Locally invasive tumors in cats are challenging to treat because of the proximity of some tumors to structurally important anatomy. Feline injection site-associated sarcomas (FISAS) behave more aggressively than naturally occurring sarcomas of nonspecific etiology and are characterized by invasive and rapid growth into surrounding tissues. Although distant metastasis affects 10% to 28% of cats, local recurrence is the most common cause of mortality in 14% to 45% of cats following complete excision and up to 70% of cats following incomplete excision. Radical excision offers the best prognosis but may be difficult to achieve given anatomic constraints and advanced stage disease at presentation. Adjuvant radiation therapy decreases recurrence and prolongs remission; however, most cats succumb to recurrent disease despite treatment. Feline oral squamous cell carcinomas (FOSCC) account for 60% to 70% of feline malignant oral tumors and are invasive of surrounding soft tissue and bone. Although FOSCC metastasizes to regional lymph nodes in up to 35% of cases, most cats die from local disease before developing clinically detectable metastasis. Treatment of FOSCC is rarely curative, and recurrence rates of 38-80% have been observed within months of aggressive surgery.
Local therapies have been attempted, but median survival times range from 2 to 4 months with a 1-year survival rate of less than 10%.5-9 Since both FISAS and FOSCC lack a clear fascial border and often invade extensively into surrounding tissues, local disease is the primary source of significant morbidity and cause for euthanasia.2,4,8 To improve quality of life and extend survival, an effective local treatment for these tumors is needed to prevent tumor recurrence that is also unlikely to adversely affect normal tissues or cause systemic toxicosis.

Carboplatin is a platinum-containing chemotherapeutic agent that has shown some efficacy when given IV to tumor-bearing cats.10-12 Although local delivery of platinum-based chemotherapeutic agents has been investigated to enhance regional control of tumors and decrease toxicity associated with systemic administration, little is known regarding tissue concentrations of carboplatin being effective against any feline tumors.13-27 Routes of local administration include subcutaneous infusion, intratumoral injection, and sustained-release carrier systems.13-16 Although intratumoral injection of carboplatin in cats with squamous cell carcinoma of the nasal plane has been reported,17 cisplatin and carboplatin-impregnated calcium sulfate hemihydrate (C-ICSH) beads are the most studied of any local chemotherapy delivery systems in small animals.18-22,28,29 C-ICSH beads comprise a biocompatible and biodegradable carrier system for sustained release of carboplatin at sites of gross tumor and marginal or radical extirpation.18-25,29 Implantation of C-ICSH beads results in antitumor effects in dogs, horses, and exotic species without clinically significant systemic or local toxicity and is safe in healthy cats.18,23,29 However, doses for carboplatin administration are currently based on maximally acceptable systemic toxicity using body surface area or glomerular filtration rate,12,30 not target tissue concentrations, and few veterinary studies21,24-27 have attempted to evaluate in vitro the local concentration of drug required to achieve antitumor effects by inhibition of 50% (half-maximal inhibitory concentration [IC50]) of tumor cell activities.

While cells grown as monolayers in 2-D culture are used as simple model systems to evaluate drug efficacy, mounting evidence suggests in vitro analyses of cancer cells should occur in 3-D models. While traditional 2-D cell cultures may be hypersensitive to chemotherapy and overestimate the in vivo response of a 3-D tumor to pharmacological compounds, tumor cells cultured in 3-D permit interactions between cells and the extracellular environment more analogous to in vivo tumor tissues.31-35 Cell-microenvironment interactions are essential for cell differentiation, proliferation, and migration. Multidimensional culture platforms such as hydrogels offer opportunity to recapitulate elements of the tumor microenvironment and study the role of cell-matrix interactions in predicting drug response.31,32

Although the in vitro sensitivities of 2-D cultured cells to chemotherapeutic agents have been evaluated in some small animal tumors, no published studies have evaluated small animal tumor cells in 3-D biomaterial culture systems.21,24-27 The goal of this study was to assess FISAS and FOSCC tumor cells in a 3-D gelatin hydrogel culture system to determine chemosensitivity to carboplatin. We hypothesized that IC50 values of carboplatin against FISAS and FOSCC cells measured at 72 hours in 3-D culture would fall within the range of carboplatin concentrations known to elute from C-ICSH beads over the same time period.13,25

