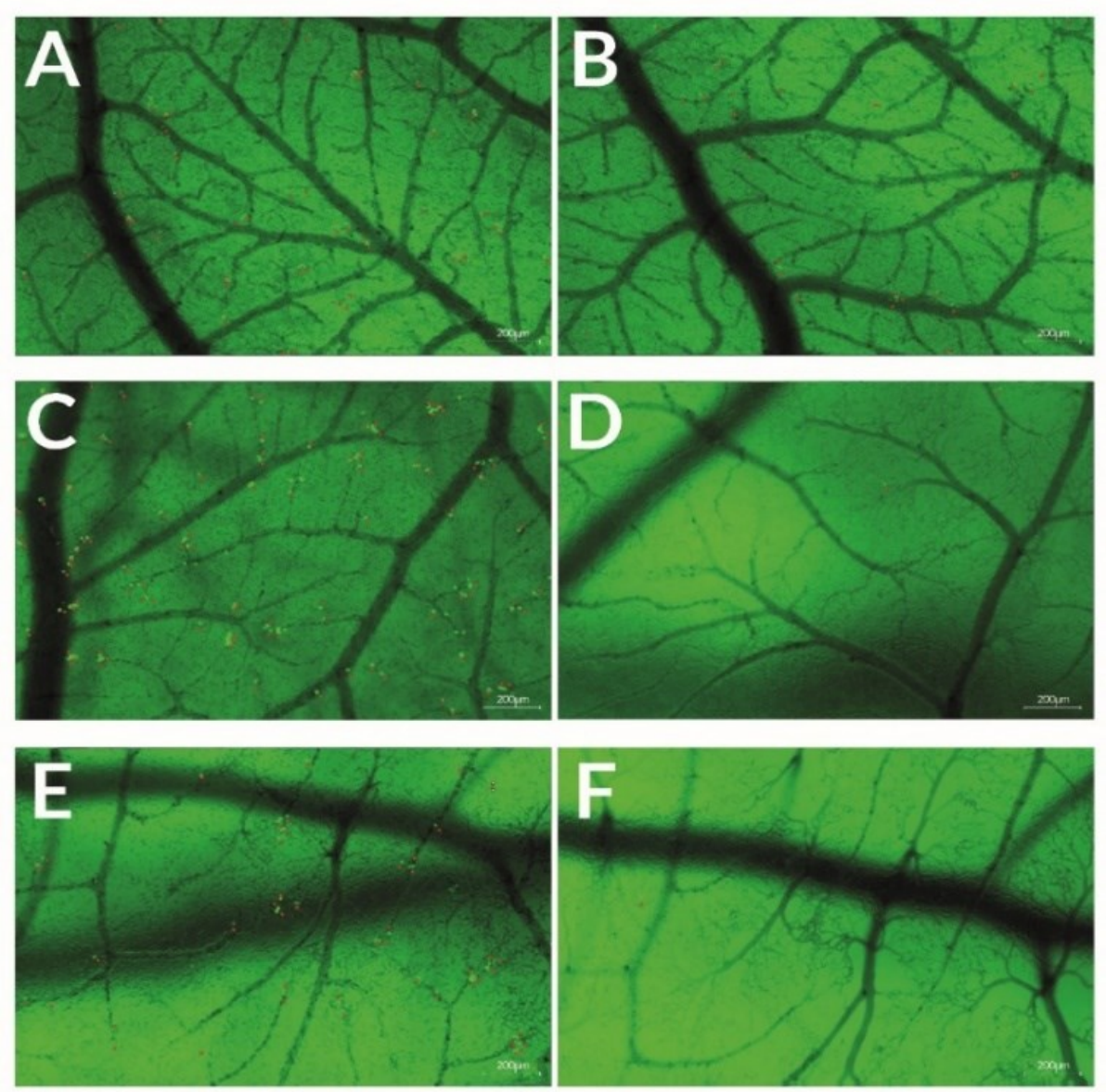

Fig. 8. Evaluation of polyethylene glycol–liposomal and conventional doxorubicin's inhibitory effects on cancer cell extravasation in the chorioallantoic membrane. Fluorescently labelled canine osteosarcoma D-17 cancer cells: A – in untreated control cells at t1, the time of cell injection and 0 h; B – in these cells at t2, after 24 h; C – in conventional doxorubicin–treated cells at t1; D – in these cells at t2; E – in PEG-liposomal doxorubicin–treated cells at t1; F – in these cells at t2. Dyed cancer cells are visible as light green dots. Scale bars 200 µm

