Ex Vivo Viability of Canine and Feline Sarcomas: A Pilot Study

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CLINICAL RELEVANCE

Assay-based chemotherapeutic protocols are common in human gynecologic oncology, most notably for patients with ovarian or breast cancer. The current study examines ex vivo incubation conditions necessary for the assessment of sarcomatous tumor response to potential chemotherapeutic drugs. Slices of sarcomatous tumors were incubated in one of two culture media. Viability indices were measured and compared across time and between media. Neither medium was sufficient to support the growth of sarcomatous tumor tissue slices based on the indices studied. It is likely that sarcomatous tumors require a different approach for ex vivo assessment than their epithelial counterparts. Our long-term goal is to incubate tumor slices with chemotherapeutic agents to predict the in vivo tumor response based on the maintenance or loss of slice viability within this system.

INTRODUCTION

Soft tissue sarcomas (STSs) account for approximately 15% and 7% of all skin and subcutaneous tumors in dogs and cats, respectively.1 Canine STSs include tumors arising from fibrous, vascular, adipose, smooth and striated muscle, and nerve tissue, whereas feline STSs often arise from subcutaneous injection sites (injection-site sarcomas [ISSs]). Canine STSs and feline ISSs share many histologic and biologic characteristics, including local invasion, relatively low metastatic rates, heterogenous microscopic features, and a pseudocapsule of compressed tumor cells. Due to high recur-
mor slices could be maintained for 24 hours and would be maintained better in Hepatocyte Incubation Medium (HIM; XenoTech, LLC, Lenexa, KS) than in Waymouth culture medium (WCM; Sigma-Aldrich, St. Louis, MO). Our long-term goal is to incubate tumor slices with chemotherapeutic agents to predict the in vivo tumor response based on the maintenance or loss of slice viability within this system. Such incubation could pave the way to pre-treatment determination of tumor chemosensitivity, thus sparing patients the morbidity, and clients the expense, associated with ineffective chemotherapeutic administration. In a manner similar to the growing use of pharmacogenomics for rational optimization of drug therapy, a tissue-slice chemosensitivity assay is expected to identify not only whether patients will respond to chemotherapy but also which chemotherapeutic agents they will respond to most profoundly.

**MATERIALS AND METHODS**

The current study was modeled after Fisher et al.’s protocol for liver slices. Viability indices of potassium and ATP measurement as well as histopathology were chosen based on previous studies. WCM was supplemented with 10% fetal calf serum (Atlanta Biologicals, Atlanta, GA), 10 mL/L antibiotic-antimycotic solution (Mediatech, Herndon, VA), 2.24 g/L sodium bicarbonate, 50 µg/mL gentamycin sulfate (VWR International, Sewance, GA), and filtered sterilized distilled water. HIM was supplemented with 50 µg/mL gentamycin sulfate and 10 mL/L antibiotic-antimycotic solution. Nine tumors previously diagnosed as sarcomas or with a high index of suspicion that had not previously been exposed to chemotherapy were obtained via surgery; immediately placed in filtered, sterilized, ice-cold V7 preservation medium (the chemicals used in this medium were obtained from Spectrum...
Chemicals, Gardenia, GA; recipe from Vitron, LLC, Tucson, AZ); and refrigerated until slicing. An 8-mm stainless steel handheld coring tool (Vitron) was used to obtain tissue cylinders of a uniform diameter. A Brendel/Vitron tissue slicer (Vitron) was used to slice the cylinders in a uniform thickness (based on weight of 20 to 30 µg). Oxygenated, ice-cold V7 preservation medium was run through the system during slicing, and the slices were returned to cold V7, where they were visually sorted as acceptable or nonacceptable. Slices were rejected for incubation if they appeared to have undergone extensive trauma (incompletely cut or shredded), as these variations may alter measurable viability indices. Time 0 slices were obtained first. Each slice was blotted, weighed, and placed in a microcentrifuge tube containing either 1 mL of distilled water for potassium measurement or 1 mL of 10% trichloroacetic acid (Sigma-Aldrich, St. Louis, MO) for ATP measurement or placed on a small square of Manila file folder (to prevent curling) and then into a scintillation vial with 2 mL of 10% buffered formalin. Samples from the first four tumors were homogenized for ATP measurement before placement into microcentrifuge tubes. Homogenization before freezing was not possible for subsequent tumors; therefore, samples from tumor 5 through 9 were frozen intact. Microcentrifuge tubes were immediately placed in a −80°C freezer; formalin vials were left at room temperature until evaluation.

Three slices for each of the remaining time points (4, 8, and 12 hr for tumors 1 through 4; 4, 8, 12, and 24 hr for tumors 5 through 9) were blotted, weighed, and placed on cylindrical stainless steel mesh rollers. Grossly similar slices were grouped together to minimize variation within each vial. Each roller held three slices placed in a uniform fashion to correlate pre- and post-incubation weights. The slice closest to the vial lid was used for potassium measurement, the middle slice for ATP measurement, and the third slice for histopathology.

Incubation racks were then placed into scintillation vials containing either 1.7 mL of WCM (all nine tumors) or 1.7 mL of HIM (tumors 5 through 9 only). All scintillation vials were capped with a lid containing a 2-mm hole for gas exchange. Scintillation vials were placed in a dynamic organ culture incubator (Vitron) at 37°C and received a gas mixture of 95% oxygen and 5% carbon dioxide for respective time periods. After incubation, the slices were again blotted and individually weighed before being placed into the appropriate solution for potassium or ATP measurement or histopathologic evaluation, as described above.