Materials and Methods

Methacrylamide-functionalized gelatin synthesis

Methacrylamide-functionalized gelatin (GelMA) was synthesized by first dissolving gelatin (gelatin from porcine skin, gel strength 300, type A [G2500]; Sigma-Aldrich) in PBS at 60°C. Methacrylic anhydride (Sigma-Aldrich) was added dropwise, and the reaction was allowed to proceed for 1 hour with stirring at 400 RPM. The reaction mixture was then quenched with PBS, transferred to 12- to 14-kDa dialysis tubing (Fisherbrand regenerated cellulose dialysis tubing, 12- to 14-kDa molecular weight cutoff, Thermo Fisher Scientific, Waltham Massachusetts), and dialyzed for 1 week against deionized water with daily solvent exchange. The dialyzed reaction mixture was then frozen and lyophilized to yield a white solid.31 Nuclear magnetic resonance was used to analyze the extent to which the gelatin backbone was functionalized with methacrylamide groups. GelMA with a degree of functionalization of 41% was used in this study.

Cell lines and culture conditions

Immortalized FISAS (Kaiser) and FOSCC (SCCF3) cell lines derived from histologically confirmed primary tumors were generously provided (David Vail, University of Wisconsin [FISAS], and Thomas Rosol, Ohio State University [FOSCC]).30 Cells were initially grown in continuous culture at 37°C and 5% CO2 in complete Dulbecco modified Eagle’s medium (Mediatech Inc) supplemented with 10% fetal bovine serum (Mediatech Inc) and 1% penicillin-streptomycin (Thermo Fisher Scientific Inc) in 75-cm2, standard screw-cap, sterile cell culture flasks (Thermo Fisher Scientific Inc). Cells were successively passaged by a standard trypsinization process for expansion using specific reagent (Gibco TrypL Express Enzyme [1X], phenol red; Thermo Fisher Scientific Inc). For 3-D cultures, 10% wt/vol hydrogels were formed with methacrylamide-functionalized gelatin (GelMA, 41% degree of functionalization with methacrylamide groups) dissolved in PBS at 65°C. For each cell line, approximately 5 X 106 cells/mL (100,000 cells/hydrogel) was resuspended in GelMA solution, and the solution was homogeneously mixed. After the addition of lithium acylphosphonate as a photoinitiator at 0.1% wt/vol, the prepolymer solution was cast into custom circular Teflon molds with a diameter of 5 mm and a thickness of 1 mm and photopolymerized by exposure to UV light at a wavelength of 365 nm.
and intensity of 5.69 mW/cm² for 30 seconds. Custom Teflon molds were prepared by the University of Illinois machine shop from 2.2-cm X 2.8-cm X 1-mm rectangular pieces of Teflon. Cell-seeded hydrogels were transferred to low adhesion 48-well plates (Thermo Fisher Scientific Inc) and incubated in cell culture medium at 37 °C and 5% CO₂ in a plate shaker.

Three hydrogels (replicates) were evaluated for each carboplatin concentration (0, 150, 300, 450, and 600 μM) in 3 experiments (repeats) carried out on different days. Medium was replaced the next day with medium containing the differing concentrations of carboplatin. Three hydrogels incubated without drug (0 μM) served as controls, and 3 hydrogels without cells and incubated in culture medium served as blank controls for background subtraction in viability assays. Medium was exchanged for medium with (treated hydrogels) or without (untreated hydrogels) carboplatin every 3 days.

