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LMWHs and DOACs Modulate Cancer Cell Procoagulant Activity and Viability: Comparison with Quercetin

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Abstract

Tissue factor (TF)-expressing cancer cells and their extracellular vesicles (CaCe-dEVs) are key drivers of cancer-associated hypercoagulability and vascular dysfunction. While low-molecular-weight heparins (LMWHs) and direct FXa inhibitors are standard therapies for cancer-associated thrombosis, their direct effects on cancer cell procoagulant potential and endothelial responses remain incompletely defined. This study compared the impact of LMWHs (enoxaparin, tinzaparin), apixaban, and quercetin on cancer cell viability, thrombin generation, and CaCe-dEVs–induced endothelial injury. Pancreatic (BXPC3) and breast (MCF7) cancer cells and their vesicles were analyzed for TF expression and thrombin generation. Human umbilical vein endothelial cells (HUVEC) were pretreated with each agent prior to vesicle exposure. Cell viability, thrombin generation, and endothelial morphology were assessed using standard assays and microscopy. Tinzaparin and quercetin significantly reduced cancer cell viability, whereas enoxaparin and apixaban showed no cytotoxicity. None of the agents affected HUVEC viability. All suppressed TF-mediated thrombin generation, with tinzaparin being most effective in BXPC3 cells. Quercetin consistently protected endothelial cells from CaCe-dEVs–induced dysfunction, while LMWHs and apixaban did not prevent endothelial damage. These findings suggest that LMWHs, apixaban, and quercetin modulate tumor-driven hypercoagulability beyond anticoagulation, with quercetin and tinzaparin showing additional cytotoxic potential. Such dual effects may reduce thrombosis risk while impacting tumor progression, meriting further investigation.

Keywords: low-molecular-weight heparin; quercetin; apixaban; thrombin generation; cancer-associated thrombosis; tissue factor

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1. Introduction

Hypercoagulability is frequent in cancer patients and is associated with tumor aggressiveness, resistance to therapy, and cancer-associated thrombosis [1–3]. Cancer cells expressing tissue factor (TF) trigger thrombin generation, leading to fibrin network formation, creating protective clot "shields" that contribute to treatment resistance [4]. Newly diagnosed, treatment-naïve patients with cancer often show elevated markers of endothelial activation (e.g., VCAM-1, VWF, circulating endothelial cells), highlighting endothelial dysfunction as an early driver of tumor vascular remodeling [5–7].

Vascular remodeling promotes direct contact of cancer and endothelial cells, inducing "education" of the latter to support tumor growth, neoangiogenesis, and metastasis. Tumor-neighboring endothelial cells display plasticity and may undergo endothelial-to-mesenchymal transition [8]. Cancer cell–derived extracellular vesicles (CaCe-dEVs) are key mediators of this process, transferring oncogenic molecules such as microRNAs, proteins, and metabolites [9]. Endothelial cells exposed to CaCe-dEVs acquire a procoagulant phenotype [10].

Low-molecular-weight heparins (LMWHs) and oral direct inhibitors of activated factor X (FXa) (apixaban, edoxaban, rivaroxaban) are cornerstone agents in the prevention and treatment of cancer-associated thrombosis [11]. Multiple lines of evidence from modeling and translational studies indicate that LMWHs, beyond inhibiting thrombin generation, can interfere with cancer cell biology and exert anticancer activity [12–16], including inhibition of angiogenesis and tumor progression [13,14]. Direct FXa inhibitors have also been reported to display anticancer effects, with supratherapeutic apixaban reducing proliferation and promoting apoptosis in experimental settings [17–19]. Beyond anticoagulants, quercetin - a dietary flavonoid - exhibits anti-inflammatory, antioxidant, and pro-apoptotic effects, and protects endothelial cells from hypoxia-reoxygenation injury [20,21]. Our previous studies showed that BXPC3 pancreatic and MCF7 breast cancer cells, and their CaCe-dEVs, express TF and induce thrombin generation, with BXPC3 demonstrating higher procoagulant activity [10,22,23]. Targeting TF and reversing endothelial "education" by CaCe-dEVs may therefore offer novel therapeutic strategies.

Building on this background, the present study aimed to determine whether LMWHs and DOACs (a) exert direct effects on cancer cells, beyond the inhibition of thrombin generation, by modulating cancer cell procoagulant activity and cell viability, and (b) protect endothelial cells from the deleterious effects of exposure to CaCe-dEVs. To benchmark these effects, we performed a comparative analysis with quercetin, used here as a reference agent with proven antioxidant and antitumor properties, and quantified treatment-associated changes in key procoagulant and viability-related endpoints.

2. Materials and Methods

Cell Culture

Human pancreatic adenocarcinoma cells (BXPC3) and estrogen receptor-positive breast adenocarcinoma cells (MCF7) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). These cell lines, differing in biological behavior and procoagulant potential, were selected to model high (BXPC3) and low (MCF7) procoagulant activity.

Using a previously published and validated experimental model [10], cells were cultured in 96-wells plate in RPMI-1640 media at 50 cells/µL (100 µL/well) and incubated at 37 °C in a humidified 5% CO_2 atmosphere until ~80% confluence before experimentation. Primary human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Levallois-Perret, France) and cultured in endothelial basal medium-2 (EBM-2, Clonetics) supplemented with 2% fetal bovine serum. Second-passage of HUVEC were seeded in 96-wells plate at 50 cells/µL (100 µL/well) and incubated at 37 °C in a humidified 5% CO_2 atmosphere until ~80% confluence before experimentation.

Cancer Cell-Derived Extracellular Vesicle Isolation and Quantification

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The CaCe-dEVs were isolated from conditioned media of confluent BXPC3 or MCF7 cultures via differential centrifugation to remove cells and debris as described previously [10]. The TF-dependent procoagulant activity of CaCe-dEVs in the culture supernatants was quantified using the ZYMUPHEN™ MP-Activity kit (Hyphen Biomed, Neuville-sur-Oise, France) according to a predefined protocol.

Endothelial Cell Exposure to CaCe-dEVs

In preliminary experiments, HUVEC (5×10^5 cells per 25-cm^2 flask) were incubated with increasing concentrations of EVs derived from BXPC3 or MCF7 cells (120, 240, and 360 nM) for 24, 48, or 72 h. In preliminary experiments, the CaCe-dEVs at concentrations of 120 or 240 nM, incubated for 24, 48, or 72 h, did not induce any detectable changes, morphological changes or TF expression in endothelial cells [24]. In contrast, exposure of HUVEC to 360 nM of CaCe-dEVs for 72 h induced marked morphological changes of the endothelial cells. Consequently, for all subsequent experiments, HUVEC were incubated with CaCe-dEVs at 360 nM for up to 72 h, following the validated protocol [10]. HUVEC were exposed to 360 nM BXPC3- or MCF7-dEVs, resuspended in 1 mL EBM-2, and incubated in 25 cm^2 flasks for 72 h.

