Evaluation of a 3D osteosarcoma model: Experimental studies and mathematical modelling of drug response

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Abstract

Osteosarcoma remains a significant clinical challenge, with poor outcomes in metastatic cases and slow progress in the development of novel therapies. In this work, a previously developed 3D osteosarcoma model, comprising bone-mimicking macroporous alginate scaffolds with hydroxyapatite particles and a perfusion bioreactor, was evaluated as a tool for anticancer drug testing. Murine osteosarcoma cells (K7M2-wt) were seeded onto the scaffolds (15×10^6 cells *per* cm^3) and cultured statically for 1 day. In two experimental series, scaffolds were then exposed to doxorubicin (1 μg cm^{-3}) under perfusion for 1 or 3 days (40 μm s^{-1} superficial velocity), modelling post-resection conditions with predominantly individual or loosely aggregated cells. In two additional series, the same treatments were applied after 7 days of perfusion, when compact spheroids had formed, mimicking established tumours. Histological analysis and MTT assay were used to assess cell morphology and metabolic activity. Short-term treatment had negligible effects, while prolonged exposure significantly reduced metabolic activity of individual cells, but spheroid-like structures exhibited increased doxorubicin resistance. To rationalize the results from the first two series, a mathematical model was developed describing drug transport, intracellular accumulation, cell proliferation, and cell death. The model captured the initial treatment phase, but overestimated the effect of prolonged treatment, suggesting additional resistance mechanisms. Overall, the results demonstrate the potential of this 3D model for anticancer drug evaluation.

Keywords: K7M2-wt cell line; perfusion bioreactor; alginate scaffold; doxorubicin; anticancer drug testing; spheroids.

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1. INTRODUCTION

During past decades, the incidence of malignant bone tumours has shown a slight increase [1]. This is also the case for osteosarcoma, a primary malignant bone tumour that most commonly affects adolescents and young adults during the period of pubertal growth but also occurs in older patients [2] with an overall annual incidence of approximately 3.4 cases *per* million people [3]. The current way of treating osteosarcoma includes neoadjuvant chemotherapy followed by surgical resection of primary tumour and all clinically detected metastases. After surgery, adjuvant chemotherapy is given to eliminate any remaining cancer cells, which may persist and contribute to relapse and poor clinical outcomes. Chemotherapy is administered in multiple cycles with chemotherapeutic drugs given over one or several consecutive days, followed by a recovery period before the next cycle [3]. The drugs currently in use have remained largely unchanged for 30 to 40 years and attempts to optimize standard therapy over the past 20 years have not yielded significant improvements [4]. Therefore, there is a strong need for the development of new therapies, particularly for patients diagnosed with metastases and those experiencing relapse. However, the validation of new drugs is a time-consuming process, and many potential drug candidates fail in clinical trials, often due to the limitations of experimental

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preclinical models [5]. Among these, animal testing and cell cultures grown in a monolayer (two-dimensional (2D) cell cultures) are still widely used in preclinical research and can provide valuable insights into drug response. However, animal testing can be very costly, raises ethical concerns, and the results are not always reproducible or reliably translatable to humans [6]. In contrast, 2D cell cultures enable rapid, simple, and highly reproducible testing; however, they do not faithfully represent the complex *in vivo* environment in which osteosarcoma develops and progresses. Three-dimensional (3D) osteosarcoma cell culture models offer an additional level of complexity and can more closely mimic the *in vivo* tumour environment, including cell-cell and cell–microenvironment interactions, thereby enabling a more comprehensive evaluation of anticancer drug efficacy [7]. For example, 3D multicellular tumour spheroids, which are aggregates formed by cancer cells, represent a relatively simple model in which cells secrete extracellular matrix (ECM) and exhibit behaviour similar to that observed *in vivo* [8]. They can be cultured under static conditions or, for improved mass transport, in microfluidic devices and biomimetic bioreactors [9]. Furthermore, to better mimic the tissue ECM, spheroids can be directly encapsulated within biomaterial scaffolds, or individual cells can be seeded onto scaffolds and allowed to subsequently form aggregates. Scaffolds for osteosarcoma models aim to imitate the structure and composition of bone tissue ECM, which consists of an organic phase (predominantly collagen) and a mineral, inorganic phase (predominantly hydroxyapatite). Accordingly, composite scaffolds combining polymers and mineral particles offer an attractive approach for 3D cell cultures, as demonstrated by alginate-calcium phosphate scaffolds, which supported osteosarcoma cell growth [10]. In addition to having a suitable composition, scaffolds are typically highly porous with interconnected pores of at least 100 μm in diameter, to enable effective osteosarcoma cell adhesion and efficient nutrient transport, thereby supporting cell survival and function [11-13]. Mass transport within such scaffolds can be enhanced using perfusion bioreactors that provide also relevant hydrodynamic shear stresses [14,15]. It has been shown that perfusion can efficiently mimic functional features observed *in vivo*, promote cell aggregation and spheroid formation, and thereby contribute to the development of a more physiologically relevant tumour model, supporting reliable testing of anticancer compounds [16,17].

3D models are also powerful tools for quantitatively analysing experimental results through the mathematical modelling, particularly in relating anticancer drug transport and cellular responses [18]. A comprehensive understanding of pharmacokinetic and pharmacodynamic processes is crucial for linking drug exposure to these responses [19]. However, establishing a quantitative relationship remains challenging, as cytotoxic effects often occur with a delay and involve complex intracellular cascades that are difficult to capture with simple models [20,21]. To address these challenges, various modelling approaches have been proposed. Some models assume that cell death is directly related to extracellular drug concentration, while others relate it to the area under the concentration-time curve (AUC) or use a Hill-type dependence [19]. In contrast, Eliaz and coworkers assumed that the cytotoxic potency of a drug is a function of the intracellular drug amount in a critical cellular compartment, rather than the drug concentration in the surrounding medium. In this model, cell death begins after a lag phase, as observed experimentally, and the total number of viable cells at any given time is determined by the balance between cell proliferation and drug-induced cell death [22]. However, these studies were performed in cell monolayers, while relatively few studies focus on more relevant 3D systems integrating drug transport, intracellular accumulation, and cellular response.

The aim of the present study was to investigate the potential of a previously developed 3D osteosarcoma cell culture model based on the use of macroporous composite alginate hydrogel with mineral particles as a cell carrier in a perfusion bioreactor, for assessing the effects of anticancer drugs [10]. In the present work, hydroxyapatite particles were used as a mineral phase, while doxorubicin was selected as a model drug. Specifically, the drug was applied both to scaffolds immediately after cell seeding and to cultured cell-seeded scaffolds after spheroid-like structures had formed. This approach allowed us to evaluate the effects of doxorubicin on individual and loosely aggregated cells simulating the post-surgical scenario with residual cancer cells, as well as on spheroid-like structures formed during perfusion culture, representing neoadjuvant chemotherapy conditions. Four experimental series were performed, in which doxorubicin was used at a concentration and treatment regime relevant for clinical application (1- and 3-day exposure). Finally, to rationalize the experimental results, an attempt was made to develop and apply a mathematical model describing doxorubicin transport, intracellular accumulation, and cell proliferation and death within the perfused scaffolds based on the model proposed in literature [22].