**Cell viability assays**

**Luminescence**

Three hydrogels (replicates) were evaluated for each carboplatin concentration (0, 150, 300, 450, and 600 μM) in 3 experiments (repeats) carried out on different days. The effects of carboplatin on FISAS and FOSCC cell viability were assessed via the CellTiter-Glo luminescence assay (Promega) 72 hours after carboplatin treatment. The hydrogels were transported to another well plate, CellTiter-Glo reagent and medium (1:1) were applied directly to wells, and the well plate was agitated on a shaker, protected from light, at room temperature for 1 hour. Reagent and medium mix were transferred to white-bottomed 96-well assay microplates (Corning Inc), and luminescence signal was measured with a microplate reader (Synergy HTX Multi-Mode microplate reader; BioTek Instruments Inc). Luminescence was correlated to ATP produced by cells using specialized software BioTek Gen5 Data Analysis software (BioTek Instruments Inc) 24 hours after the hydrogels were made for the control and blanks groups and 72 hours posttreatment for the control, blanks, and all carboplatin treatment groups. Cell viability at each concentration of carboplatin was compared with that of untreated control cells. IC₅₀ values were defined for each cell line as the carboplatin concentrations that inhibited 50% of FISAS and FOSCC tumor cellular metabolic activities, respectively.

**Confocal microscopy**

The growth of tumor cells treated with 300 or 600 μM of carboplatin was compared with untreated control cells (0 μM) using live-cell morphology imaging analysis performed at 3, 7, and 14 days after treatment with carboplatin. These doses were chosen because they approximated the median and maximum carboplatin concentrations shown to elute from C-ICSH beads. For each time point, medium in the wells containing hydrogels was replaced with a solution containing calcine and ethidium homodimer-1 (EthD-1) dyes (LIVE/DEAD viability/cytotoxicity kit; Invitrogen) and PBSS. The hydrogels were incubated for 30 minutes at 37 °C, washed with PBSS, and scanned with a laser scanning confocal microscope (LSM 700 laser scanning confocal microscope; Carl Zeiss Microscopy) using an EC Plan-Neofluar 10×/0.30 objective lens. Excitation was provided by 555 nm (EthD-1) and 488 nm (calcine) diode-stable solid-state lasers. The Z-stack acquisition range was 614 μM to visualize the entire depth of the hydrogels. Cell imaging software (Imaris, Bitplane software) was used to obtain cell counts and calculate percentages of live and dead cells.

### Statistical analysis

Doses corresponding to IC₅₀ were computed for inhibitory dose-response data using the Hill nonlinear variable slope model preprogrammed in GraphPad Prism 8.2 (GraphPad Software Inc). Measured response values for each cell line were adjusted for background luminescence using blank hydrogels and independently normalized. Dose concentrations were log₁₀ transformed and the nonlinear Hill slope and IC₅₀ were estimated for each repeat experiment, each replicate within each repeat, and all samples. Luminescence data from each cell line were analyzed as a nested ANOVA using the PROC GLIMMIX command in SAS. Luminescence was log₁₀ transformed (as for the IC₅₀). The categorical independent variables were dose, repeat, and replicates within repeats (replicate[repeat]). The Tukey honestly significant difference test was used to determine pairwise differences if an independent variable was significant. Confocal data from each cell line were log₁₀ transformed and analyzed as doubly repeated measures (repeat, replicate) using the PROC GLIMMIX command. The model included day, dose, and dose X day, and the random parameter was the ordinal order of day and dose. Pairwise differences were determined with Tukey and Sidak adjustment and the denominator degrees of freedom with Satterthwaite adjustment. Normal probability plots were produced for the residuals and Studentized residuals. For all analyses, values of P ≤ 0.05 were considered statistically significant.

### Results

Twenty-four hours after seeding, mean luminescence for the control groups was 1.51 X 10⁵ (range, 1.04 X 10⁵ to 2.09 X 10⁵) for FISAS cells and mean 2.77 X 10⁵ (range, 2.12 X 10⁵ to 2.80 X 10⁵) for FOSCC cells. For blank groups, mean luminescence was 3.93 X 10² (range, 2.26 X 10² to 5.94 X 10²) for FISAS cells and 1.07 X 10² (range, 7.0 X 10¹ to 1.45 X 10²) for FOSCC cells. Luminescence for the control groups of both cell lines increased over 72 hours, and mean luminescence was 2.88 X 10⁵ (range, 1.86 X 10⁵ to 4.38 X 10⁵) for FISAS control cells and 4.86 X 10⁵ (range, 4.37 X 10⁵ to 4.99 X 10⁵) for FOSCC control cells. For blank groups, mean luminescence after 72 hours was 3.84 X 10² (range 1.28 X 10² to 8.79 X 10²) for FISAS cells and 8.8 X 10¹ (range, 6.7 X 10¹ to 1.23 X 10²) for FOSCC cells. For both cell lines, luminescence values decreased significantly with each
increasing carboplatin concentration in the treatment groups (Figure 1).