Pretreatment of HUVEC with Antithrombotic Agents

HUVEC (5×10^5 cells per 25-cm^2 flask) were pretreated for 24 h with low or high concentrations of the LMWHs enoxaparin (1 or 2 anti-Xa IU/mL), tinzaparin (1 or 2 anti-Xa IU/mL), or the direct specific FXa inhibitor apixaban (1 or 2 µg/mL), or quercetin (166.5 or 333 µM), or PBS (control), all diluted in EBM-2 medium. After 24 h, cells were washed three times with PBS, and subsequently, 360 nM of CaCe-dEVs suspended in EBM-2 medium were added for incubation periods of up to 72 h. No residual anti-Xa activity from enoxaparin or tinzaparin, nor any measurable apixaban, was detected in the washing solution after the third wash of cells previously exposed to these antithrombotic agents.

Thrombin Generation Assay

Thrombin generation was assessed according to a previously published validated experimental procedure [4] using the calibrated automated thrombogram (CAT) with the Thrombinoscope system and Thrombinoscope software (Diagnostica Stago, Asnières-sur-Seine, France) as follows: BXPC3, MCF7, or HUVEC (pretreated or not by agents and/or exposed to CaCe-dEVs) were seeded in 96-well plates (100 µL at 50 cells/µL). After 24 h of incubation, the wells were washed three times with warm PBS, and 80 µL of normal platelet-poor plasma (PPP) purchased by Diagnostica Stago (Asnières-sur-Seine, France) was then added. Thrombin generation was initiated by adding 20 µL of a triggering solution containing $CaCl_2$ and fluorogenic substrate according to the manufacturer's instructions. The thrombogram parameters lag-time, peak of thrombin (Peak), and endogenous thrombin potential (ETP) were analyzed.

Viability and Proliferation Assays

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay: HUVEC (5×10^3 cells/well) were incubated with CaCe-dEVs for 72 h. Cell viability was assessed using the MTT assay (Roche Diagnostics, Mannheim, Germany), and absorbance was measured at 570 nm with a reference wavelength of 750 nm. The assay specifically measures mitochondrial reductase activity in metabolically active cells and allows for the evaluation of subtle changes in endothelial cell function following CaCe-dEVs exposure.

Crystal Violet Assay: Cancer cells (5×10^3 cells/well) were treated with test agents and incubated up to 72 hours before staining with crystal violet (Sigma-Aldrich, St. Louis, MO, USA). Absorbance was measured at 570 nm. This assay was used for cancer cell analysis to quantify total adherent cell biomass, capturing both proliferative capacity and treatment-induced detachment.

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Propidium Iodide (PI) Staining: HUVEC were exposed to CaCe-dEVs for 72 h. Following exposure, cells were harvested, washed with cold PBS, and incubated with propidium iodide (PI) solution according to the manufacturer's instructions. Samples were then immediately analyzed by flow cytometry (FACS), and the percentage of PI-positive cells was quantified as a measure of necrotic cell rate.

Tissue Factor Expression

The TF expression was analyzed by flow cytometry using the murine anti-human TF antibody conjugated to FITC (ref 4508CJ, Sekisui Diagnostics, London, UK) and by ELISA using the ZYMUPHEN™ MP-TF kits (Hyphen Biomed, Neuville-sur-Oise, France) according to the manufacturer's instructions.

Phosphatidylserine Expression

Phosphatidylserine (PS) expression was evaluated by flow cytometry using Annexin V conjugated to phycoerythrin (Sigma-Aldrich, St. Louis, MO, USA) and by ELISA using ZYMUPHEN MP Activity™ kits (Hyphen Biomed, Neuville-sur-Oise, France) according to the manufacturer's instructions.

Microscopy

Cell morphology was visualized using an IX83 Olympus inverted microscope with a LUCPLFLN 20× PH objective and an ORCA-Flash 4.0 LT camera (Hamamatsu Photonics, Hamamatsu, Japan). Images were acquired and analyzed using CellSens Dimension software (v1.16, Olympus, Hamburg, Germany).

Statistical Analysis

Data are presented as mean ± standard deviation (SD) from 6 independent experiments. Normality was confirmed, and comparisons were made using paired one-way ANOVA. A *p-value* < 0.05 was considered statistically significant. Analyses were conducted using SPSS (v21.0; IBM Corp., Armonk, NY, USA).

3. Results

CaCe-dEVs Induced Alterations in the Procoagulant State of Endothelial Cells

Expression of TF by endothelial cells exposed to CaCe-dEVs. Native HUVEC did not express any detectable levels of TF. Exposure of HUVEC to CaCe-dEVs induced significant TF expression. Upon treatment with BXPC3-dEVs, HUVEC exhibited markedly higher TF levels as compared to those treated with MCF7-dEVs (25 ± 2 pM vs. 0.9 ± 0.2 pM, respectively; $p<0.05$) (Figure 1A).

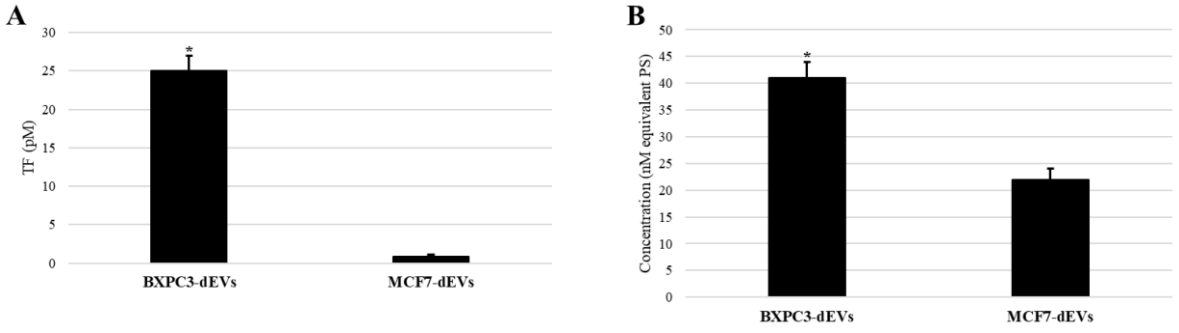

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Figure 1. Expression of Tissue factor (TF) (Frame A) and procoagulant phospholipid (Frame B) by HUVEC following exposure to CaCe-dEVs. Native HUVECs did not express detectable TF and expressed less than 5 nM PS. *p<0.05 Comparing HUVEC exposed to BXPC3-dEVs versus HUVEC exposed to MCF7-dEVs.