1. MATERIALS AND METHODS

2. 1. Materials

For the preparation of macroporous scaffolds, low-viscosity sodium alginate (A3249) was purchased from AppliChem (Germany), hydroxyapatite (HAp; particle size 2.5 μm, surface area ≥ 80 m^2 g^{-1}) and calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) were obtained from Sigma-Aldrich (USA), isopropanol was purchased from Zorka Pharma (Serbia), and disodium ethylenediaminetetraacetate dihydrate (Na_2-EDTA$\cdot 2H_2O$) was supplied by HiMedia Laboratories Pvt. Ltd. (India). High glucose Dulbecco's Modified Eagle's medium with L-glutamine (DMEM) was obtained from Biological Industries (Israel), fetal bovine serum (FBS) from Gibco (Brazil) and penicillin/streptomycin solution from Sigma-Aldrich (Israel). Phosphate-buffered saline (PBS), trypan blue solution and trypsin-EDTA solution were obtained from Sigma-Aldrich (USA) and doxorubicin from Ebewe (Austria). MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-dyphenyl tetrazolium bromide) was purchased from Sigma (USA), dimethyl sulfoxide (DMSO) from Fisher Scientific (UK), formaldehyde solution (35–37%) from NRK Inženjering (Serbia) and hematoxylin-eosin (H&E) staining solution from BioGnost (Croatia).

2. 2. Cell culture

Murine osteosarcoma cells K7M2-wt (ATCC® CRL-2836™, USA) were cultured in T-flasks in complete culture medium (cDMEM), containing DMEM, 10% fetal bovine serum and 1% penicillin-streptomycin solution under standard conditions (37 °C, 5% CO_2 in a humidified atmosphere). When the confluence of about 80-90% was reached, the cells were detached by using trypsin /EDTA solution. Briefly, the attached cells in T-flasks were washed twice with 5 cm^3 PBS and treated with 1 cm^3 of trypsin/EDTA solution for 5 min at 37 °C, which was then neutralized with cDMEM. The resulting cell suspension was collected, transferred to a centrifuge tube, and centrifuged at 1200 rpm for 5 min to obtain a cell pellet. The pellet was then resuspended and used for cell counting and preparation of the cell suspension for seeding.

2.3. Production of macroporous composite alginate scaffolds

Macroporous composite alginate scaffolds were produced according to a previously established procedure [23]. Hydroxyapatite powder (HAp) was dispersed in a 0.33 wt.% $CaCl_2 \cdot 2H_2O$ solution using an ultrasonic probe (750 W, 30 s). The obtained dispersion (10 wt.% HAp; 0.30 wt.% $CaCl_2 \cdot 2H_2O$) was mixed with 2.5 wt.% Na-alginate solution under intensive stirring to avoid early gelation, resulting in a final mixture containing 2 wt.% Na-alginate, 2 wt.% HAp, and 0.06 wt.% $CaCl_2 \cdot 2H_2O$. The mixture was poured into a previously activated dialysis tubing (2.5 cm in diameter, 15 cm in length), activated by boiling in 10 mM Na_2-EDTA$\cdot 2H_2O$ for 30 min and subsequently immersed in 3.97 wt.% $CaCl_2 \cdot 2H_2O$ solution under slow stirring for 24 h. Following replacement with a fresh solution, incubation continued for another 24 h to ensure complete gelation. The obtained composite cylinders were then rinsed with distilled water, dried, placed in a freezing container filled with isopropanol and frozen overnight at a controlled rate down to −70 °C. The frozen samples were lyophilized (−70 °C, 0.011 mbar, 24 h) in a freeze-dryer (Beta 2-8 LDplus, Martin Christ Gefriertrocknungsanlagen GmbH, Germany) and then sterilized by UV irradiation for 30 min.

2. 4. Cell seeding

Prior to cell seeding, the obtained dry composite cylinders were immersed in complete cell culture medium and incubated for 1 h. After incubation, the cylinders were cut into disc-shaped scaffolds approximately 4.5 mm thick, which were then placed in 24 well plates, covered with 125 mm^3 of complete culture medium, each, and left in an incubator for 3 h. After the rehydration, the dimensions of each scaffold were measured using a vernier caliper. The final scaffolds had an average diameter of 9.0 ± 0.3 mm and average thickness of 4.4 ± 0.4 mm (determined for n = 18). For cell seeding, each scaffold was placed in a well of non-adherent 12-well plates and the cell suspension (167×10^6 cells *per* cm^3) was poured onto the upper scaffold surface (42.6 ± 5.4 mm^3), so to obtain a nominal cell density of 15×10^6 cells *per* cm^3 of scaffold volume. The plates were then placed in the incubator for 1 h, after which 2.5 cm^3 of complete medium was added to each well to fully submerge the scaffolds. The scaffolds were then incubated at 37 °C in a humidified atmosphere containing 5 % CO_2 and cultured for 24 h to ensure initial cell adhesion and adjustment to the scaffolds.

Cell seeding efficiency (*E*) was calculated as the difference between the initial number of cells in the suspension and the number remaining in the medium 24 h after seeding, divided by the initial cell number. Cell numbers were determined by the trypan blue exclusion method using a hemocytometer and an inverted optical microscope Olympus CKX53 (Olympus, Japan). It should be noted that this calculation assumes negligible cell proliferation in the medium or on the well bottom during the initial 24 h of static culture, which may not be entirely accurate. Therefore, the actual seeding efficiencies may be somewhat higher than the calculated values.

2. 5. Perfusion bioreactor culture and doxorubicin treatment

In all experiments, following the initial 24 h static culture, perfusion cultures were established in "3D Perfuse" perfusion bioreactor systems (Innovation Center of the Faculty of Technology and Metallurgy, University of Belgrade, Serbia) (Figure 1a). In brief, each cell-seeded scaffold was placed in a separate bioreactor chamber formed by a piece of silicone tubing (12 mm in diameter, 30 mm in length) and each bioreactor chamber was then connected to a separate recirculation loop including a medium reservoir, silicone tubing for medium recirculation and gas exchange, as well as two syringes for medium exchange. Each system was filled with 15 cm³ of cDMEM and placed in a humidified incubator (37 °C, 5% CO_2). In all experiments, the systems were continuously perfused by a multichannel peristaltic pump (Shenzhen, China) at a flow rate of 0.27 cm³ min^{-1} corresponding to the superficial velocity of 40 μm s^{-1}. Four experimental series were performed in which doxorubicin was added to the medium in the reservoir at a concentration of 1 μg cm^{-3}. In the first two experimental series the treatment started immediately after the systems were established and lasted for one and three days, respectively, in order to assess the drug effects on individual and loosely aggregated osteosarcoma cells. In the remaining studies, the cell-seeded scaffolds were cultured under continuous medium perfusion for 7 days to allow for the spontaneous formation of spheroid-like structures, after which they were treated with doxorubicin for one and three days. In the case of the three-day treatments, the treatment medium was completely replaced daily with a fresh dose of doxorubicin added to the reservoir. In all experiments, untreated perfused cultures served as controls. For each experimental group, at least two scaffolds (and in some cases three) were used in each experiment. All experiments involving three-day treatments of individual and loosely aggregated cells, and spheroid-like cultures were independently repeated twice.

