Comparison of the Inhibitory Effect of PEG-Liposomal and Conventional Doxorubicin on Migration and Extravasation Efficacy on Canine Osteosarcoma Cell Line- in Vitro and Ex Ovo Studies.

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Abstract

The chick chorioallantoic membrane (CAM) assay has long been used to study the effects of drugs on angiogenesis or evaluate cancer cell invasiveness by quantifying in vivo rates of cancer cell extravasation. Extravasation plays a crucial role in the metastatic cascade, whereby circulating cancer cells derived from the primary tumor cross the endothelial barrier to reach the target metastatic site. Accordingly, we adapted an ex ovo model to study the anti-extravasation efficiency of anticancer drugs. The drugs investigated include conventional and PEG-liposomal doxorubicin. The conventional form is commonly used in chemotherapy protocols for canine appendicular osteosarcoma (OSA), although it has no specific biodistribution and a low therapeutic index. For this reason, this study compared the effects of conventional and PEG-liposomal doxorubicin on cytotoxicity and migration inhibition in the in vitro environment. Cytotoxicity was evaluated by the MTT assay, Annexin V staining and the Draq 7 test; the inhibition of migration was analyzed using the scratch assay test. Moreover the inhibitory effect of study drugs on cancer cell extravasation was analyzed in the in vivo conditions, on the ex ovo model. The results of experiments performed showed that PEG-liposomal doxorubicin has a higher inhibitory effect on the in vitro migration of canine OSA ($p \leq 0.05$). Ex ovo research revealed both drugs elicited a high efficiency for inhibiting the extravasation of canine OSA ($p < 0.0001$). Therefore PEG-liposomal doxorubicin may be considered as a potentially useful anti-metastatic agent in canine osteosarcoma due to its inhibitory effect on both the migration and extravasation of the D-17 cell line.

Introduction

Metastasis involves the spread of cancer cells from the primary tumor to surrounding tissues and distant organs. It is the primary cause of cancer morbidity and mortality. Although most cancer cells are rapidly destroyed in circulation, either by the immune system or hemodynamic forces, some of them form metastatic foci.(Janssen et al. 2017) There are four main steps in the metastatic cascade: intravasation, transit in the blood or lymphatic system, extravasation, and adaptation to the secondary site.(Hapach et al. 2019) The ability of cells to extravasate into the surrounding tissue, by degrading the basement membrane and extracellular matrix, has long been considered a major rate-limiting step in metastatic process.(Luzzi et al. 1998) In it, a single cancer cell undergoes trans endothelial migration, forming invasive invadopodia to mediate translocation of the tumor cell from the vessel lumen into tissue.(Kim et al. 2016) The metastatic process may begin at the time of primary tumor detection, or metastases can occur many years after primary cancer treatment. Preventing metastatic dissemination seems an appropriate way to reduce metastatic disease and potentially prolong a patient's life. Although metastatic diseases are responsible for about 90% of cancer deaths, most cancer research does not involve *in vivo* metastatic studies,(Seyfried et al. 2014) which are necessary to understand the metastatic process. (Anderson et al. 2019)

Appendicular osteosarcoma (OSA) is the most common primary bone tumor in dogs and is both locally aggressive and highly metastatic.(Straw et al. 1990) Standard treatment, which consists of local control via amputation followed by adjuvant chemotherapy (carboplatin, cisplatin, and doxorubicin), results in a

median survival time range of 277 to 479 days.(Selmic et al. 2014; Skorupski 2016) While less than 10% of patients have metastatic disease (stage III) identified on initial presentation, 90% of patients will ultimately succumb to metastatic disease. Metastases disseminate to lungs, bones, regional lymph nodes, or internal organs (spleen, liver).(Selvarajah and Kirpensteijn 2010),(O'Brien et al. 2004) Doxorubicin is a drug of choice for treating canine osteosarcoma; however, its use is limited by severe side effects such as cardiotoxicity (dysrhythmia and heart failure).(Szewczyk et al. 2015) PEG-liposomal doxorubicin has been tested for efficacy and safety in patients with metastatic breast cancer.(Lien et al. 2014) The long-acting form of doxorubicin, encapsulated in liposomes, was designed to reduce side-effects while preserving antitumor efficacy by altering tissue distribution and pharmacokinetics. (Olusanya et al. 2018)

The aim of this study was to compare the inhibitory effect of conventional and PEG-liposomal doxorubicin on the migration and extravasation of a canine osteosarcoma cell line *in vitro* and e*x ovo*.