Expression of procoagulant phospholipids by endothelial cells exposed to CaCe-dEVs. Native HUVEC released less than 5 nM of EVs expressing phosphatidylserine (PS). Upon exposure to BXPC3- or MCF7-derived EVs, the concentration of PS-expressing EVs in the conditioned media increased to 41 ± 3 and 22 ± 2 nM, respectively, after 72 h (Figure 1B).

Procoagulant activity of endothelial cells exposed to CaCe-dEVs. Native HUVEC did not induce any detectable thrombin generation in normal PPP. The HUVEC exposed to BXPC3-dEVs or MCF7-dEVs triggered thrombin generation. The thrombin generation lag-time was significantly shorter in HUVEC exposed to BXPC3-dEVs compared with those exposed to MCF7-dEVs, whereas ETP and Peak did not differ significantly. Data are summarized in Figure 2, and Table 1 shows representative thrombin generation curves induced by endothelial cells exposed to BXPC3-dEVs and MCF7-dEVs.

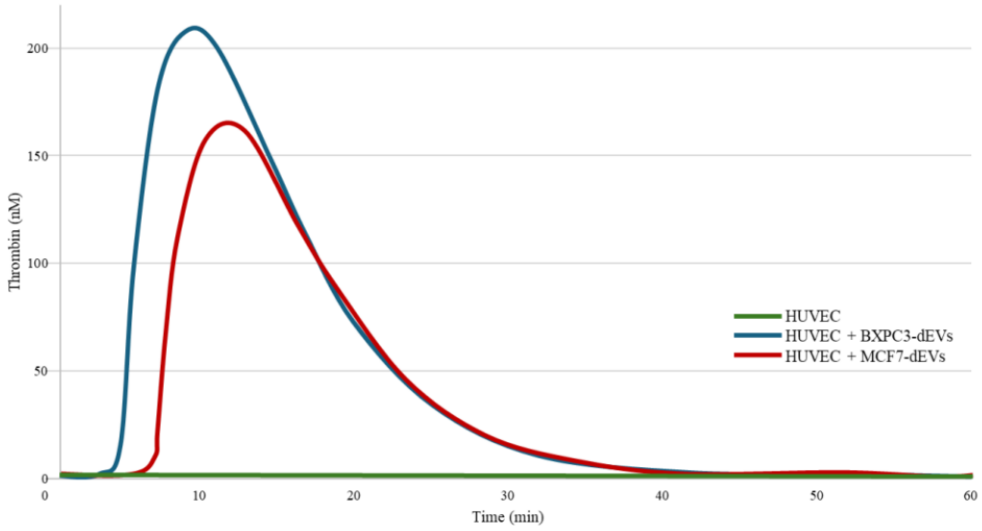

Figure 2. Representative thrombin generation curves measured by calibrated automated thrombography (Thrombinoscope) in normal human platelet-poor plasma in contact with HUVEC following exposure to BXPC3-dEVs or MCF7-dEVs. Parameters derived from the curves include lag-time, peak of thrombin, and endogenous thrombin potential. Data are representative of six independent experiments.

Table 1. Thrombin generation parameters measured by calibrated automated thrombography (Thrombinoscope) in normal human platelet-poor plasma in contact with HUVEC following exposure to BXPC3-dEVs or MCF7-dEVs. Parameters derived from the curves were lag-time, peak of thrombin, and endogenous thrombin potential (ETP). Results are presented as mean ± standard deviation from six independent experiments. Native HUVEC did not induce detectable thrombin generation within 40 min. *p<0.05 versus HUVEC + BXPC3-dEVs.

	Native HUVEC	HUVEC + BXPC3-dEVs	HUVEC + MCF7-dEVs
Lag-time (min)	> 40	4.0 ± 0.2	6.0 ± 0.1*
ETP (nM•min)	0	1603 ± 367	1476 ± 437
Peak (nM)	0	214 ± 95	167 ± 82

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CaCe-dEVs Induced Alterations in the Functional State of Endothelial Cells

Effect of CaCe-dEVs on the morphology and the survival of endothelial cells. The confluence of HUVEC exposed to CaCe-dEVs for 72 h was significantly lower (20%) than that of native HUVEC (90%; $p<0.05$). Native HUVEC exhibited significantly lower necrotic cell rates compared to those exposed to CaCe-dEVs, either BXPC3-dEVs or MCF7-dEVs (2.84% versus 7.21%, 5.12% respectively; $p<0.05$). Otherwise, no differences were observed in necrotic cell rates in HUVEC exposed to BXPC3-dEVs or MCF7-dEVs ($p>0.05$).

Phase-contrast microscopy showed clear morphological changes in HUVEC after exposure to CaCe-dEVs compared with native cells. Figure 3 depicts representative images of the morphological alterations of HUVEC induced by exposure to CaCe-dEVs. Native HUVEC (Figure 3, frame A) displayed the expected phenotype of quiescent endothelial cells with predominantly adherent, well-spread cells of elongated/spindle to polygonal morphology and a relatively homogeneous distribution across the field, with only rare rounded/refractile elements. Following exposure to BXPC3-dEVs (Figure 3, frame B), HUVEC largely remained adherent and elongated, but they appeared less uniform, with increased morphological heterogeneity, more refractile/rounded cells, and occasional small aggregates consistent with mild cellular stress or partial detachment in a subset of cells. Exposure to MCF7-dEVs (Figure 3, frame C) produced the most marked alterations, characterized by a higher frequency of rounded, bright/refractile elements and clusters, with evidence of reduced spreading and focal disruption of the adherent monolayer, suggesting greater impairment of cell adhesion and viability compared with both native HUVEC and BXPC3-dEVs–treated cells.