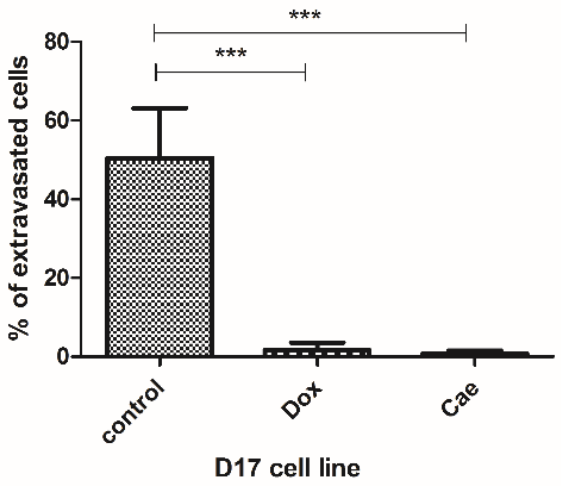

Fig. 9. Bar graphs showing the influence on the extravasation rate of Caelyx polyethylene glycol–liposomal doxorubicin and conventional doxorubicin on the canine D-17 osteosarcoma cell line after 24 h of incubation
*** – very high statistical significance at $P < 0.001$. Results are presented as mean ± standard error of the mean

The extravasation rates in chick embryos treated with conventional and liposomal doxorubicin were 4.3% and 1.5%, respectively. Nevertheless, it is important to indicate that the obtained result could be partially influenced by the cytotoxic effect of the tested drugs, because IC_{50} doses of both doxorubicin preparations were used, and the result therefore cannot be simply interpreted only as the inhibition of cancer cell extravasation efficacy. The aim of this study design was to more closely resemble the impact of the drugs injected intravenously in the *in vivo* environment on this particular stage of the metastatic cascade, which can be easily observed using the CAM model.

Discussion

Osteosarcoma is a common primary bone tumour in dogs, unfortunately with very high metastatic potential (22). This study intended to compare non-liposomal conventional and liposomal doxorubicin's effects on the canine D-17 metastatic OSA cell line. So far, therapies of OSA have included amputation or limb sparing surgery with adjuvant therapy like chemotherapy and/or radiotherapy. However conventional cytostatic drugs lack specific biodistribution, non-liposomal doxorubicin among them, which has a low therapeutic index and causes multi drug resistance in cancer cells (6). Liposomes as drug delivery systems may reduce the negative side effects and increase the therapeutic efficacy. Compared with conventional drugs, liposomes can directly deliver drugs to specific cells or tissues, thereby greatly reducing the adverse effects (29). In a community-based observational study, Salzberg *et al.* (19) indicated that PEG-liposomal doxorubicin is a well-tolerated active agent in patients with metastatic breast cancer. To the best of the authors' knowledge, there are no studies on the application of PEG-liposomal doxorubicin in canine metastatic OSA.

The presented study first evaluated the cytotoxicity after 24 h of conventional and PEG-liposomal doxorubicin on canine metastatic OSA using the MTT assay. The resulting IC_{50} after 24 h for PEG-liposomal doxorubicin was more than fourfold higher than it was for the conventional form, which may be explained by the slow release rate of free PEG-liposomal doxorubicin after 24 h. Haghiralsadat *et al.* (9) showed that the liposomal formulations indicated a lower inhibition of cell growth than conventional doxorubicin after 24 h, with which our results are in agreement. Doxorubicin becomes available after the liposomes extravasate and enter the tissue compartment (8).

In the next step, the accuracy of the IC_{50} doses was confirmed by flow cytometry with Draq 7 and Annexin V. The results obtained indicated that doxorubicin induced D-17 cell death mainly through apoptosis. There is no current consensus between scientists on the exact doxorubicin mechanism responsible for cancer cell death, and two possible models are indicated: apoptosis and necrosis (18). The specific form of cell death is governed by the drug concentration, the treatment duration and the (7). One of the advantages of the key pharmacological properties of PEG-liposomal doxorubicin was the drug's ability to suppress anti-apoptotic pathways and further amplify apoptotic activity (18). The apoptotic mechanism of doxorubicin-induced cell death for D-17 canine OSA cells presented in this study matches one posited in our previous research (16).

Cell migration is one of the key steps in the complex metastatic cascade (30). To assess the inhibitory effect of non-liposomal conventional and PEG-liposomal doxorubicin on canine OSA migration, a wound-healing assay was performed, and it indicated significantly ($P \leq 0.01$ and $P \leq 0.001$) stronger inhibiting effects of the PEG-liposomal doxorubicin than of the conventional preparation after 12 and 24 h incubation (Figs 6 and 7). The wound-healing assay serves as a valuable tool to quantify cell migratory capability following exposures to various chemotherapeutic compounds (28).