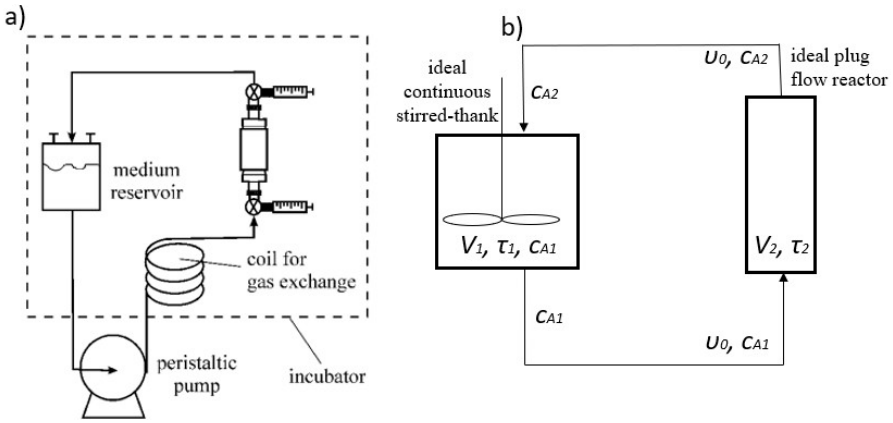

Figure 1. *Perfusion bioreactor system consisting of a bioreactor chamber, tubing for medium recirculation, a medium reservoir, and a peristaltic pump that provides continuous medium flow: a) schematic presentation of the experimental system (modified with permission from [24]); b) model of the system consisting of a plug flow reactor with volume V_2 and residence time τ_2, and an ideally stirred vessel with volume V_1 and residence time τ_1; υ_0 represents the volumetric flow rate through the system, while c_{A1} and c_{A2} are the doxorubicin concentrations in the vessel (and at the reactor inlet) and at the reactor outlet, respectively*

2. 6. Analytical methods

2. 6. 1. MTT assay

The MTT assay was used before and after bioreactor cultures to assess the cell metabolic activity within the scaffolds. An MTT stock solution (5 mg cm^{-3} in PBS) was diluted with cDMEM to a final concentration of 0.5 mg cm^{-3}. One scaffold or a half of a scaffold was covered with 2 cm³ of MTT solution (0.5 mg cm^{-3}) and incubated for 3.5 h at 37 °C. After

incubation, the scaffolds were cut in small pieces, and the formed formazan crystals were dissolved in 4 to 7 cm³ DMSO using an optimized sequential procedure (sequential additions of 1 to 1.5 cm³ every 20 min). After each extraction, the DMSO was collected separately and replaced with fresh DMSO; finally, the absorbance was measured in the combined collected solutions at 570 nm using a UV-1800 spectrophotometer (Shimadzu, Japan), with DMSO as the blank.

2. 6. 2. Histological analyses

After removal from the bioreactor chambers, the scaffolds (either entire or halves) were immersed in 4 % pH-neutral buffered formaldehyde (10 % formalin) for at least 24 h followed by dehydration through a graded series of ethanol solutions (70, 96 and 100 %), clearing in xylene, and embedding in paraplast (Sakura Tissue-Tek TEC, CA, USA). The embedded scaffolds were sectioned using a microtome (Leica Reinhart, Austria; Leica SM 2000 R, Germany) to obtain 5 µm-thick cross-sections, which were then stained with H&E [25]. Formed spheroid-like structures were analysed by scanning the cross-sections using an Olympus DP70 camera connected to a light microscope (Olympus BX51, Japan) and evaluated for the number as well as the size and shape using AperioImageScope (Leica Biosystems Imaging, Inc., USA, https://www.leicabiosystems.com/digital-pathology/manage/aperio-imagescope/) and ImageJ software (https://imagej.net/ij/) respectively.

2. 6. 3. Statistical analysis

Data were analysed using one-way ANOVA, followed by Welch's two-sample t-tests for comparisons between groups (two-tailed) using Microsoft Excel software (Microsoft Corp., USA). All quantitative data are expressed as a mean ± standard deviation. Statistical significance is accepted as p <0.05.

2. 7. Mathematical modelling of doxorubicin transport and effects on cells

A mathematical model of doxorubicin transport and accumulation in cells coupled with cell proliferation and death was derived with the aim to quantify the effect of doxorubicin treatment on individual cells in the 3D osteosarcoma cell culture model. The case of a 3-day doxorubicin treatment was modelled, in which the medium was replaced, and a fresh dose of doxorubicin was added at the beginning of each day. The experimental recirculation system (Figure 1a) was modelled as a plug flow reactor connected with an ideally stirred vessel (Figure 1b). During each pass through the scaffold, represented by the reactor, doxorubicin is taken up by the cells, resulting in a decrease in drug concentration in the medium. The medium then enters the vessel, where perfect mixing is assumed, before returning to the scaffold.

The scaffold is represented as a plug flow reactor with a volume corresponding to the void volume of the scaffold pores. The doxorubicin concentration (c_A) decreases along the reactor length due to cellular accumulation at a rate ($-r_A$), yielding Equation (1):

$$-\mathrm{d}c_A = -r_A \, d\tau_2 \tag{1}$$

where τ_2 is the reactor residence time. Accumulation of doxorubicin in cells was assumed to follow the first-order kinetics, Equation (2) based on the experimental data in literature [22]:

$$-\frac{\mathrm{d}n_A}{\mathrm{d}t} = kc_A N_{cell} \tag{2}$$

where n_A is the total intracellular amount of doxorubicin accumulated in all cells, k is the uptake rate constant and N_{cell} is the total number of cells. Dividing Equation (2) by the volume of the plug flow reactor (V_2) yields an expression for the accumulation rate ($-r_A$), which can be then substituted into Equation (1) to provide a differential Equation (3):

$$-\frac{\mathrm{d}c_A}{c_A} = \frac{kN_{cell}}{V_2}\mathrm{d}\,\tau_2 \tag{3}$$

Integration of Equation (3) provides the change in drug concentration during a single pass through the scaffold, Equation (4):

$$c_{A2} = c_{A1} e^{\frac{-kN_{cell}\tau_2}{V_2}} \tag{4}$$

where c_{A1} and c_{A2} represent the doxorubicin concentration in the medium at the reactor inlet and the outlet, respectively.

In the reservoir, the medium is perfectly mixed, resulting in a governing equation for the change in doxorubicin concentration, Equation (5):

$$\left(c_{A2}\text{-}c_{A2}\right)=\frac{\mathrm{d}c_{A1}}{\mathrm{d}\tau}\tau_1 \tag{5}$$

where τ_1 is the residence time of medium in the reservoir (including the tubing volume). The total number of viable cells in the scaffold depends on time (τ) and is determined by the balance between the rates of cell proliferation and cell death induced by doxorubicin accumulation described by differential Equation (6) [22]:

$$\frac{\mathrm{d}N_{\mathrm{cell}}}{N_{\mathrm{cell}}}=\left(k_s\text{-}k_u nA_{\mathrm{Ai}}\right)\mathrm{d}\tau \tag{6}$$

where k_s is the rate constant of cell proliferation, k_u is the rate constant of cell death, and n_{Ai} is the intracellular amount of doxorubicin per cell. However, according to literature [22], a lag time of 3 hours was assumed, during which the cells proliferate and the intracellular amount of doxorubicin increases without any apparent effect. Thus, during this period, Equation (6) reduces to Equation (7):

$$\frac{\mathrm{d}N_{\mathrm{cell}}}{N_{\mathrm{cell}}}=k_s\mathrm{d}\tau \tag{7}$$

Change in the total intracellular amount of doxorubicin accumulated in all cells within a scaffold (n_{A}) over time is calculated from the mass balance of doxorubicin in the reactor, as given by Equation (8):

$$\left(c_{A1}\text{-}c_{A2}\right)\nu_0=\frac{\mathrm{d}n_{\mathrm{A}}}{\mathrm{d}\tau} \tag{8}$$

where ν_0 is the medium flow rate. The amount of drug per cell is then obtained by dividing this value by the total number of viable cells in the scaffold and adding it to the previously accumulated intracellular amount ($n_{\mathrm{Ai,prev}}$), as described by Equation (9):

$$n_{\mathrm{Ai}}=\frac{\mathrm{d}n_{\mathrm{A}}}{N_{\mathrm{cell}}}+n_{\mathrm{Ai,prev}} \tag{9}$$

The resulting system of ordinary differential equations was solved numerically in Python using the Runge-Kutta method [26]. The procedure started by solving Equation (5) for one pass through the reactor, lasting τ_2. The amount of doxorubicin consumed during that pass was calculated by Equation (8) and the resulting internal doxorubicin amount per cell was determined by Equation (9). The number of viable cells was then calculated using Equation (7) for the first 3 hours, and Equation (6) thereafter. Finally, the new doxorubicin concentration c_{A1} after passing through the reservoir was calculated using Equation (5) and the entire calculation process was repeated for each subsequent pass through the reactor (*i.e.,* scaffold).