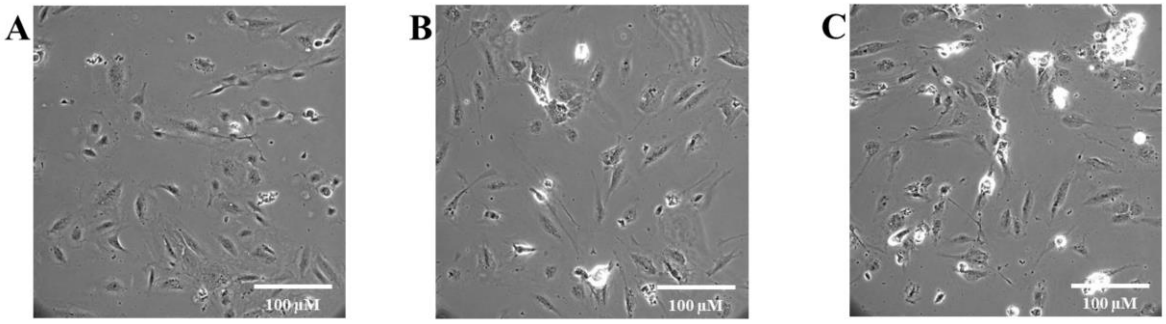

Figure 3. Morphological changes of HUVEC induced by exposure to CaCe-dEVs. (A) Native HUVEC; (B) HUVEC exposed to BXPC3-dEVs; (C) HUVEC exposed to MCF7-dEVs. Representative images from one of six independent experiments.

Effect of CaCe-dEVs on the proliferation of endothelial cells. Exposure of HUVEC to CaCe-dEVs significantly reduced cell proliferation. Preliminary experiments showed that, following incubation with 360 nM BXPC3-dEVs or MCF7-dEVs, the maximal reduction in HUVEC proliferation was reached after 24 h of exposure, indicating a plateau of the inhibitory effect. At 72 h, proliferation was reduced by 52 ± 5% and 37 ± 6% in the HUVEC exposed to BXPC3-dEVs and MCF7-dEVs, respectively ($p<0.05$).

Modulation of Cancer Cell–Driven Thrombin Generation by LMWHs, Apixaban, and Quercetin

In BXPC3 cells, enoxaparin (1 or 2 anti-Xa IU/mL) did not significantly modify lag-time, ETP, or Peak compared with untreated controls. In MCF7 cells, enoxaparin at 1 anti-Xa IU/mL had no significant effect, whereas 2 anti-Xa IU/mL significantly reduced ETP (−24%) and Peak (−36%).

Tinzaparin at 1 anti-Xa IU/mL did not significantly affect thrombin generation in either cell line. At 2 anti-Xa IU/mL, tinzaparin significantly prolonged the lag time by 1.1-fold in both BXPC3 and MCF7 cells. In BXPC3 cells, this was accompanied by a 19% reduction in Peak, while the 8% decrease

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in ETP did not reach statistical significance. In MCF7 cells, 2 anti-Xa IU/mL tinzaparin significantly reduced ETP (−22%) and Peak (−32%).

Apixaban (1 or 2 μg/mL) did not significantly modify thrombin generation parameters in BXPC3 cells. In MCF7 cells, 1 μg/mL had no effect, whereas 2 μg/mL significantly prolonged the lag time (1.2-fold) and reduced ETP (−23%) and Peak (−30%).

Quercetin at 166.5 μM did not significantly affect thrombin generation in either cell line. At 333 μM, quercetin significantly prolonged the lag time in BXPC3 (1.2-fold) and MCF7 cells (1.6-fold). In BXPC3 cells, this was associated with a significant 11% reduction in Peak, while the 8% decrease in ETP was not statistically significant. In MCF7 cells, the higher concentration significantly reduced both ETP (−31%) and Peak (−41%) (Table 2).

Table 2. Effect of pre-treatment of cancer cells with enoxaparin, tinzaparin, apixaban or quercetin on their ability to trigger thrombin generation in normal human platelet-poor plasma, assessed by calibrated automated thrombography (Thrombinoscope). Data are shown as mean ± standard deviation from six independent experiments. *Low concentrations:* enoxaparin and tinzaparin, 1 anti-Xa IU/mL; apixaban, 1 μg/mL; quercetin, 166.5 nM. *High concentrations*: enoxaparin and tinzaparin, 2 anti-Xa IU/mL; apixaban, 2 μg/mL; quercetin, 333 nM. **p*<0.05 versus control; $*p*<0.05 versus enoxaparin, tinzaparin, or apixaban.

BXPC3					
Low concentrations	**Control**	**Enoxaparin**	**Tinzaparin**	**Apixaban**	**Quercetin**
Lag-time (min)	1.93 ± 0.1	1.71 ± 0.0	1.99 ± 0.1	1.82 ± 0.1	2.27 ± 0.1
ETP (nM•min)	1009 ± 4.7	969 ± 28.1	1003 ± 29.5	976 ± 20.1	945 ± 37.5
Peak (nM)	119 ± 1.5	121 ± 1.71	120 ± 2.9	122 ± 1.8	108 ± 4.9
High concentrations	**Control**	**Enoxaparin**	**Tinzaparin**	**Apixaban**	**Quercetin**
Lag-time (min)	2.33 ± 0.2	2.06 ± 0.2	3.00 ± 0.5*	2.06 ± 0.1	2.72 ± 0.1*
ETP (nM•min)	1452 ± 50	1426 ± 90	1336 ± 50	1529 ± 100	1340 ± 50
Peak (nM)	161 ± 10	169 ± 6	131 ± 7*	177 ± 20	144 ± 6*
MCF7					
Low concentrations	**Control**	**Enoxaparin**	**Tinzaparin**	**Apixaban**	**Quercetin**
Lag-time (min)	11.49 ± 0.1	10.46 ± 1.52	10.9 ± 0.0	11.90 ± 1.43	12.16 ± 0.83
ETP (nM•min)	652.11 ± 47.4	674.22 ± 23.86	687.24 ± 9.41	671.81 ± 97.11	789.24 ± 38.11
Peak (nM)	41 ± 5	43.63 ± 6.64	50.40 ± 0.48	51.93 ± 16.63	67.21 ± 1.03
High concentrations	**Control**	**Enoxaparin**	**Tinzaparin**	**Apixaban**	**Quercetin**
Lag-time (min)	11.84 ± 0.5	12.78 ± 1.1	13.33 ± 0.5*	14.33 ± 1.2*	18.61 ± 1*
ETP (nM•min)	945 ± 0.5	717 ± 0.5*	735 ± 0.5*	729 ± 7*	649 ± 0.5*$
Peak (nM)	73 ± 7	47 ± 5*	50 ± 5*	51 ± 5*	43 ± 5*$

Impact of LMWHs, Apixaban, and Quercetin on Cancer Cell Viability

Tinzaparin significantly reduced cell viability in both cell lines. At 1 anti-Xa IU/mL, viability decreased by 37% in BXPC3 cells and 18% in MCF7 cells. Increasing the concentration to 2 anti-Xa IU/mL further reduced viability by 46% and 29%, respectively ($p<0.05$ vs untreated controls).