Materials And Methods

Preparation of osteosarcoma cell line

The commercial canine osteosarcoma cell line D-17 (ATCC, USA) (derived from an osteosarcoma metastasized to the lungs) was cultivated in Eagle's Minimum Essential Medium (EMEM) (ATTC, USA) with the addition of heat-inactivated fetal bovine serum (FBS), penicillin–streptomycin (50 IU ml), and amphotericin B (2.5 mg ml) under standard conditions (5% of CO_2, 95% of humidity, 37 °C). Cells in a logarithmic growth phase with 90% confluence were harvested using 0.025% trypsin (Sigma Aldrich, USA) and reseeded. The number of living and necrotic cells was determined by staining with 0.2% trypan blue solution (Sigma Aldrich, USA) and analyzed with an automatic cell counter (Countess II, Thermo Fischer, USA).

In vitro experiments

MTT viability test

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used as a quantitative test of cell viability. Actively respiring cells convert the water-soluble MTT to an insoluble purple formazan dye that can be detected spectrophotometrically. The test also established the half maximal inhibitory concentration (IC_{50}) and the inhibitory concentration causing 20% cell death (IC_{20}).

D-17 cells were seeded onto 96-well cell culture plates (Sigma Aldrich, USA), and after achieving 90% confluency, the liposomal doxorubicin (Caelyx®, Janssen-Cilag Internionall, Belgium) and conventional doxorubicin (Adriblastina®, Pfizer, USA) were added in decreasing concentrations (µg/ml): 250, 100, 50, 25, 10, 5, 2.5, 1, 0.5. Cells were incubated with test substances in standard conditions. After 24h, 10 µl of the MTT labeling reagent (final concentration 0.5 mg/ml) was added to each well. Within 4 hours, each well was filled with 100 µl dimethyl sulfoxide (DMSO) (Sigma Aldrich, USA). Optical densities of the

supernatant were read at 555 nm using a microplate spectrophotometer (Tecan Infinite, USA). Absorbance was normalized using untreated control cells and used to calculate differences in cell viability. The test was repeated three times for result reliability.

Annexin V and Draq 7 for quantification of apoptotic and necrotic cell death

Analysis of necrosis and apoptosis by flow cytometry (Annexin V and Draq7 test) was performed to confirm results obtained from the colorimetric test.

The Annexin V and Draq 7 assay allows the quantitative analysis of live, early, and late apoptotic and dead cells. In the experiment, Draq 7(Biolegend) was used instead of propidium iodide (PI) and 7-AAD, as Draq 7 has no emission overlap with doxorubicin..

Briefly, cells were seeded onto 6-well cell culture plates (Sigma Aldrich, USA) in EMEM serum-free medium (Sigma Aldrich, USA). After 24h, when cells reached 90% confluence, the medium was removed, and cells were treated with IC_{50} doses (assessed with the MTT assay) of PEG-liposomal and conventional doxorubicin. After 24h of incubation, media with substances were transferred to cytometry tubes (Abcam, UK) and cells were flushed three times with cold Phosphate-Buffered Saline (PBS) (Sigma Aldrich, USA), according to the manufacturer's instructions. Previously prepared binding buffer was added to each sample. Samples were divided into four groups:

1. Unstained
2. Annexin V 450 (Abcam, UK)
3. Draq 7 (Abcam, UK)
4. Annexin V and Draq 7

Samples with Annexin V were incubated in the dark for 10-15 minutes at room temperature. Tubes were stored on ice and analyzed within 1 hour. The percentages of viable, apoptotic, and necrotic cells were analyzed by flow cytometry using the BD FACS Aria^{TM} II flow cytometer (BD Biosciences, San Jose, USA). Data were analyzed using the FACS Diva software version 6.1.3 (BD Biosciences, San Jose, USA). The experiment was repeated three times to confirm results.