3. RESULTS

In this study, a previously developed 3D osteosarcoma cell culture model based on macroporous composite alginate/HAp scaffolds and perfusion bioreactor, was evaluated for anticancer drug testing with doxorubicin selected as a representative drug. Similarly, to the previous study [10], osteosarcoma cells were successfully seeded onto the scaffolds achieving a high seeding efficiency of about 86±8 %. As previously reported, after 1 day of static culture, individual cells and loose aggregates could be observed, which further formed denser, spheroid-like structures over 7 days of cultivation under medium perfusion. This establishes a robust model system for studying the effects of doxorubicin on both individual cancer cells and more mature, tumour-like aggregates. Accordingly, four experimental series were performed with doxorubicin applied at a concentration of 1 μg cm $^{-3}$, a dosage commonly used in studies of its effects on osteosarcoma cells [27-28]. The treatment regimens, lasting either 1 or 3 days, were inspired by clinically relevant protocols. In the first two experimental series, doxorubicin was applied immediately after establishing the bioreactor culture (*i.e.* one day after cell seeding and static culture) to examine its effects on individual cells. In the third and fourth series, the treatment began after 7 days of bioreactor culture to assess the effects on spheroid-like structures.

3. 1. Effects on individual and loosely aggregated cells

In the first two experimental series doxorubicin was applied for 1 and 3 days upon the set-up of perfusion cultures, respectively, and the resulting cultured scaffolds were examined for cell metabolic activity by the MTT assay and morphological appearance by histological analyses. Untreated cultures served as controls in both cases. The MTT assay showed that the cell metabolic activity in the initial scaffolds (n = 7) in both experimental series was similar, as the absorbance values of the dissolved formazan solutions were not statistically different; therefore, these initial control values are presented as a single average. However, absorbance values from cultured scaffolds were normalized to their respective initial scaffolds (Figure 2).

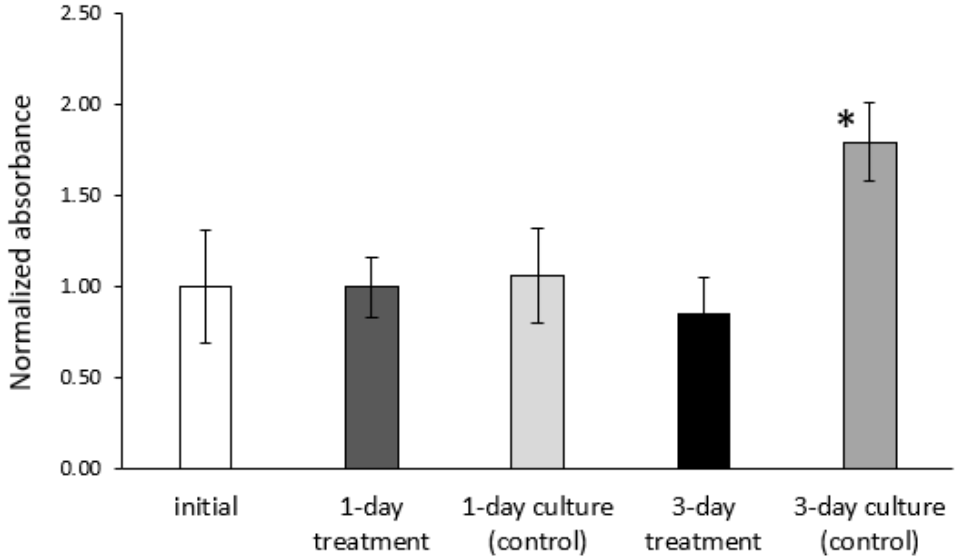

Figure 2. *Absorbance values normalized to the corresponding values determined for initial scaffolds cultured statically for 24 h after seeding, shown for initial scaffolds (n = 7), scaffolds treated with doxorubicin for one day (n = 3), scaffolds treated with doxorubicin for three days (n = 5), and respective untreated control scaffolds (n = 2 and n = 4, respectively) (*indicates a statistically significant difference between the 3-day control group and each of the other groups: 3-day treatment, 1-day treatment, and initial scaffolds)*

The results shown in Figure 2 demonstrate that the 1-day treatment did not induce any adverse effect on the cell metabolic activity. In contrast, following the 3-day treatment, the metabolic activity remained similar to that of the initial scaffolds, while the untreated control exhibited approximately a twofold increase in metabolic activity over the 3-day perfusion culture.

Figures 3 and 4 show H&E-stained histological cross-sections of doxorubicin-treated scaffolds for 1 and 3 days, respectively, and corresponding untreated controls. Individual cells (indicated by black stars) and loose cell aggregates (indicated by black arrows) are clearly visible within the pores of control scaffolds as well as scaffolds treated with doxorubicin for one day. The alginate matrix appears light purple, while hydroxyapatite particles are seen as dark-purple spots. Histological analysis was consistent with the results obtained by the MTT assay. In specific, when comparing the controls after 1 and 3 days of perfusion culture, apparently a higher number of denser aggregates, along with individual cells, were observed in the latter culture (Figs 3b and 4b, respectively). A 1-day doxorubicin treatment did not induce any visible effect, as the histological cross-sections of treated and control scaffolds appeared similar (Figure 3). In contrast, after 3 days of treatment, individual cells could rarely be observed, with mostly relatively dense aggregates remaining (indicated by black triangles) (Figure 4a). These results demonstrate that the 3-day treatment was effective against cells directly exposed to the drug, whereas cells within compact spheroid structures were less affected, likely due to the protective effects of three-dimensional cellular organization and possibly reduced drug diffusion into the spheroid interior.