The *ex ovo* CAM model was used next to assess the *in vivo* effect of both studied drugs on OSA cells. The CAM model has been described as an economical, rapid and reproducible technique to evaluate cancer cell invasiveness. It enables rates of cancer cell extravasation to be quantified and is a reliable model for studying anti-metastatic drug responses (13). It should be emphasised that it is an experimental metastasis model performed with mammalian cells in an avian microenvironment and should be treated as a single step of the metastatic process yielding results which do not illuminate the whole metastatic cascade. There is no single preclinical model that reflects the metastatic process in patients with cancer (3). The

CAM model suits the 3Rs (reduction, replacement and refinement) approach in preclinical experimentation. "Reduction" minimises animal use by extracting more information from the same number of animals. "Replacement" uses animals with reduced potential for pain perception (in this case, chick embryos before day 16 of incubation). "Refinement" minimises animal suffering and increases animal welfare during experimentation. *Ex ovo* models overcome some of the limitations of the extensively used zebrafish model. Zebrafish grow optimally at a temperature of approximately 30°C, which is too low for mammalian cancer cell proliferation. Chick embryos need incubation at 37°C, which is the ideal temperature condition for *in vivo* cancer cell proliferation (25). This research used a model that provides cells of warm-blooded animals with appropriate conditions to sustain them. It guarantees the presence of fluid flow, the extracellular matrix, and suitable pH and interstitial pressure (4). Moreover, the *ex ovo* CAM assay enables a straightforward observation of chick microvessels and enables performance of microvascular research on a large surface of the CAM available for experimental intervention. In comparison to the murine model, the short incubation time of the chick embryo of 20–21 days in the *ex ovo* model allows a quick screening test (4). It is worthwhile to note that the *ex ovo* model requires high manual skill to perform injections into the microscopic vessels of the CAM, and confirmation that cells were properly injected intravenously needs to be carried out manually every time.

Kim *et al.* (13) stated that cancer cell extravasation is a crucial step in cancer metastasis which occurs within 24 h of cell injection, and they showed that most cancer cells injected at t = 0 h remained in vessels (intravascular) for the next 15 min and extravasated afterwards, with only <5% of cells still present inside the vessels of the CAM and not yet extravasated after 24 h had elapsed (13). As these results suggested, we conducted our experiment over 24 h, the appropriate time for cancer cell extravasation. In the first step, we calculated the extravasation efficacy of D-17 cells, which was on average 48%. To the best of our knowledge, the *ex ovo* model has not previously been used to determine the extravasation efficacy of the canine D-17 cell line or how it was influenced by PEG-liposomal doxorubicin. After 24 h, we observed that 4.3% and 1.5% of cancer cells extravasated in chick embryos treated with IC_{50} doses of non-liposomal and liposomal doxorubicin, respectively. The result confirms the satisfactory inhibitory effect of both tested drugs on cell extravasation. Despite the difference between conventional and liposomal doxorubicin percentages being appreciable, it was statistically nonsignificant. The dose of PEG-liposomal doxorubicin in the research was comparable to the dose used in a preclinical trial of doxorubicin entrapped in sterically stabilised liposomes in dogs with spontaneously arising malignant tumours. A group of 51 dogs received PEG-liposomal doxorubicin at the dose of 0.75 mg/kg (26). In the presented study, PEG-liposomal doxorubicin was given at the dose of 0.6 mg/kg. The dose was calculated on the average weight of a chick embryo on the 12th day of incubation, which is approximately 5 g, the known drug concentration of 30μg/mL and the volume of the drug injected (100 μL). *In vitro* and *ex ovo* studies revealed that both study drugs could be considered effective antimetastatics for canine OSA.

The main limitation of the experimental design that should be considered while interpreting the presented results is the use of only one cancer cell line. However, there is only one canine OSA cell line available in the ATCC and European Cell Culture Collection.

The present study provides crucial information about PEG-liposomal doxorubicin. It shows an inhibitory effect of the liposomal form of doxorubicin on the proliferation, migration and extravasation of canine D-17 OSA cells in *in vitro* and *in vivo* tests. The results of the *in vitro* and *ex ovo* studies indicate that PEG-liposomal doxorubicin could be considered a useful drug for metastatic canine OSA; however, further *in vivo* studies on dogs with metastatic OSA are needed to confirm this thesis.

Conflict of Interests: Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: According to the Polish Act of December 2019 and Directive 2010/63/EU as transposed into current Polish legislation, the approval of the Animal Ethics Commission was not required.

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