Scratch assay test

The *in vitro* wound-healing assay was performed to assess the inhibition of cancer cell migration by incubating with conventional and PEG-liposomal doxorubicin. Cells were seeded on 6-well cell culture plates (Sigma Aldrich, USA). After 24 hours, when a 100% confluence was reached, the medium was removed, and Mitomycin C (Abcam, UK) was added to the medium at a concentration of 10 µg/ml and incubated at 37°C. After 3 hours, medium with Mitomycin C was replaced with serum-free medium containing an IC_{20} dose (assessed by MTT test) of liposomal doxorubicin, IC_{20} dose of conventional doxorubicin, and serum free medium as a control. The monolayer was wounded by scratching the surface with a pipette tip (100µl) (Eppendorf, Germany). Images of cells invading the scratch were

captured at indicated time points (t_1=0, t_2=24h) with an inverted microscope (Primovert, Zeiss, Germany) and pictures were analyzed using the Zen Pro 2012 program (Zeiss, Germany). The rate of migration was assessed by calculating the total distance between the edges of the scratch. Measurements were obtained at 100 points for each sample.

Ex ovo assay

Ex ovo models are 3R preclinical models used to determine the extravasation efficacy of cancer cells.(Kim et al. 2016) For the experiment, 120 fertilized white Ross 308 ("Marylka", Poland) were incubated at 39°C and 70% humidity. On the third day of incubation, eggshells were opened with a driller and chick embryos removed and put into 100ml, 85x85 mm black polystyrene weigh boats (Global scientific, UK) and covered with plastic Petri dishes (Bio-Rad, USA) (Fig. 1). On that day, embryo rotation in the incubator stopped. Embryos were stacked and transported in batches using plastic boxes with water on the bottom. The water layer prevented embryos from drying (Fig.2). On the fourth and fifth day, embryos were examined for survival, and dead embryos were removed from the incubator.

On the 12th day of incubation, 1.0×10^5/ml D-17 were dyed with Cell Tracker green CMFDA (5-chloromethylfluorescein diacetate) dye (Life Technologies, UK) and stored on ice. Solutions of study drugs were prepared at the IC_{50} concentrations, 29 µg/ml and 6 µg/ml of PEG- liposomal doxorubicin in concentrate and conventional doxorubicin, respectively. Chick embryos were divided into 3 groups (n=10 embryos in each group):

1. Control group – injection of dyed cells without test compounds
2. PEG liposomal doxorubicin group – injection of dyed cells and liposomal doxorubicin at IC_{50}
3. Conventional doxorubicin group – injection of dyed cells and conventional doxorubicin at IC_{50}

Embryos from each group were injected with 100 µl cell suspension. After injection, the presence of stained cancer cells in CAM circulation was confirmed under the wide-field fluorescence microscope (Zeiss Examiner. Z1, Germany) using the 10X objective. Then, 100 µl liposomal doxorubicin (IC_{50}), 100 µl of conventional doxorubicin (IC_{50}), or 100 µl of saline was injected into embryos in each study group. Intravenous injections of dyed cells and test compounds were introduced into the CAM using the stereoscopic microscope (IBIDI, Germany) with 50µm diameter microneedles controlled with an automatic pipette puller, PC-100 (Narishige, Japan). Following injection, a sterile silicon ring (7 mm external diameter, 6 mm internal diameter, and 1 mm thick; Zegir PTHU, Poland) was placed on the superficial surface of the CAM to limit the evaluation area. To analyze the efficacy of extravasation, all cells within each silicon ring were counted at the starting time (t_1 = 0) and after 24 hours (t_2 = 24h), and captured with a camera (Zeiss AxioCam MR, Germany) microscope examiner, 4X objective and 1X zoom. Extravasation efficacy from each silicone ring was calculated according to the formula:

$$\text{extravasation efficacy} = \frac{number\ of\ cells\ (t2)}{number\ of cells\ (t1)} * 100\%$$

Dead embryos were excluded. After the experiment all embryos were euthanized.