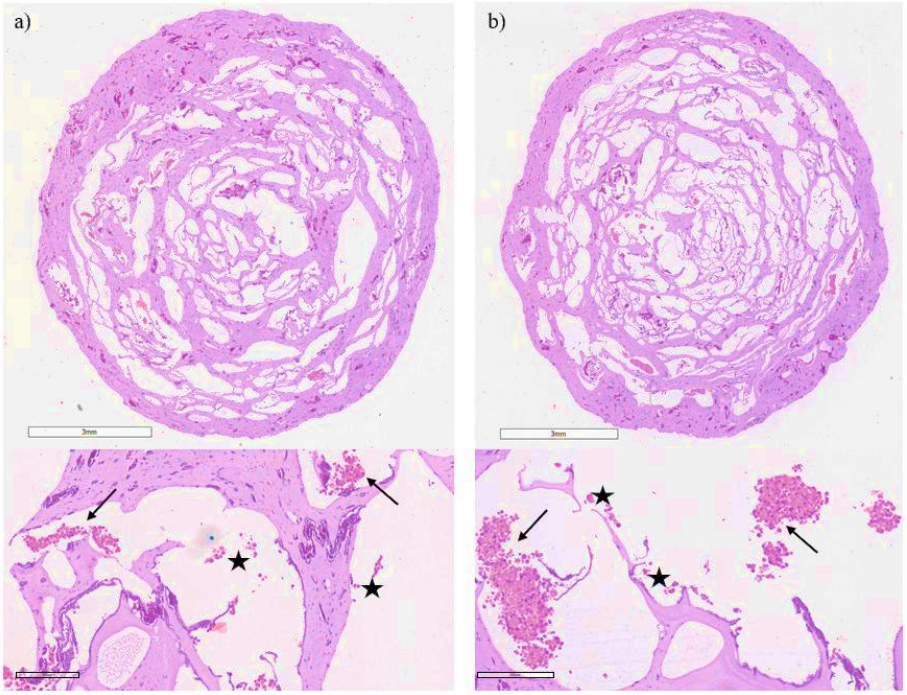

Figure 3. *Histological cross-sections of scaffolds after 1-day perfusion culture: a) treated with doxorubicin; b) control (untreated); black stars indicate individual cells, while arrows mark regions with loosely aggregated cells (upper panels: entire cross-sections: scale bar = 3 mm; lower panels: representative regions at higher magnification, scale bar = 200 µm; H&E stain)*

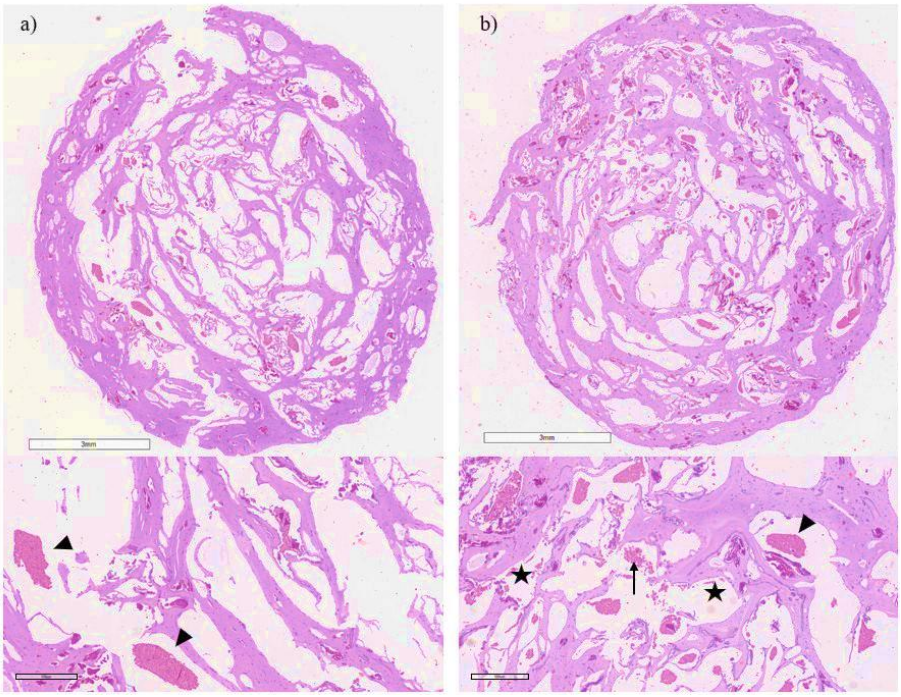

Figure 4. Histological cross-sections of scaffolds after 3-day perfusion culture: a) treated with doxorubicin; b) control (untreated); triangles indicate spheroid-like structures, black stars indicate individual cells, and arrows mark regions with loosely aggregated cells (upper panels: entire cross-sections: scale bar=3 mm; lower panels: representative regions at higher magnification, scale bar=500 µm; H&E stain)

3. 2. Effects on spheroid-like structures

The third and fourth experimental series aimed to evaluate the effectiveness of 1- and 3-day doxorubicin treatments (1 µg cm^{-3}), respectively, on compact spheroid structures formed after 7 days of perfusion culture. The 1-day treatment

did not affect either the metabolic activity or the morphological appearance of the cell spheroids, as shown by the MTT assay and histological analysis of all samples (Supplementary material, Figures S1 and S2, respectively). These findings indicate that this exposure time was insufficient to inhibit cell activity within spheroid structures similarly to the results of 1-day treatment of the initial scaffolds.

In contrast, the 3-day treatment produced some effects on the cells, as indicated by the MTT assay, which showed a slightly lower, though not statistically significant, metabolic activity in the treated samples compared to the untreated controls (Figure 5). High standard deviations in all results should be noted.

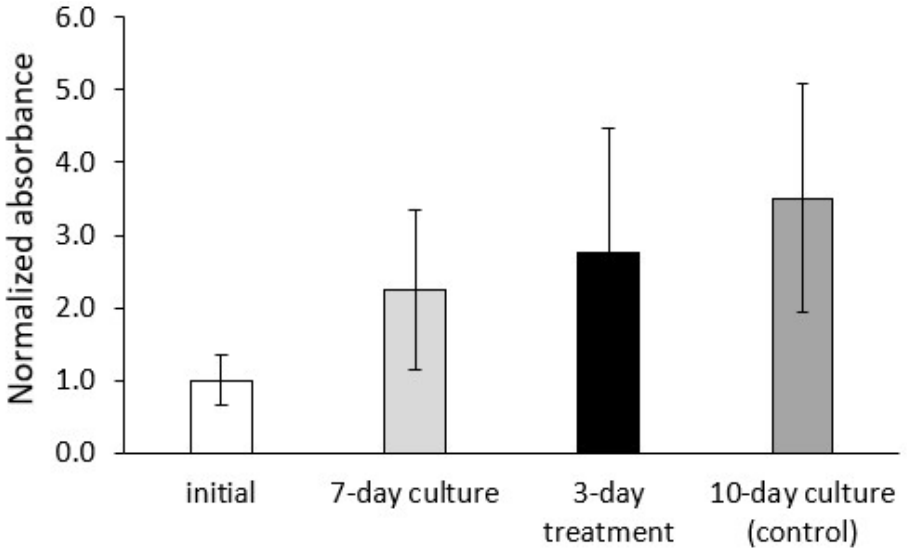

Figure 5. *Absorbance values normalized to the initial scaffolds cultured statically for 24 h after seeding, shown for: initial scaffolds (n = 3), scaffolds after 7 days of perfusion (n = 4), scaffolds treated with doxorubicin for 3 days following 7 days of perfusion (n = 5), and control scaffolds cultured under perfusion for 10 days (n = 4)*

Histological cross-sections of scaffolds before the doxorubicin treatment (*i.e.* after one day of static culture followed by 7-day perfusion, Figure 6a) show only compact spheroid-like aggregates within the scaffolds. This indicates that, during this cultivation period, the loosely aggregated and individual cells, clearly visible in control samples at earlier time points (Figures 3 and 4) underwent self-organization and extracellular matrix production, resulting in formation of dense spheroid-like structures.

Viable, compact spheroid-like structures with apparently the same morphology are also visible in scaffolds that underwent a 3-day doxorubicin treatment, as well as in the controls cultured for 10 days under perfusion in total (Figures 6b and 6c, respectively). However, due to the random distribution of spheroids and the variability introduced by random sectioning planes, it was not feasible to perform thorough statistical analyses of spheroid number and size across different groups within the scope of this study.