For FISAS cells treated with 150 µM carboplatin, mean luminescence at 72 hours was $1.56 \times 10^5$ (range, $9.97 \times 10^4$ to $1.94 \times 10^5$). Mean luminescence was $6.03 \times 10^4$ (range, $4.07 \times 10^4$ to $1.11 \times 10^5$) for cells treated with 300 µM carboplatin, $4.30 \times 10^4$ (range, $2.39 \times 10^4$ to $6.00 \times 10^4$) for cells treated with 450 µM carboplatin, and $2.59 \times 10^4$ (range, $1.41 \times 10^4$ to $3.50 \times 10^4$) for cells treated with 600 µM carboplatin. Therefore, for carboplatin treatment groups of FISAS cells compared with control groups at 72 hours, luminescence decreased significantly by 45.8%, 79.1%, 85.1%, and 91.0% after treatment with 150, 300, 450, and 600 µM carboplatin, respectively.

For FOSCC cells treated with 150 µM carboplatin, mean luminescence at 72 hours was $2.85 \times 10^5$ (range, $2.52 \times 10^5$ to $3.30 \times 10^5$; $P < 0.001$). Mean luminescence was $1.36 \times 10^5$ (range, $1.17 \times 10^5$ to $1.65 \times 10^5$; $P < 0.001$) for cells treated with 300 µM carboplatin, $6.34 \times 10^4$ (range, $3.82 \times 10^4$ to $1.55 \times 10^5$; $P < 0.001$) for cells treated with 450 µM carboplatin, and $1.98 \times 10^4$ (range, $1.44 \times 10^4$ to $2.71 \times 10^4$; $P < 0.001$) for cells treated with 600 µM. Therefore, for carboplatin treatment groups of FOSCC cells compared with control groups at 72 hours, luminescence decreased significantly by 41.4%, 72.0%, 87.0%, and 96.0% after treatment with 150, 300, 450, and 600 µM carboplatin, respectively.

The IC$_{50}$ values for FISAS and FOSCC cells ranged from 123 to 171 µM and 155 to 190 µM, respectively, for each tumor type as determined by luminescence assay and extrapolation from a survival curve (Figure 2). The coefficient of variation for log(IC$_{50}$) was 9.86% for FISAS experiments and 2.94% for FOSCC experiments. Due to the small variance for FOSCC experiments, F tests for replicates and repeats were significant. For FISAS experiments, due to a larger variance the replicates did not differ, but IC$_{50}$ values for the first repeat were less than for the next 2 repeats, which were similar ($P < 0.01$).

Live cell morphology images of treated and untreated control cells in 3-D hydrogels from FISAS and FOSCC cell lines were obtained using laser scanning confocal microscopy at 3, 7, and 14 days.
Cells of both cell lines in control groups showed > 95% viability and high proliferation potential at all times using the LIVE/DEAD viability/cytotoxicity kit, which discriminates live and dead cells as green or red fluorescent cells, respectively. With the use of confocal microscopy, cells of both FISAS and FOSCC cell lines treated with 300 and 600 µM carboplatin showed dose-dependent and time-dependent decreases in viability (Figures 3 and 4). For each cell line, the pairwise differences were the same for the Sidak and Tukey adjustments. The mean number of untreated FISAS and FOSCC cells at 3, 7, and 14 days and the number of viable cells in treated groups from both cell lines were summarized (Table 1). These data showed that cellular viability for both cell lines at a carboplatin concentration of 300 µM was approximately 50%, 25%, and 5% at 3, 7, and 14 days, respectively. Cellular viability for both cell lines at a carboplatin concentration of 600 µM was 25%, 10%, and < 5% at 3, 7, and 14 days, respectively. Overall, treatment with carboplatin significantly decreased FISAS and FOSCC cell survival over 14 days.