The test was repeated three times for reliability.

Statistical analyses

Statistical analyses of the wound healing assay and *ex ovo* experiment were performed by one-way ANOVA and post-hoc Tukey tests (GraphPad Prism 5.0, USA). With each data point represented as mean ± SD or mean ± SEM, significance of $p \leq 0.05$ (*) was assigned as relevant, while $p \leq 0.01$(**) and $p \leq 0.001$ (***) were assigned as highly relevant.

Results

In vitro experiments

Cells viability

For the canine osteosarcoma cell line (D-17), cells viability was determined (Fig.3). Moreover IC_{20} and IC_{50} doses of PEG-liposomal doxorubicin (Caelyx ®) and conventional doxorubicin were administered after 24 hours.(Tab.1).

	IC_{20} after 24h	IC_{50} after 24h
PEG-liposomal doxorubicin	3.608 µg/ml	28.862 µg/ml
Conventional doxorubicin	0.76 µg/ml	6.090 µg/ml

Tab.1 MTT assay results after 24 hours incubation of PEG-liposomal doxorubicin and conventional doxorubicin on the D-17 cells

Quantification of apoptotis and necrosis

Analysis of necrosis and apoptosis by flow cytometry confirmed results obtained from the colorimetric test. D-17 were treated with study drugs at IC_{50} doses: 28,862 µg/ml and 6,090 µg/ml for PEG-liposomal and conventional doxorubicin, respectively. Both study drugs exhibited similar cytotoxic effects (on early, late apoptosis, and necrosis) (Fig. 4) but conventional doxorubicin triggered cancer cell death mostly through the apoptotic pathway (*$p \leq 0.05$) (Fig. 5).

Assesment of cell migration

The ability of conventional and PEG-liposomal doxorubicin to inhibit D-17 cell migration was determined using an *in vitro* wound healing assay. As shown in Fig. 5, conventional and PEG-liposomal doxorubicin effectively inhibited the migration of all cells, in comparison to the control treatment. Moreover, test drugs visibly impacted cancer cell morphology, growth, and proliferation. The “wound” was not rebuilt (cells

migration was inhibited by drugs), but also, the cell monolayer of was noticeably decimated (Fig. 6). Both drugs inhibited the migration of cancer cells, with a significantly higher (p 0.05) inhibition in cells treated with PEG-liposomal doxorubicin in comparison to those treated with conventional doxorubicin (Fig 7).

Ex ovo experiment

Each area was evaluated, and all intravasated and extravasated cells were counted (Fig.8). After the injection at t=0, cells were averaged. After 24h, in the group receiving doxorubicin, there was a single chick embryo out of 7 with no cancer cells. In the group with PEG-liposomal doxorubicin, 3 out of 7 chick embryos remained free of cancer cells after 24h of incubation.

The results are summarized in Figure 9, showing the high efficiency of both drugs at inhibiting the extravasation of D-17 cells after 24h (***p <0.0001).

The survival rate of embryos in each group differed significantly. Mortality was much higher in the group of embryos that received conventional doxorubicin (13 out of 21 were dead after 24h conventional doxorubicin incubation) in comparison to the control group (5 out of 21 were dead 24h after cell injection) and the PEG-liposomal doxorubicin group (10 out of 21 were dead within 24h PEG-liposomal doxorubicin incubation).