3. 3. Mathematical modelling of doxorubicin transport and effects on cells

To quantify the effects observed in the 3-day doxorubicin treatment of initial scaffolds a mathematical model was derived to describe the doxorubicin transport within the recirculating perfusion bioreactor system, intracellular drug accumulation, and the resulting change in the cell number over time. Model parameters were adopted based on the values obtained from the present experiments and data taken from literature (Table 1). In specific, the initial doxorubicin concentration used in the model corresponded to that applied experimentally (c_A = 1.84 μmol dm^{-3}). The initial number of cells (N_{cell}) was estimated as the average seeding value multiplied by the average seeding efficiency, as determined experimentally, resulting in 3,648,375 cells per scaffold. The plug flow reactor volume (V_2) was calculated as the total pore volume in the scaffold, based on a measured porosity of approximately 40 % from scaffold cross-sections. The reservoir volume V_1 corresponding to the medium volume in the system loop was calculated as a difference between the total medium volume added to the system and the scaffold pore volume.

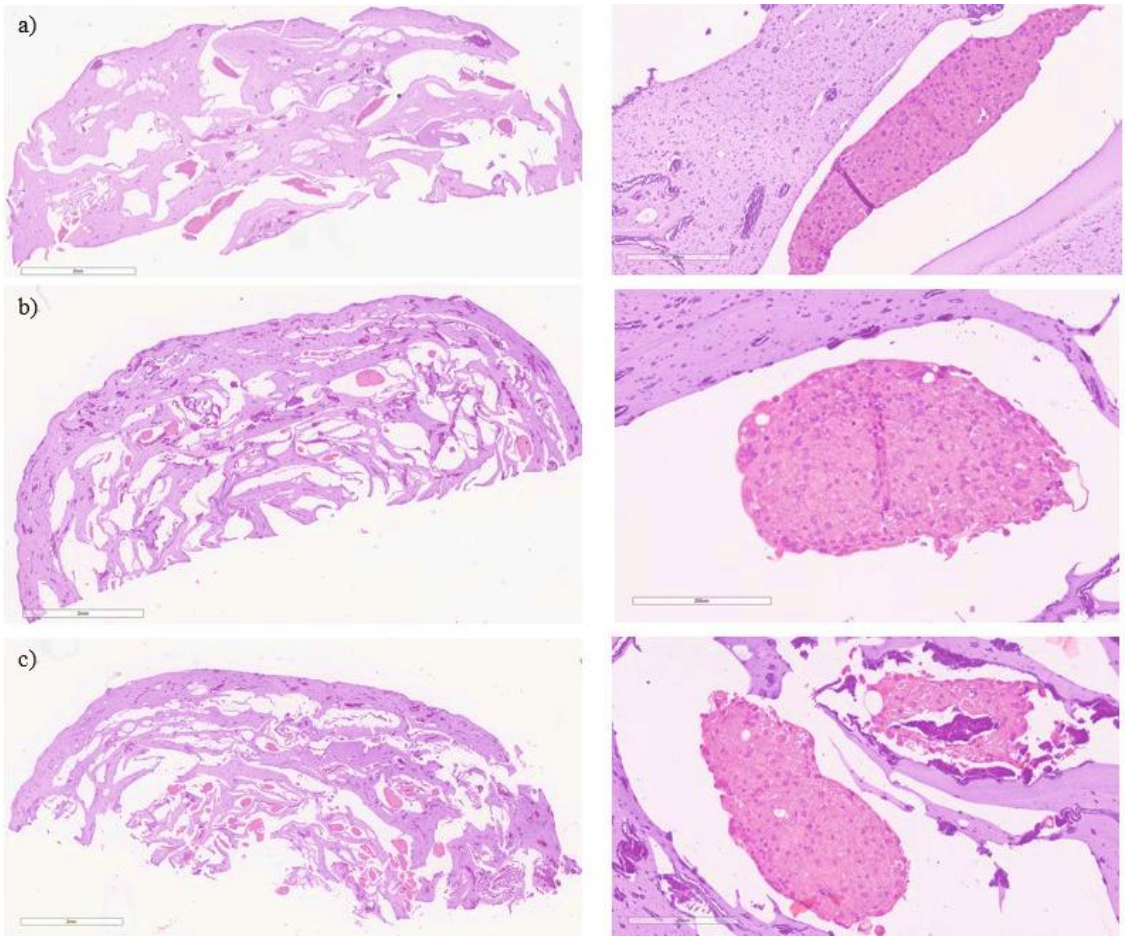

Figure 6. *Histological cross-sections of scaffolds: a) cultured under bioreactor conditions for 7 days; b) cultured under bioreactor conditions for 7 days and then treated with doxorubicin for three days; (c) control (untreated) scaffold cultured under bioreactor conditions for a total of 10 days (left panels: half cross-sections, scale bar = 3 mm; right panels: representative regions at higher magnification, scale bar = 200 μm; H&E stain)*

The medium flow rate was 0.27 $cm^3\ min^{-1}$. The remaining model parameters were derived or adopted from literature data. The uptake rate constant k was calculated using Equation (3) based on experimental data reported in literature [22] for B16F10 melanoma cells treated with doxorubicin within a concentration range that includes the value used in the present study, 1.84 μmol dm^{-3} (Supplementary material, Figure S3). The cell death rate constant, k_u, was adopted from literature as 1.04×10^{11} $(mol\ min)^{-1}$ [22]. The cell proliferation rate constant, k_s, was determined 3.73×10^{-4} min^{-1} from the cell doubling time provided in the ATCC product datasheet for the K7M2-wt cell line.

Table 1. *Parameters used in the mathematical model*

Parameter	Value
N_{cell}	3,648,375
c_A / μmol dm^{-3}	1.84
V_1 /cm^3	14.887
V_2 / cm^3	0.11286
ν_0 / $cm^3\ min^{-1}$	0.27
τ_1 /min	55.14
τ_2 / min	0.42
k / $dm^3\ min^{-1}\ cell^{-1}$	3.68×10^{-12}
k_u / $mol^{-1}\ min^{-1}$	1.04×10^{11}
k_s / min^{-1}	3.73×10^{-4}

The system of ordinary differential equation was solved as described in section 2.7. with time intervals equal to the bioreactor residence time τ_2 = 0.42 min. The simulation provides time-dependent profiles of drug concentration,

intracellular drug accumulation, and changes in the cell number over time. During the first 24 h of treatment the model predicts a gradual decline in doxorubicin concentration in the medium due to continuous accumulation of the drug in the cells during repeated passes through the scaffold (Figure 7a). At the same time, the intracellular amount of doxorubicin per cell increases, reaching 1.1 fmol *per* cell after 180 min (Figure 7b), which is comparable to the 1.2 fmol reported by Eliaz *et al.* [22] for the same initial timeframe. According to the authors and as adopted in the model, only cell proliferation occurs during this time, resulting in a gradual increase in cell number. After this period, drug induced cell death is included in the model; however, the overall cell population remains nearly constant during the first day of exposure, with the model predicting an overall increase in the viable cell number of only 7 % (Figure 7c). This result is consistent with the experimental MTT analysis results, which showed negligible differences in metabolic activity before and after the treatment in the first experimental series (Figure 2).