In contrast, neither apixaban (2 μg/mL) nor enoxaparin (2 anti-Xa IU/mL) significantly affected cell viability.

Quercetin (333 μM) significantly reduced viability by 55% in BXPC3 cells and 39% in MCF7 cells ($p<0.05$ vs untreated cells) (Figure 4). Kinetic analyses demonstrated that the maximal effects of tinzaparin and quercetin on cancer cell viability were observed after 72 h of exposure (data not shown).

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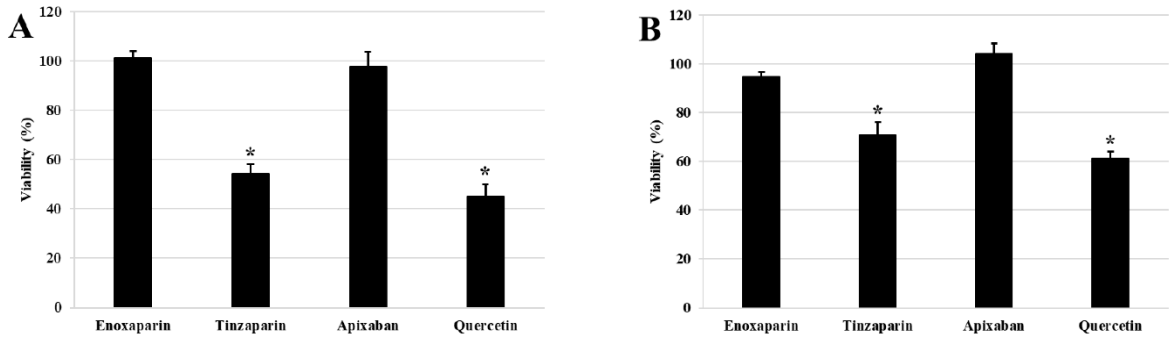

Figure 4. Cell viability after exposure to antithrombotic agents or quercetin. (A) BXPC3 and (B) MCF7 cell viability following 72 h incubation with enoxaparin or tinzaparin 2 anti-Xa IU/ml, apixaban (2 μg/ml), or quercetin (333 μM). Viability is expressed as a percentage of the untreated control, as described in Materials and Methods. Data are presented as mean ± SD from six independent experiments. *p<0.05 versus untreated control.

Effects of Antithrombotic Agents and Quercetin on HUVEC Morphology and Protection Against CaCe-dEVs–Induced Alterations

Treatment of native HUVEC with enoxaparin, tinzaparin, apixaban, or quercetin at the tested concentrations did not significantly affect cell viability compared with untreated controls. Morphological analysis revealed distinct patterns of endothelial remodeling. HUVEC treated with enoxaparin or tinzaparin displayed an elongated, spindle-shaped morphology with prominent cytoplasmic extensions and increased cell density relative to untreated cells (Figure 5, frames A–C). Apixaban-treated HUVEC exhibited a comparable elongated phenotype, although cytoplasmic projections were less pronounced (Figure 5, frame D). In contrast, quercetin-treated HUVEC appeared more rounded, with limited cytoplasmic extensions, closely resembling the morphology of untreated controls (Figure 5, frame E).

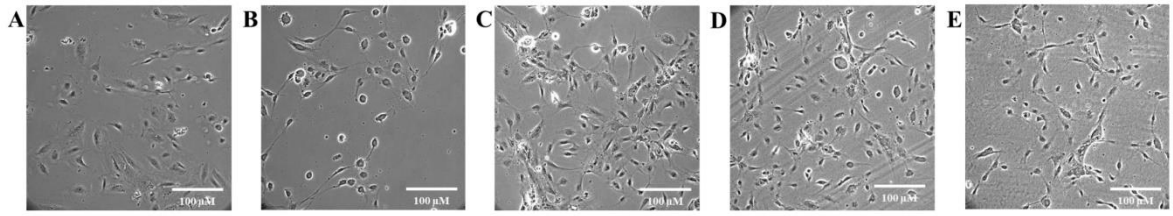

Figure 5. Morphological changes in HUVEC treated with enoxaparin or tinzaparin (2 anti-Xa IU/mL), apixaban (2 μg/mL), or quercetin (333 μM). (A) HUVEC native, (B) HUVEC treated with enoxaparin, (C) HUVEC treated with tinzaparin, (D) HUVEC treated with apixaban, (E) HUVEC treated with quercetin. Representative images from one of six independent experiments.

Pre-treatment with enoxaparin or tinzaparin (1 or 2 anti-Xa IU/mL) did not significantly attenuate the morphological alterations induced by subsequent exposure to BXPC3-dEVs or MCF7-dEVs. As shown in Figures 6 (frame B and C) and 7 (frame B and C), respectively, the morphology of pre-treated HUVEC was comparable to that of cells exposed to BXPC3-dEVs or MCF7-dEVs alone.

In contrast, HUVEC pre-treated with apixaban and then exposed to BXPC3-dEVs (Figure 6, frame D) or MCF7-dEVs (Figure 7, frame D) exhibited morphological alterations of lesser magnitude compared with cells treated with CaCe-dEVs alone, suggesting a partial protective effect.

HUVEC pre-treated with quercetin and subsequently exposed to BXPC3-dEVs (Figure 6, frame E) or MCF7-dEVs (Figure 7, frame E) displayed a mixed phenotype, combining features of quercetin exposure and CaCe-dEVs–induced injury. These cells showed marked elongation, increased cytoplasmic protrusions, and disrupted cell–cell contacts. Although morphological changes were

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slightly attenuated compared with CaCe-dEVs–treated cells, the overall protective effect of quercetin appeared limited.

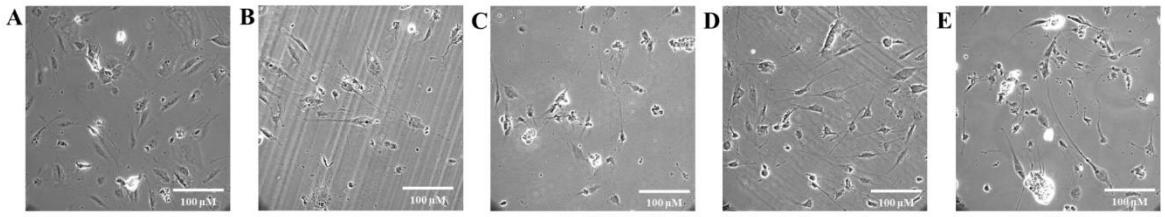

Figure 6. Morphological changes in HUVEC pre-treated with enoxaparin or tinzaparin (2 anti-Xa IU/mL), apixaban (2 μg/mL), or quercetin (333 μM) and then exposed to BXPC3-dEVs. (A) HUVEC native exposed to BXPC3-dEVs, (B) HUVEC pre-treated with enoxaparin and exposed to BXPC3-dEVs, (C) HUVEC pre-treated with tinzaparin and exposed to BXPC3-dEVs, (D) HUVEC pre-treated with apixaban and exposed to BXPC3-dEVs, (E) HUVEC pre-treated with quercetin and exposed to BXPC3-dEVs. Representative images from one of six independent experiments.