Discussion

We described the use of a 3-D gelatin hydrogel culture system to characterize the chemosensitivity of FISAS and FOSCC cell lines to carboplatin in vitro. When a luminescence assay was used to determine the IC50 values for both tumor cell types in 3-D culture, mean IC50 values at 72 hours for FISAS and FOSCC were within concentrations known to elute from C-ICSH beads.20,21 Luminescence data for both cell lines indicated that treatment with 150 to 600 µM carboplatin resulted in dose-dependent decreases in viability of tumor cells. When confocal microscopy was used to quantify and qualify the cellular responses in the control and treatment groups, treatment with carboplatin concentrations derived from elution of C-ICSH beads resulted in both dose-dependent and time-dependent decreases in viability of FISAS and FOSCC cells.20,21 In treatment groups of both cell lines, the proportion of viable cells was substantially decreased 3 to 14 days after exposure to carboplatin compared with untreated control groups. Although in vitro sensitivity testing cannot model in vivo pharmacokinetics or pharmacodynamics, it is the accepted method to determine efficacy of chemotherapeutic agents for a particular tumor type in people and provides valuable insight regarding inherent susceptibility or resistance of tumor cells to agents before clinical use.24–27,31–35,39 The method classically assesses the effects of various concentrations of chemotherapeutic drugs on cells derived from tumors of interest in 2-D culture systems.21,24–27 However, as 2-D cell culture systems are incapable of recapitulating the heterogeneous and dynamic parameters of multicellular tumor tissue, 3-D systems may be more suitable and reliable.

Figure 3—Laser confocal images of FISAS cells stained with calcein AM (green, live cells) and ethidium homodimer-1 (EthD-1; red, dead cells). Untreated cells (A), cells treated with 300 µM (B), and cells treated with 600 µM (C) carboplatin at 3 days. Untreated cells (D), cells treated with 300 µM (E), and cells treated with 600 µM (F) carboplatin at 14 days. Note the decrease in live cells staining green and increase in dead cells staining red with increasing carboplatin dose and time. Scale = 200 µm.
Multidimensional culture platforms such as hydrogels were developed to mimic tissue mechanics and biochemical composition more accurately than 2-D monolayer systems and provide a more representative pathophysiological environment. Hydrogels also mimic several mechanical properties of mammalian tissue, such as pore size, volume of distribution, density, and permeability, crucial for determining drug diffusion in subcutaneous tissues and are a proven surrogate for mammalian tissue.

To the authors’ knowledge, ours is the first report of use of a 3-D cell culture system to determine the sensitivity of cells of any feline tumor to a particular chemotherapeutic agent. We chose a hydrogel-based 3-D culture system utilizing GelMA for its excellent biocompatibility, biodegradability, and extensive use as a tissue engineering platform. GelMA is a semisynthetic hydrogel obtained by the functionalization of gelatin with methacrylic anhydride and provides cells with an aqueous biological environment that supports adhesion, growth, and proliferation. GelMA can be quickly photocross-linked to provide shape fidelity and stability at physiological temperatures and mechanical properties that can be fine-tuned to mimic extracellular matrix. GelMA is also transparent, which facilitates evaluation by microscopy.

Confocal microscopy permits visualization and characterization of cells in 3-D without degradation of the hydrogel and is helpful when working with thick specimens to eliminate out-of-focus light. Live-cell staining using confocal microscopy is not dependent on cell proliferation and can characterize live and dead cells directly in the hydrogels. In our study, confocal microscopy revealed dose-dependent and time-dependent decreases in cellular viability. Because tumor beds among cats will vary in size, vascularity, severity of inflammation, and rate and proportion of fluid exchange, previous research concluded that concentrations of carboplatin eluted from C-ICSH beads in any in vivo environment would be expected to fall within the range of the reported

Table 1—Numbers (X 10^3) of viable feline injection site-associated fibrosarcoma (FISAS) and feline oral squamous cell carcinoma (FOSCC) cells at 3, 7, and 14 days following no treatment (control) and treatment with 300 and 600 µM carboplatin.