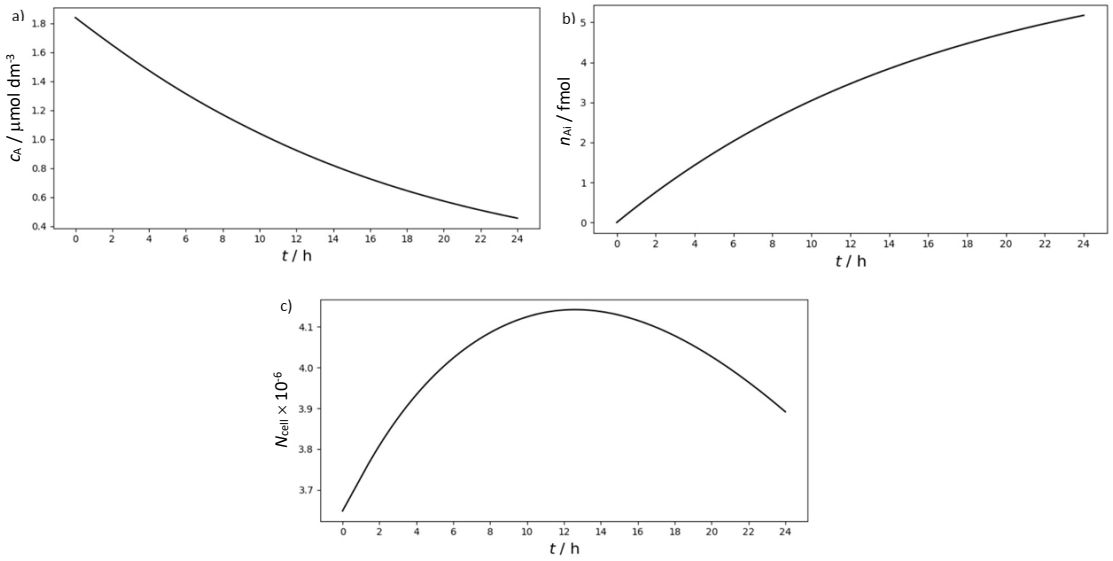

Figure 7. *Predicted profiles of: a) drug concentration in the medium, b) intracellular drug accumulation and c) viable cell number during the first day of the doxorubicin treatment*

On the second day, the medium is replaced and a fresh dose of doxorubicin is added, and this procedure is then repeated on the third day. Doxorubicin concentration profiles for all 3 days are shown in Figure 8.

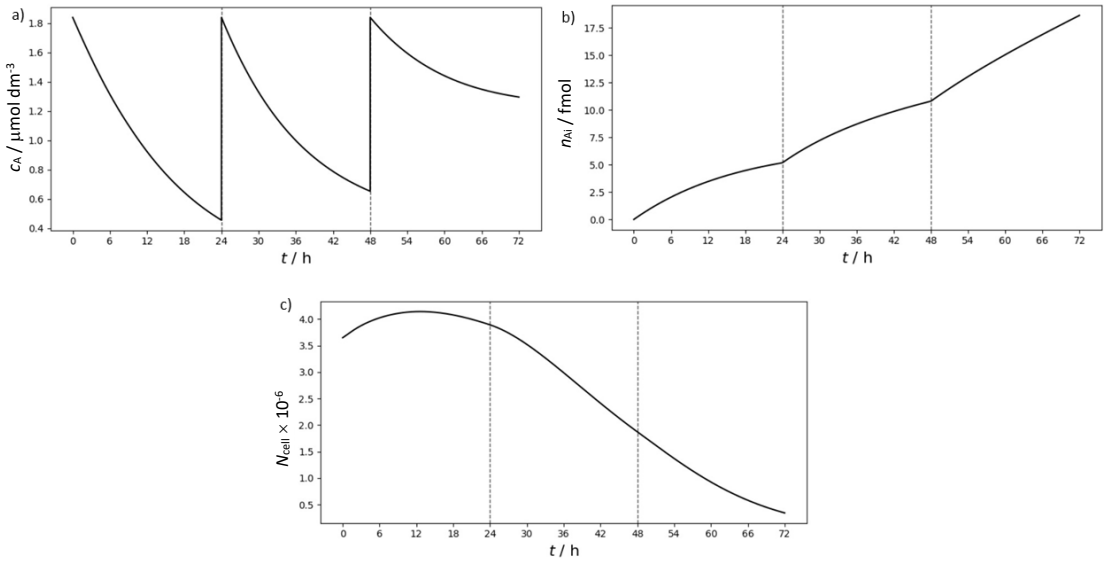

Figure 8. *Predicted profiles of: a) drug concentration in the medium, b) intracellular drug accumulation and c) viable cell number during the 3 days of doxorubicin treatment*

Each subsequent dose of doxorubicin is distributed among fewer cells, whereas the intracellular amount of accumulated doxorubicin continues to increase. Consequently, the cell death rate increases, resulting in a progressively faster decline in the cell population. By the end of the third day, the model predicts approximately 3.4×10^5 remaining cells or approximately 9 % of the initial cell population. However, this prediction is not consistent with the experimental results. The MTT assay demonstrated only an approximately 15 % decrease in metabolic activity after the 3-day treatment (Figure 2), suggesting that the model overestimated the effect of doxorubicin. Histological cross-sections also confirmed the presence of viable cells after the treatment although mostly in dense aggregates (Figure 4a).

4. DISCUSSION

The present study builds on a previously developed 3D osteosarcoma cell culture model based on macroporous composite alginate scaffolds with mineral particles and a biomimetic perfusion bioreactor, with the modification that the mineral component in this work consists only of hydroxyapatite [10]. As previously confirmed, this model provides a suitable environment for bone cancer cell cultivation without negative effects on the cells, supporting cell growth and proliferation under bone tissue-like conditions. Individual and loosely aggregated cells observed after one day of initial static culture, progressively formed more compact, spheroid-like structures during 7 days of cultivation under continuous medium flow (0.27 cm^3 min^{-1}; superficial velocity of 40 µm s^{-1}) (Figure 6). This structural evolution enables the establishment of a robust model system for studying the effects of anticancer drugs on both individual cancer cells and more mature, spheroid-like structures. Doxorubicin was selected as a representative chemotherapeutic and applied at 1 µg cm^{-3} for one and three days. The results showed that the 1-day treatment was not effective for either individual and loosely aggregated cells or compact spheroid-like structures (Figures 2 and S1). The observed lack of effect is consistent with previous studies on 3D tumour spheroids, including U2OS osteosarcoma spheroids, which showed that metabolic activity did not significantly change across all tested doxorubicin concentrations (10, 20, 30 and 40 µM) compared to untreated controls [8]. In those studies, significant inhibition was observed only after 3 to 5 days of exposure**.** In comparison, the concentration used in the present study (~1.84 µM) is substantially lower, which may contribute to the negligible effect observed after one day of treatment.

A 3-day drug treatment, inspired by clinically relevant exposure protocols [3] was effective in the case of individual and loosely aggregated cells, as confirmed by the MTT assay (Figure 2), which showed reduced metabolic activity in treated samples. In addition, histological analysis revealed that individual cells were rarely observed, with mostly relatively dense aggregates remaining (Figure 4). The same treatment applied to compact spheroid-like structures formed during preceding seven days of bioreactor cultivation did not induce any significant effect although treated samples showed a slight trend toward reduced metabolic activity and a slight increase in the fraction of smaller spheroids (Figure 5). Similar findings were reported in a study using a 3D perfused tumour spheroid model, where 5-fluorouracil treatment (1 µg cm^{-3}) for 48 and 96 h did not significantly reduce the cell number or tumour volume. In contrast, in 2D cultures, cell number decreased by about 50 % after 48 or 96 h of exposure [16]. Also, in osteosarcoma models using the MG-63 bone cancer cell line cultured under perfusion conditions, liposomal doxorubicin at concentrations equivalent to the 2D IC_{50} (~0.1 µg cm^{-3}) exhibited minimal cytotoxic effects, even after prolonged exposure. Cell viability remained at 100 % after 3 days of treatment and was still ~79 % after 7 days, in contrast to both 2D cultures (~55 and ~13 %, respectively) and static 3D models (~72 and ~27 %, respectively) [29]. These results demonstrate increased drug resistance in 3D systems, particularly under perfusion, compared to 2D monolayer cultures where the cells are directly exposed to the drug. Also, these results, together with those obtained in the present work, highlight the time-dependent effect of drug exposure, where the delayed cytotoxic effect may be attributed to the time required for intracellular drug accumulation, as well as to limited drug penetration into spheroid structures [8]. In compact spheroid structures, localized regions with reduced drug transport efficiency may exist, and the outer cell layers may consume doxorubicin before it can reach all the cells, preventing the drug from being lethal to the entire cell population. In addition, cells located within the spheroid interior may exhibit reduced proliferative and metabolic activity, which could further decrease their sensitivity to doxorubicin [30].