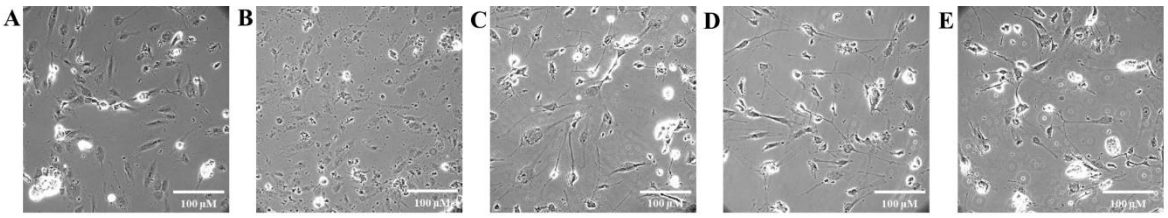

Figure 7. Morphological changes in HUVEC pre-treated with enoxaparin or tinzaparin (2 anti-Xa IU/mL), apixaban (2 μg/mL), or quercetin (333 μM) and then exposed to MCF7-dEVs. (A) HUVEC native exposed to MCF7-dEVs, (B) HUVEC pre-treated with enoxaparin and exposed to MCF7-dEVs, (C) HUVEC pre-treated with tinzaparin and exposed to MCF7-dEVs, (D) HUVEC pre-treated with apixaban and exposed to MCF7-dEVs, (E) HUVEC pre-treated with quercetin and exposed to MCF7-dEVs. Representative images from one of six independent experiments.

Effects of Enoxaparin, Tinzaparin, Apixaban, and Quercetin on HUVEC-Driven Thrombin Generation Following CaCe-dEVs Exposure

Exposure of native HUVEC to enoxaparin, tinzaparin, apixaban, or quercetin at the tested concentrations did not significantly modify thrombin generation compared with untreated controls.

Pre-treatment of HUVEC with apixaban (2 μg/ml) prior to exposure to BXPC3-dEVs significantly reduced the Peak and ETP. The lag-time showed only minor, non-significant changes.

Pre-treatment with quercetin (333 nM) before exposure to BXPC3-dEVs resulted in a significant prolongation of lag-time and a significant reduction in Peak and ETP compared with BXPC3-dEVs exposure alone.

In contrast, pre-treatment of HUVEC with apixaban or quercetin prior to exposure to MCF7-dEVs did not significantly alter lag-time, ETP, or Peak relative to MCF7-dEVs exposure alone (Table 3).

Table 3. Impact of HUVEC pre-treated with apixaban (2 μg/mL) or quercetin (333 μM) and exposed to BXPC3- or MCF7-dEVs on thrombin generation. Data are shown as mean ± standard deviation from six independent experiments. *$p<0.05$ versus HUVEC exposed to BXPC3-dEVs *$p<0.05$ versus HUVEC/BXPC3-dEVs *++p<0.05* versus native HUVEC.

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	Not exposed to CaCe-dEVs			Exposed to BXPC3-dEVs			Exposed to MCF7-dEVs		
	Native HUVEC	HUVEC + Apixaban	HUVEC + Quercetin	HUVEC	HUVEC + Apixaban	HUVEC + Quercetin	HUVEC	HUVEC + Apixaban	HUVEC + Quercetin
Lag-time (min)	11.5 ± 2.9	12 ± 1.2	13 ± 1.0	4.0 ± 0.2^{++}	5.0 ± 0.9	6.0 ± 1.0^{*++}	6.0 ± 0.1^{*++}	6.0 ± 0.5	6.5 ± 1.0
ETP (nM•min)	910 ± 150	940 ± 45	1002 ± 69	1603 ± 367^{++}	1250 ± 120	1011 ± 60^{++}	1476 ± 437^{++}	1360 ± 77	1250 ± 190
Peak (nM)	60.7 ± 15	71 ± 8	75 ± 10	214 ± 95^{++}	169 ± 6$^{\$}$	177 ± 5$^{\$}$	167 ± 82^{*++}	165 ± 50	159 ± 22

4. Discussion

This study focuses on the modulation of the procoagulant potential of cancer cells and the prevention of endothelial procoagulant transformation upon exposure to TF-expressing CaCe-dEVs. It provides original evidence to formulate a mechanistic approach to the interactions of LMWHs with the biology of cancer cells and endothelial cells. The data presented herein show that the procoagulant potential of cancer cells is decreased upon their exposure to the LMWH tinzaparin but not to enoxaparin. The specific direct FXa inhibitor apixaban induced a decrease in the mild procoagulant potential of the breast cancer cells MCF7 but not of the highly aggressive BXPC3. Quercetin, a flavonoid with metabolic activity, also decreased the procoagulant potential of both BXPC3 and MCF7 cells. Pretreatment of endothelial cells with apixaban or quercetin also partially prevented morphological changes induced by CaCe-dEVs, underscoring their potential to modulate cancer-associated hypercoagulability beyond inhibition of thrombin generation.

Exposure of endothelial cells to CaCe-dEVs induced TF expression, likely originating from the endothelial cells themselves, as previously shown [10]. Contact with TF-bearing vesicles may further enrich endothelial TF. Exposure also triggered morphological changes, reduced proliferation, and increased mortality of endothelial cells. These morphological changes of endothelial cells were associated with enhanced TF expression and amplification of thrombin generation. Compared with native endothelial cells, those adjacent to tumors exhibit structural and functional abnormalities [8,25,26], a phenotype reproduced in our study following CaCe-dEVs exposure. Surviving cells appeared elongated with extensive cytoplasmic extensions, suggesting enhanced motility and migration, resembling “cancer-associated endothelial cells” [8]. Tumor-associated endothelial cells are known to exhibit abnormal gene expression, chromosomal instability, aneuploidy, and cytogenetic abnormalities [27,28]. Further mechanistic studies are needed to clarify how membrane-level interactions with CaCe-dEVs trigger these alterations. Collectively, these findings identify CaCe-dEVs as key drivers of endothelial procoagulant transformation and warrant translational research to define biomarkers and therapeutic targets for monitoring endothelial dysfunction and thrombotic risk in cancer patients. This phenomenon likely constitutes an amplification loop of hypercoagulability, activating endothelial cells both locally within the tumor microenvironment and distantly.