<table>
<thead>
<tr>
<th>Condition</th>
<th>FISAS cells</th>
<th>FOSCC cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 3</td>
<td>Day 7</td>
</tr>
<tr>
<td>Untreated</td>
<td>331^a</td>
<td>503^a</td>
</tr>
<tr>
<td>300 µM carboplatin</td>
<td>156^c</td>
<td>132^d</td>
</tr>
<tr>
<td>600 µM carboplatin</td>
<td>19^e</td>
<td>51^e</td>
</tr>
</tbody>
</table>

Values with different superscripts are significantly (P ≤ 0.05) different.

Figure 4—Laser confocal images of FOSCC cells stained with calcein AM (green, live cells) and EthD-1 (red, dead cells). Untreated cells (A), cells treated with 300 µM (B), and cells treated with 600 µM (C) of carboplatin at 3 days. Untreated cells (D), cells treated with 300 µM (E), and cells treated with 600 µM (F) of carboplatin at 14 days. Note the decrease in live cells staining green and increase in dead cells staining red with increasing carboplatin dose and time. Scale = 200 µm.
minimum and maximum in vitro concentrations.\textsuperscript{19–21} We chose to dose cells with 300 and 600 \(\mu M\) carboplatin as these doses approximated the median and maximal concentrations reportedly released from C-ICSH beads in vitro.\textsuperscript{19–21}

Cell viability at each concentration of carboplatin was also assessed using a luminescence assay and was compared with the viability of untreated control cells. The 72-hour IC\(_{50}\) values of cells of the FOSCC SCCF3 cell line and FISAS Kaiser cell line in 3-D culture were 2 to 5 times greater than the 48- and 72-hour IC\(_{50}\) values previously reported for these same cell lines in 2-D culture.\textsuperscript{21} Possible explanations for these differences lie in the contrast between 2-D and 3-D culture systems. Hydrogel-based 3-D culture systems allow for cell-cell and cell-matrix interactions that permit cell growth and potentially facilitate chemoresistance.\textsuperscript{31–35,42,43} Additionally, cells cultured in 2-D are subjected to significantly different surface topologies and local mechanical stiffness than experienced in soft tissue, leading to alterations in cell physiology due to cytoskeletal rearrangements causing artificial polarity and aberrant gene and protein expression that may cause hypersensitivity to chemotherapeutic agents.\textsuperscript{13,34} Proteins involved in metabolism, cell stress response, structural integrity, signal transduction, and cellular transport are all expressed at elevated levels in 3-D versus 2-D cultures, potentially altering chemosensitivity.\textsuperscript{13,34}

In vivo efficacy of a local chemotherapy delivery system depends not only on the concentration of chemotherapy released but also on how long the drug is in contact with the tissues and the diffusion of the agent in tissues.\textsuperscript{12–16,21–29} It is not known for what duration significant concentrations of any chemotherapeutic should be maintained in tissue. The systemic dose of carboplatin recommended to treat tumor-bearing cats is 200 to 240 mg/m\(^2\) IV every 3 to 4 weeks, resulting in a mean peak plasma concentration of only 23 mg platinum/L (119 \(\mu M\) carboplatin) and a mean residence time of 110 minutes.\textsuperscript{40} The specific tissue distribution and duration of drug exposure in specific tissues following a single IV dose or subcutaneous infusion of carboplatin in cats are not known and likely vary within and among cats.\textsuperscript{12,39} However, the mean concentration of carboplatin reportedly eluted from a single C-ICSH bead at 72 hours (1,367 \(\mu M\) carboplatin, 265 mg/L platinum) is 8 times the maximum IC\(_{50}\) of FISAS cells and 7.2 times the maximum IC\(_{50}\) of FOSCC cells reported at 72 hours in the present study, indicating that implantation of as few as one bead may be sufficient to treat tumors in affected cats.\textsuperscript{20} Understanding the minimum dose of bead or other carboplatin-impregnated substrate required for adequate suppression of tumor cell growth is important for surgical planning, particularly as the destructive tissue effects of tumors such as FOSCC may result in little area available for bead or other depot implantation.\textsuperscript{6–9} Carboplatin is also a radiation sensitizer that increases tumor cell toxicity when given IV in combination with radiation, and a chemoradiation protocol combining local carboplatin treatment with radiation therapy may also result in local radiosensitivity.\textsuperscript{46} Clinical trials are needed to investigate the diffusion kinetics of carboplatin delivery systems and tumor response to multimodal therapies in affected cats.\textsuperscript{15,17,18,29} Based on IC\(_{50}\) data from the present study, treatment of cats affected by FISAS or FOSCC with local delivery of carboplatin should achieve minimum tissue concentrations of at least 150 to 200 \(\mu M\) carboplatin, concentrations known to elute from C-ICSH beads. Delivery systems such as C-ICSH beads capable of releasing 300 to 600 \(\mu M\) carboplatin to local tissues could result in > 50% tumor cell suppression, as evidenced by our confocal data.