To further understand the experimental results and provide a quantitative basis for analyzing drug response, we developed a mathematical model adapted from literature [22] to describe doxorubicin transport, intracellular accumulation, and changes in cell number during the 3-day treatment of individual and loosely aggregated cells in the recirculating perfusion bioreactor system. The model proposed by Eliaz and coworkers [22], grounded in their experimental findings, assumes that the cytotoxic effect of doxorubicin is governed by the intracellular drug amount rather than the extracellular concentration, with the viable cell number determined by the balance between the cell proliferation rate and the drug-induced cell death rate after a lag phase [22].

Our model adequately described the first day of treatment when the cell distribution is relatively homogeneous and spheroid-like structures are not yet developed. The model included the delayed cytotoxic effect as a fixed lag phase, as observed experimentally, followed by a balance between cell proliferation and death rates. This resulted in apparently similar predicted viable cell number as that in the initially seeded samples, which was consistent with experimentally assessed same metabolic activity in these two sample groups. Thus, the model grasped the effect of a short-term drug exposure of individual cells. Still, it should be noted that a delayed cytotoxic effect can be incorporated into the model using approaches other than a fixed lag phase. For example, a transit compartment model incorporates a series of intermediate steps that allow for a more realistic description of the delay between drug exposure and effect, which may provide greater flexibility for modelling long-term drug responses [31].

During the subsequent 2 days of treatment, intracellular doxorubicin accumulation continued to increase following the daily replacement of the medium and addition of a fresh doxorubicin dose. The number of viable cells progressively decreased, and although the model does not explicitly include a saturation effect or a predefined survival plateau, unlike Hill-type or peak concentration models [19], it captured the persistence of viable cells after 3 days of treatment. However, the model predicted a substantially stronger treatment effect than that indicated by the experimental MTT results, which may be explained by the fact that model does not capture emergence of additional drug resistance mechanisms, such as possible drug efflux, intracellular redistribution, saturation of drug uptake or other adaptive cellular responses, making it challenging to establish a quantitative relationship between drug exposure and cellular response [20,21]. As a result, intracellular drug accumulation continuously increases in all viable cells equally during repeated dosing, leading to an overestimation of drug induced cell death. Still, the applied model offers a possibility to directly incorporate biological constraints, such as a maximum drug capacity per cell or intrinsic resistance in certain cell populations, to more accurately reflect observed survival plateaus. At the same time, the experimentally observed formation of spheroid-like structures, in which cells were better protected from drug exposure, would require an additional model term to account for the dynamics of drug diffusion and accumulation within these aggregates. These improvements would allow a more realistic description of doxorubicin accumulation and cytotoxicity in long-term perfusion cultures and could improve the prediction of viable cell numbers after prolonged treatments.

5. CONCLUSION

The present work successfully utilized a previously developed 3D osteosarcoma cell culture model based on macroporous composite alginate scaffolds and biomimetic perfusion bioreactor for studies of anticancer drug effects. Specifically, it was shown that the 3D model can be used to simulate both a post-surgery situation with residual cancer cells and neoadjuvant chemotherapy in a simplified model of a solid tumour. Doxorubicin applied at a concentration of 1 μg cm^{-3} for 1 and 3 days was shown to induce a delayed cytotoxic effect on individual cells, while it had minor effects on cell-aggregates and spheroid-like structures. Only after the 3-day exposure, individual cells were rarely observed. The mathematical model, incorporating doxorubicin transport, intracellular accumulation, and cell proliferation and death within the perfused scaffolds, adequately described the limited response observed after 1 day of treatment but overestimated the effect of the 3-day treatment. This work represents one of the rare attempts to adapt and utilize a mathematical model for describing drug effects in a 3D cell culture. However, the model did not account for the formation of cell aggregates, which may have protected cells from the drug. Incorporating this aspect could be an important direction for future model development.

Overall, the 3D osteosarcoma cell culture model proved to be a robust platform for further studies of anticancer drug effects, while the developed mathematical model provides a foundation for future enhancements, including the incorporation of more complex cellular resistance mechanisms and terms describing drug diffusion and accumulation within spheroids.

SUPPLEMENTARY MATERIAL

Additional data are available electronically at https://www.ache-pub.org.rs/index.php/HemInd/article/view/1733, or from the corresponding author on request.

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Evaluacija 3D modela osteosarkoma: eksperimentalna studija i matematičko modelovanje odgovora na lek

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(Naučni rad)

Izvod

Osteosarkom i dalje predstavlja značajan klinički izazov, sa lošim ishodima u slučaju metastaza i sporim napretkom u razvoju novih terapija. U ovom radu je prethodno razvijen 3D model osteosarkoma, koji se sastoji od makroporoznih alginatnih nosača sa česticama hidroksiapatita, koji oponašaju koštano tkivo, i protočnog bioreaktora, procenjen kao alat za ispitivanje antitumorskih lekova. Ćelija mišjeg osteosarkoma (K7M2-wt) zasejane su na nosače (15×10^6 ćelija po cm^3) i gajene jedan dan u statičnim uslovima. U dve eksperimentalne serije nosači su potom izloženi doksorubicinu (1 μg cm^{-3}) u protočnim uslovima tokom 1 ili 3 dana (površinska brzina 40 μm s^{-1}), čime su simulirani uslovi nakon resekcije tumora, sa pretežno pojedinačnim ili slabo agregiranim ćelijama. U dve dodatne serije isti tretmani primenjeni su nakon 7 dana protočne kulture, kada su se formirali kompaktni sferoidi, čime je simuliran razvijen tumor. Histološka analiza i MTT test korišćeni su za procenu morfologije ćelija i njihove metaboličke aktivnosti. Kratkotrajni tretman imao je zanemarljiv efekat, dok je produžena izloženost značajno smanjila metaboličku aktivnost pojedinačnih ćelija, dok su sferoidne strukture pokazale povećanu otpornost na doksorubicin. Radi objašnjenja rezultata dobijenih u prve dve eksperimentalne serije, razvijen je matematički model koji opisuje transport leka, intracelularnu akumulaciju, proliferaciju ćelija i njihovo odumiranje. Model je uspešno opisao početnu fazu tretmana, ali je precenio efekat produžene izloženosti, ukazujući na mogućnost postojanja dodatnih mehanizama rezistencije. Sveukupno, rezultati potvrđuju potencijal ovog 3D modela za ispitivanje antitumorskih lekova.

Ključne reči: K7M2-wt ćelijska linija; protočni bioreaktor; alginatni nosač; doksorubicin; testiranje antitumorskih lekova; sferoidi