In the second part of the study, we examined the effects of LMWHs, apixaban, and quercetin on cancer cell procoagulant activity and the activation of endothelial cells by CaCe-dEVs. Quercetin was included as a non-anticoagulant control with known metabolic and cytotoxic effects on cancer cells [29].

Exposure of cancer cells to enoxaparin, tinzaparin, and apixaban significantly reduced their capacity to initiate and amplify thrombin generation. Quercetin produced a similar effect. However, results varied between the two cell lines. The panel of anticoagulants exhibited differential inhibitory effects. While MCF7 cell procoagulant activity was susceptible to all agents (both LMWHs, apixaban, and quercetin), BXPC3 cell activity was only inhibited by tinzaparin and quercetin.

Tinzaparin and quercetin also reduced cancer cell viability, whereas enoxaparin and apixaban had no effect. These reductions were specific to cancer cells, as endothelial viability remained unaffected. The cytotoxic effects of tinzaparin and quercetin were more pronounced in pancreatic BXPC3 cells than in breast cancer MCF7 cells. Such variable effects likely depend on cancer cell heterogeneity, signaling pathways, and the mechanisms of action of anticoagulants. This variability in cytotoxic response may be partially explained by the dual pharmacological profile of quercetin.

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Quercetin does not function solely as an antioxidant; its activity is concentration-dependent [30,31]. At low concentrations, it acts as a reactive oxygen species (ROS) scavenger, reducing intracellular oxidative stress. Conversely, at the higher concentrations used in the present study, quercetin is known to exert a pro-oxidant effect [30,31]. This shift leads to a sharp increase in intracellular ROS, which can induce mitochondrial dysfunction and ultimately apoptosis—a mechanism frequently implicated in its anticancer properties [29,32]. We hypothesize that the concentration of quercetin utilized here exploits the already elevated basal ROS levels in cancer cells, pushing them over a critical threshold and triggering cell death via this pro-oxidant pathway.

The differential impact on HUVEC cells versus cancer cell lines is particularly noteworthy. While the same pro-oxidant mechanism may be at play in endothelial cells, their non-transformed nature equips them with more robust antioxidant defenses [33]. Upon exposure to quercetin, HUVEC cells may activate compensatory pathways, such as the NRF2-mediated stress response, to manage the oxidative load, reduce inflammation, and promote survival, possibly after a transient cell cycle arrest [33]. In cancer cells, one would anticipate a distinct sub-G1 population, indicative of apoptosis. In contrast, HUVEC cells could show a temporary arrest, likely in the G1 phase, followed by recovery. The observed morphological changes in HUVEC cells support this concept. However, this hypothesis warrants further investigation through detailed cell cycle analysis.

Based on this ensemble of our findings, we conclude that:

(a) LMWH-induced cytotoxicity does not represent a uniform class effect but rather reflects specific properties of individual agents.

(b) Cancer cell aggressiveness influences sensitivity, with BXPC3 cells being more affected than MCF7.

(c) Effects on survival are distinct from effects on procoagulant potential, indicating a lack of direct correlation.

(d) Inhibition of procoagulant activity by tinzaparin was comparable to that of quercetin.

(e) Variability in efficacy reflects the molecular characteristics of each agent more than their anticoagulant activity.

We then assessed whether pretreatment of endothelial cells with these agents could prevent CaCe-dEVs–induced procoagulant transformation. Neither LMWHs nor apixaban fully prevented deleterious morphological or proliferative changes. Previous studies indicate that tinzaparin can reduce endothelial adhesion and angiogenesis indirectly. [34,35] Under our experimental conditions, the most notable effects were observed with apixaban and quercetin. Microscopy analysis revealed slight protective changes, although these require confirmation using more sensitive imaging approaches. Apixaban partially mitigated the procoagulant shift induced by BXPC3-dEVs, but not by MCF7-dEVs. Quercetin produced a similar yet stronger protective effect. However, none of the treatments fully prevented CaCe-dEVs–induced endothelial dysfunction at the tested concentrations. The experiments were conducted in cancer cell cultures without clotting factors or natural inhibitors. This design allowed us to identify direct cellular effects of LMWHs and apixaban on cancer cells and endothelial cells. The ensemble of these observations leads to the conclusion that in the studied LMWHs, the chemical composition of the low-affinity material (LAM) for the antithrombin might play a determinant role in the modulation of cancer cell biology. The fact that the observed biological effects appearing at supratherapeutic levels underlines also the potential dissociation between the anticoagulant-specific and the multitargeted effect of the LMWHs. Based on these data, future pharmacological studies on a potential anticancer effect of heparins should focus on the low-affinity material for the antithrombin. Moreover, the rationality for the concentration of this LAM should not follow that of the clinically relevant anti-Xa activity.

Future studies should explore broader metabolic and cytoprotective mechanisms through which the LAM of LMWHs modulate the procoagulant potential of cancer cells and potentially protect endothelial cells from the deleterious effects of CaCe-dEVs.

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5. Conclusion

In conclusion, our study demonstrates that enoxaparin, tinzaparin, apixaban, and quercetin modulate the procoagulant potential of cancer cells and influence endothelial transformation induced by CaCe-dEVs. Tinzaparin and quercetin reduced tumor cell viability, suggesting an antineoplastic activity of tinzaparin beyond the specific AT-dependent inhibition of thrombin generation. Apixaban and quercetin provided partial protection against endothelial morphological and functional changes induced by exposure to CaCe-dEVs. Table 4 summarizes the ensemble of the biological effects of the LMWHs, apixaban, and quercetin on cancer cells and endothelial cells. Further mechanistic and translational studies are needed to confirm these effects and evaluate their clinical applicability.

Table 4. Summary of the effects of CaCe-dEVs and antithrombotic agents on endothelial and cancer cell functional parameters.