Discussion

Canine osteosarcoma (OSA) is the most common primary bone tumor in dogs, with high metastatic potential.(Straw et al. 1990) This study aimed to compare the inhibitory effects of conventional and liposomal doxorubicin on the extravasation of a canine osteosarcoma cell line. So far, therapies to treat osteosarcoma, including amputation, limb sparing surgery, chemotherapy, and radiotherapy, have proven unsuccessful. When conservative treatment is applicable, issues like drug choice remain problematic. Treatment failure results from old generation, cytostatic drugs lacking specific biodistribution, like conventional doxorubicin, which has a low therapeutic index and causes multi drug resistance (MDR) in cancer cells.(Boston et al. 2006) Over two decades ago, scientists proved that conventional doxorubicin may be successfully replaced with the PEG-liposomal form. For this reason, we evaluated and compared the efficacies of both conventional and liposomal doxorubicin in canine osteosarcoma. Symon et al. revealed that liposomal doxorubicin selectively accumulates in MBC cells metastasized to the bone. (Symon et al. 1999) Other studies confirmed the efficacy of PEG-liposomal doxorubicin in patients with metastatic breast cancer (MBC). In a community-based observational study, Salzberg et al. supported previous reports indicating that PEG-liposomal doxorubicin is an active, well-tolerated agent in patients with metastatic breast cancer.(Salzberg et al. 2008) However, clinical research has many limitations like low objectivity and high population diversity.(Kostis and Dobrzynski 2020) Thus, this study analyzed the inhibition of extravasation using an *ex ovo* animal model, which proved objective and reliable. Kim et al. described a rapid, reproducible, and economical technique to evaluate cancer cell invasiveness by quantifying rates of cancer cell extravasation in the chorioallantoic membrane (CAM) of chicken embryos.(Kim et al. 2016) Importantly, the CAM model suits the 3Rs (replacement, refinement, reduction)

approach in preclinical experimentation. “Replacement” describes the use of animals with relatively reduced potential for pain perception (chick embryos before day 16 of incubation). “Refinement” minimizes animal suffering and enhances animal welfare. “Reduction” minimizes animal use by obtaining more information from the same number of animals (policy regarding the use of avian embryos). Based on the many advantages of this assay, this study used the CAM model. The chicken embryo CAM has already been confirmed as a reliable model for studying anti-cancer drug responses. (Kue et al. 2014) In comparison to the widely-used zebrafish model, *ex ovo* models overcome some of it limitations. Zebrafish grow optimally at 31 °C, which is too low for mammalian cancer cell proliferation. Chick embryos require incubation at 37°C, resembling optimal conditions for *in vivo* cancer cell growth. (Tat et al. 2013) This research study used a model that provides conditions most similar to the physiology of warm-blooded living things. It provides the presence of the extracellular matrix, fluid flow, interstitial pressure, and accurate microRNA profiles and pH.(Augustine et al. 2020) All of these advantages of the CAM *ex ovo* model supported its use in our research.

The cytotoxicity of both forms of study drugs is an issue. Therefore, this study evaluated and investigated changes using a different drug formulation. Using the MTT assay, this study compared the cytotoxicity of conventional and PEG-liposomal doxorubicin on canine OSA. Haghiralsadat et al. performed an MTT assay to determine cell viability in the presence of conventional and liposomal doxorubicin.(Haghiralsadat et al. 2017) During the 24 hour period, the liposomal formulations indicated a lower inhibition of cell growth than conventional doxorubicin.(Palareti et al. 2016) Results of our study showed a similar relationship; the IC_{50} obtained for conventional doxorubicin was approximately five times lower than for the liposomal form. The difference could be simply explained by the slow-release rate of free PEG-liposomal doxorubicin in *in vitro* conditions. An MTT assay was likewise performed to estimate experimental drug doses. Although a widely used colorimetric test, some research undermines its credibility with liposomal drugs.(Angius and Floris 2015) The accuracy of IC_{50} doses was verified by another standard assay for measuring chemotherapy potency-flow cytometry with annexin V, which analyzes early and late apoptosis.(Maass et al. 2015) Toldo et al. used flow cytometry to evaluate the apoptotic efficiencies of liposomal and conventional doxorubicin.(Toldo et al. 2013) Our research revealed that annexin V flow cytometry yields a similar cytotoxic effect (including early and late apoptosis, and necrosis) with both study drugs. This research study confirmed results obtained with the photometric MTT test. However, an Annexin V and Draq 7 colorimetric test showed that conventional doxorubicin triggered cancer cell death mostly through an apoptotic pathway.