Limitations of the present study included the in vitro experimental design, as the direct translation of in vitro data to in vivo situations must be considered cautiously.\textsuperscript{47} Although 3-D cell culture systems using biomimetic hydrogels might model in vivo tissues, factors such as tumor blood flow, drug metabolism, and host and immune responses not reproduced in cell culture systems could alter the concentrations and efficacy of chemotherapeutic agents in patients.\textsuperscript{47} The IC\(_{50}\) values were calculated for FISAS and FOSCC cells at a single time point (72 hours), and evaluations at other times could differ.\textsuperscript{48} Additionally, the exchange of medium for medium with (treated hydrogels) or without (untreated hydrogels) carboplatin every 3 days and exposure times of 3, 7, and 14 days does not necessarily correlate with the length of time carboplatin concentrations from one or several beads would be sustained in vivo. Nevertheless, the data provide information on the expected duration of the effects on viability of cells exposed to carboplatin. There was minor variability within our replicated and repeated experiments. While some variability is expected among replicate and repeat experiments, it has been reported that variability may be increased when indirect cell viability assays traditionally used for 2-D cell culture investigations are extrapolated to the study of cells in 3-D culture systems.\textsuperscript{48} ATP-based assays are reported to be most suitable for hydrogel-based 3-D culture systems, and the luminescence assay used here performed most accurately in one comparative study.\textsuperscript{48} Investigators of the same study concluded that indirect assays should be supported by direct microscopic imaging of cells to assess viability using calcein AM with EthD-1 staining, as in the present study. Such reagents are used to stain living and dead cells directly in the hydrogel followed by confocal microscopy to produce Z-stack images of the whole culture system, eliminating much of the uncertainty associated with indirect assays. With rising interest in 3-D culture systems, industry professionals have begun development of high-throughput live/dead molecular probes and tissue-clearing techniques for viability assessment; however, basic colorimetric, fluorescent, and luminescent viability assays are currently used most often.\textsuperscript{48}

Future studies should leverage the approaches developed here to quantify the invasive migration of cancer cells through gelatin hydrogels and to interrogate the role of cell-matrix interactions and...
extracellular levels of hypoxia on the invasive phenotype of FISAS and FOSCC cells. Although 3-D culture systems have been described using extracellular matrix-based natural hydrogels, synthetic hydrogels, and engineered hydrogels that mimic extracellular matrix such as GeiMA, it is unknown which substrate or which concentration of GeiMA most closely approximates feline tissue. Finally, all tumor cell culture systems might select for aggressive phenotypes that show enhanced resistance to chemotherapy, and IC_{50} values may exceed concentrations required in vivo where clinical benefits may be seen at lower concentrations.

In conclusion, treatment with carboplatin had significant dose- and time-dependent effects on the viability of FISAS and FOSCC cells in 3-D culture. Three-dimensional hydrogel-based cell culture systems support the growth of feline tumor cells and may be used to predict in vivo responses to chemotherapy. These systems may be suitable for additional in vitro dose-response studies evaluating alternative chemotherapeutic agents or radiation response.

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References


42. Pepelanova I, Krupka K, Scheper T, Lavrentieva A. Gelatin-methacryloyl (GelMA) hydrogels with defined degree of functionalization as a versatile toolkit for 3-D cell culture and extrusion bioprinting. *Bioengineering (Basel).* 2018;5(3):55–69.