Parameter	Effect of CaCe-dEVs on HUVEC	Effect of Antithrombotic Agents on Cancer Cells (BXPC3 and MCF7)	Protective Effects on HUVEC Exposed to CaCe-dEVs
Procoagulant phenotype	Marked induction of tissue factor (TF) expression, increased release of phosphatidylserine (PS)-positive extracellular vesicles, and enhanced thrombin generation. BXPC3-dEVs induced stronger procoagulant responses than MCF7-dEVs.	Enoxaparin showed minimal effects, with reduction of ETP and peak thrombin only in MCF7 at 2 anti-Xa IU/mL. Tinzaparin (2 anti-Xa IU/mL) prolonged lag time and reduced peak thrombin in both cell lines. Apixaban (2 µg/mL) reduced thrombin generation parameters in MCF7 but not in BXPC3. Quercetin (333 µM) prolonged lag time and reduced peak thrombin in both cell lines, with a stronger effect in MCF7.	Pre-treatment with apixaban or quercetin partially attenuated BXPC3-dEV–induced thrombin generation (reduced peak and/or prolonged lag time). No significant protection was observed against MCF7-dEV–induced procoagulant changes.
Cell viability and proliferation	Significant reduction in proliferation (plateau at 24 h) and decreased cell viability; effects were more pronounced with BXPC3-dEVs.	Tinzaparin (2 anti-Xa IU/mL) and quercetin (333 µM) significantly reduced cancer cell viability. Enoxaparin and apixaban did not significantly affect viability.	No evidence of protection of endothelial viability or proliferation following CaCe-dEVs exposure.
Cell morphology	Disruption of endothelial monolayer integrity, reduced confluence, and increased rounded/refractile cells. Alterations were more pronounced following MCF7-dEVs exposure.	Not assessed.	Enoxaparin and tinzaparin did not prevent morphological alterations. Apixaban showed slight attenuation. Quercetin conferred limited protective effects, with a mixed morphological phenotype.

Summary of Key Findings

Parameter/ Condition	Effect of CaCe-dEVs on Endothelial Cells (HUVEC)	Effect of Antithrombotic Agents on Cancer Cells (BXPC3 & MCF7)	Protective Role on Endothelial Cells (HUVEC) Exposed to CaCe-dEVs
Procoagulant State	↑↑ **TF Expression:** Strong induction by CaCe-dEVs. BXPC3-dEVs induced much higher TF levels than MCF7-dEVs. ↑↑ **PS Exposure:**	**Variable Anticoagulant Effect:** • **Enoxaparin:** Minimal effect on BXPC3. Reduced ETP/peak in MCF7 only at 2 IU/mL. • **Tinzaparin:** Prolonged lag-time/reduced peak in both cell lines at 2 IU/mL. • **Apixaban:** No effect on BXPC3. Prolonged lag-time/reduced ETP/peak in MCF7 at 2 µg/mL. • **Quercetin:** Prolonged lag-time/reduced peak in both cell	**Partial Prevention:** • **Apixaban (Pre-treatment):** Significantly decreased the peak of thrombin generation in HUVEC exposed to BXPC3-dEVs. • **Quercetin (Pre-treatment):** Significantly prolonged lag-time and decreased peak in HUVEC exposed to BXPC3-dEVs. • Neither agent significantly

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	Increased release of PS-expressing EVs. BXPC3-dEVs had a stronger effect than MCF7-dEVs. ↑↑ **Thrombin Generation:** Induced thrombin generation. BXPC3-dEVs shortened the lag-time more than MCF7-dEVs.	lines at 333 µM. Stronger effect on MCF7.	protected HUVEC from the procoagulant effects of MCF7-dEVs.
Cell Viability & Proliferation	↓↓ **Cell Viability:** Increased necrotic cell rates (BXPC3-dEVs > MCF7-dEVs). ↓↓ **Cell Proliferation:** Significantly reduced proliferation in a time-dependent manner (max. effect by 24 h).	**Cytotoxic Effects:** • **Tinzaparin (2 IU/mL):** Significantly reduced viability (BXPC3: -46%; MCF7: -29%). • **Quercetin (333 µM):** Significantly reduced viability (BXPC3: -55%; MCF7: -39%). • **Enoxaparin & Apixaban:** No significant effect on cancer cell viability.	**Not Assessed / No Protection:** The text does not describe experiments measuring whether these agents protect endothelial cell viability or proliferation from CaCe-dEVs.
Cell Morphology	**Disrupted Morphology:** Reduced confluence, increased rounded/refractile cells, loss of uniform monolayer. MCF7-dEVs caused more marked alterations than BXPC3-dEVs.	**Not Assessed on Cancer Cells:** The text describes the effect of drugs on cancer cell viability, but not on their morphology.	**Limited Prevention:** • **Enoxaparin & Tinzaparin:** Failed to prevent morphological changes. • **Apixaban:** Showed a slight reduction in morphological changes. • **Quercetin:** Showed a limited protective effect, with cells displaying mixed characteristics.

Simplified Summary of Effects

	Effect of CaCe-dEVs ***(What the cancer vesicles do)***	**Effect of LMWHs** ***(Enoxaparin & Tinzaparin)***	**Effect of Apixaban**	**Effect of Quercetin**

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On Cancer Cells	N/A *(These come from cancer cells)*	Enoxaparin does not kill cancer cells. Tinzaparin do.	Does not kill cancer cells.	Kills cancer cells strongly.
On Cancer Cell Clotting	N/A	Reduces the clotting ability of cancer cells (especially Tinzaparin).	Reduces clotting ability (only in MCF7 breast cancer cells).	Reduces the clotting ability of cancer cells.
On Healthy Vessel Cells	Damages them: • Makes them clot. • Changes their shape. • Kills them.	Fails to protect the shape of the vessel cells.	Slightly protects the shape. Reduces clotting caused by pancreatic (BXPC3) vesicles.	Limited protection of shape. Reduces clotting caused by pancreatic (BXPC3) vesicles.

Author Contributions: **M.A.B:** Conceptualization, data curation, formal analysis, methodology, software, validation, visualization, writing – original draft, and writing – review & editing. **P.H.F.C.L.C:** Data interpretation and writing – review & editing. **E.M:** Formal analysis and methodology. **A.R:** Formal analysis and resources. **P.K:** Data interpretation, review & editing **A.A.S:** Critical review & editing. **V.B:** Critical review & editing. **M.S:** Protocol conceptualization and formal analysis. **J.F:** Data interpretation and contribution to protocol development. **P.V.D:** Protocol conceptualization, funding acquisition, data curation, formal analysis, methodology, project administration, supervision, validation, writing – original draft, and writing – review & editing. **V.T:** Review and interpretation of the results. **E.L:** Supervision, formal analysis, and writing – review & editing. **G.G:** Protocol conceptualization, funding acquisition, data curation, formal analysis, methodology, project administration, supervision, validation, writing – original draft, and writing – review & editing.

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