Researchers determined the cytotoxic effect of selected drugs and their inhibitory effect on canine OSA cell migration. Wound healing assays serve as useful tools in quantifying alterations in cell migratory capabilities following experimental manipulation with chemotherapeutic agents.(Grada et al. 2017), (Varankar and Bapat 2018) To the best of our knowledge, wound healing assays have not been performed to assess the inhibitory effect of liposomal doxorubicin on the migration of any canine OSA cell line. In this study, PEG-liposomal doxorubicin inhibited the migration of canine osteosarcoma. However, conventional doxorubicin had a significantly higher inhibitory effect on the migration of canine

OSA. We suspect that the reduced effect of the liposomal drug form could relate to the slow release of PEG-liposomal doxorubicin in *in vitro* conditions.

In vitro models cannot mimic the physiological environment of cancer cell expansion. This feature is characterized only by *in vivo* models.(Katt et al. 2016) However, compared to *in vivo* studies, *in vitro* methods usually demonstrate less variability with liposomal drugs.(Jiang et al. 2011) Accordingly, this study included an *in vivo* experiment to corroborate *in vitro* results. The *ex ovo* model enables investigation of the metastatic cascade at the time of extravasation.(Kim et al. 2016) To the extent of our knowledge, the *ex ovo* model has not previously been used to determine the efficacy of anticancer drugs in veterinary medicine. We adapted Kim's CAM research model to our experiment. In this research study, dyed cancer cells were injected into chick embryo CAM, and drugs of interest administered into the chick embryo blood circulation to estimate the inhibitory effect on cancer cell extravasation. Augustine et al. demonstrated that using cancer cells expressing green fluorescent protein allows easy detection and quantification of migrated cancer cells in *in vivo ovo* models.(Augustine et al. 2020) In our *ex ovo* experiment, green fluorescent-labeled cancer cells were visible in the vessel network, which facilitated quantification of extravasated canine OSA cells. The *ex ovo* assay, in comparison to the *in ovo* assay, guarantees a simpler network of CAM vessels. Cancer cell extravasation is a dynamic process that occurs within 24h of cell injection, as confirmed by Chen et al.(Craik 2008) During intravital imaging, Kim et al. confirmed this theory, showing that most cancer cells injected at t=0 (within 15 minutes) remain in vessels (intravasated), and after 24h, 95% of them extravasated and <5% of cells remain in the intravascular space.(Kim et al. 2016) In accordance with these results, we conducted our experiment within 24 hours of injection, the appropriate time for cancer cell extravasation. After 24 hours, we observed around 2% and 0.5% of extravasated cancers cells in chick embryos treated with conventional and liposomal doxorubicin, respectively. The trace presence of extravasated canine OSA cells after drug administration confirms their sufficient inhibitory effect on cancer cell migration.

Visible differences exist between results obtained *in vitro* and *in vivo* (*ex ovo*), which confirms pharmacokinetic dissimilarities of the conventional and liposomal drug forms. Liposomal drug release can be influenced by the presence of a vessel membrane in addition to environmental triggers, such as low pH, the presence of particular enzymes, and appropriate temperatures.(Shibata et al. 2015) The present study provides important information about PEG-liposomal doxorubicin and its inhibitory effect on the migration of D-17 cells in *in vivo*/ *ex ovo* studies

Abbreviations

AM – performed ex ovo experiments, contributed to writing the manuscript

BT- performed Annexin V and Draq7 assay

AnW- performed cell culture

SW- performed cell culture, contributed to writing the manuscript

AgW – performed cell culture

KZK- designed the study, performed statistical analyses, critically reviewed the manuscript for important intellectual content

RL- coordinated the study, critically reviewed the manuscript for important intellectual content

Conclusion

Regarding the environment, we observed different drug efficiencies. In *in vitro* conditions, both drugs inhibited the migration of cancers cells; however, PEG-liposomal displayed a significantly higher level of inhibition in comparison to the conventional drug. *In vivo* experiments also revealed that both drugs had high efficiencies for inhibiting the extravasation of D-17 cells after 24 hours ($p < 0.0001$). Both PEG-liposomal doxorubicin and conventional doxorubicin significantly inhibit the extravasation of D-17 osteosarcoma cells after 24 hours. In conclusion, the results presented show that PEG-liposomal doxorubicin may serve as a potentially useful anti-metastatic agent in canine osteosarcoma due to its inhibitory effect on both the migration and extravasation of the D-17 cell line. Accordingly, further *in vivo* studies should be conducted to confirm this hypothesis.

Declarations

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Author contributions All authors contributed to the study conception and design.

MW – performed cell culture, in vitro experiments (MTT assay, Annexin V and Draq7 assay, scratch assay), ex ovo experiments, wrote a major part of the manuscript, prepared the manuscript for publication. The manuscript is a part of the PhD thesis of Magdalena Walewska. All authors edited, read, and approved the final manuscript.

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Data availability All data generated and analyzed during this study are included in this published article.

Compliance with ethical standards The Approval of Animal Ethics Commission was not required according to the Polish act on the protection of animals used for scientific and educational purposes, which was passed in December 2019 and transposed Directive 2010/63/EU into current Polish legislation.

Conflicts of interest The authors declare that they have no conflict of interest.

Consent to participate Not applicable.

Consent for publication All authors give consent for publication.

Code availability Not applicable.

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Figures

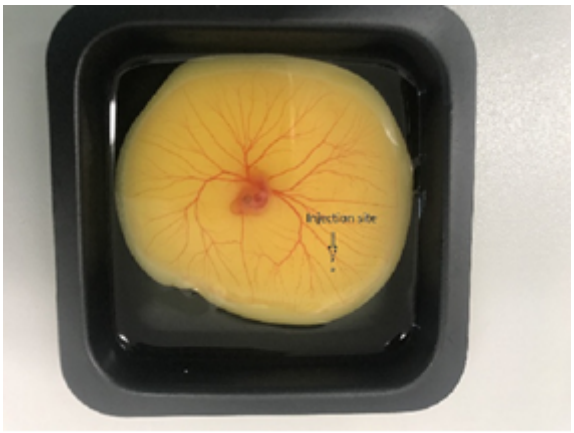

Figure 1

CAM cancer cell injection site for the efficiency of inhibiting extravasation assay.

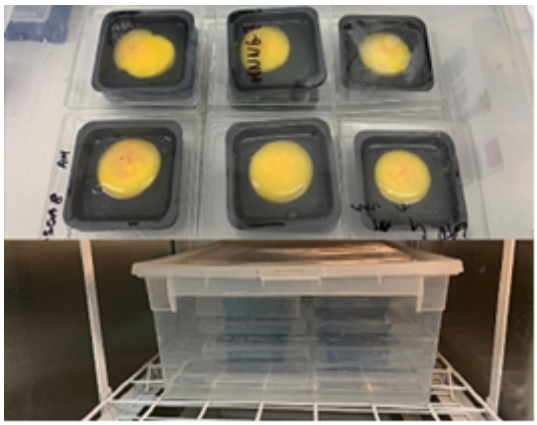

Figure 2

Embryos transported to the incubator in batches using plastic containers. Large collecting containers with water at the bottom ensured the appropriate humidity.