Values were standardized by slice wet weight. Briefly, potassium samples were thawed, vortexed, and processed with a potassium EasyLyte Plus analyzer (Medica Corp, Bedford, MA). The results were expressed as mmol/L and converted to µmol/g using slice weight and solute volume. ATP samples were homogenized or vortexed after thawing and centrifuged for 10 minutes at 3000 rpm. For each sample, 10 µL of supernatant was diluted 200× by the addition of 2 mL of water. These samples were then vortexed, run according to the ATP bioluminescence kit, and read on a Thermo Appliskan plate reader (Thermo Electron Corp, Microplate Instrumentation, Vantaa, Finland), and the results were expressed in nmol/mg.

Slices in formalin were embedded in paraffin, cut into 5-µm slices, and stained with hematoxylin and eosin. All slides were scored by one author (N. G.), and random slides were blinded and reviewed by a board-certified veterinary pathologist to ensure appropriate scoring. Samples submitted for histopathology were also scored. The histologic parameters evaluated were percentage of necrosis, extent of
pyknosis, presence of nucleoli, and mitotic index. The entire slice was scanned for all indices except mitotic index, which was counted per 10 high-power fields. Pyknosis was identified based on the presence of clumped chromatin, giving the nucleus a dark, shrunken appearance. Each slice was scored as 0, 1, or 2, indicating mild, moderate, or severe pyknosis, respectively. The presence of nucleoli was recorded as a binary response. Zero was assigned when <20% of cells had visible nucleoli, and 1 was assigned when >20% of cells had visible nucleoli. Mitotic figures were counted and expressed per 10 random high-power fields (400x).

Averages of triplicate samples were compared across time between tumors and between media for tumors. For descriptive statistics, parametric data (percentage of necrosis and mitotic index) were reported as mean and standard deviation, whereas nonparametric data were reported as the median and range (pyknosis) or as a proportion (nucleoli). Indices of viability (metabolic and histopathologic) were compared between time points using a repeated measures analysis of variance and Holm-Sidak pairwise multiple comparison procedure and between media (n = 4) using a t test and a Mann-Whitney rank sum test. Scores of baseline histopathology samples were compared with time 0 scores using a paired t test to assess the effect of overnight refrigeration of tissues before slicing. Standard software was used; values of P ≤.05 were considered significant (SigmaPlot 11.0, version for Windows 2008).

■ RESULTS

Nine tumors were obtained for evaluation (Table 1). Seven were feline ISSs, and two were canine STTs. One feline fibrosarcoma (tumor 5) was contaminated during processing and was discarded because of the potential impact of bacterial infection on indices of viability.

Potassium measurement: Differences among times were limited to time 0, which was significantly higher than all other times (P <.001; Figure 1). Potassium concentrations did not differ across time between the two media.

ATP measurement: Tumors 6 through 9 could not be homogenized before or after freezing, precluding ATP measurement. The inability to homogenize these tumors was suspected to be due to their high connective tissue content. ATP concentrations in tumors 1

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TABLE 1. Tumors Obtained for Evaluation

<table>
<thead>
<tr>
<th>Species</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog</td>
<td>HSA</td>
<td>FSA</td>
<td></td>
<td></td>
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<tr>
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<td>MXS</td>
<td>FSA</td>
<td>MFH</td>
<td>FSA</td>
<td>FSA</td>
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FSA = fibrosarcoma, HSA = hemangiosarcoma, MFH = malignant fibrous histiocytoma, MXS = myxosarcoma.

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Figure 1. Combined potassium (K) level of all tumors over time. A significant decrease was detected (P <.001) over time but not between media (P >.4).
through 4 were significantly higher at times 2 through 8 hours compared with baseline and 12 hours \((P < .001; \text{Figure 2})\).

**Histopathology:** Histologic lesions were averaged for each tumor and compared between media (Table 2). No significant changes were seen between the two media in any of the viability indices. Within-media comparisons revealed some differences across time. In both media, pyknosis increased (WCM \(P < .002; \text{HIM} P < .001; \text{Figure 3}\)) and mitotic index decreased (WCM and HIM \(P < .001; \text{Figure 4}\)) across all times compared with baseline. Necrosis in WCM slices was significantly increased at time 24 compared with times 0 and 4 \((P < .003; \text{Figure 5})\). No significant differences were seen in nucleoli over time in either medium (WCM \(P = 1.000; \text{HIM} P > .4; \text{Figure 6}\)). No significant differences were seen between the two media in any of the viability indices during this time period.

We also evaluated the effect that waiting to process the tumors may have had on viability indices. Due to logistic concerns, tumors were extracted from patients, immediately placed in ice-cold V7 preservation medium, and refrigerated overnight. Histologic viability indices were determined and compared to the time 0 indices. These results are shown in Table 3 and indicate a significant decrease in both nucleoli and mitotic index over this time period.

## DISCUSSION

Tissue slice methodology, originally described in 1923 for the liver, has been adapted to various tissues, including kidney, heart, lung, prostate, brain, and intestine. Tissue slices have proven useful for assessing cytotoxicity, pharmacologic activity, cellular metabolism, enzymatic induction, reperfusion injury, and fibrogenesis. Tissue slice–based assays have also been adapted for the investigation of tumor response to chemotherapeutic agents. Example outcome measures include caspase induction, incorporation of radioactive hydrogen into tumor cells, and gene expression of \(p73\) and \(p53\) protein levels. Reports using human tumors generally are limited to epithelial neoplasms, with no single medium most commonly cited. These reports offer evidence of the potential clinical application of the methods reported here. For example, proliferation, apoptosis, and overall viability of breast cancer tissue slices decreased in a dose-dependent manner during 4-day incubation with taxol.

STSs were chosen rather than epithelial tumors for this project because of their marked variability in response to chemotherapy, contributing to their therapeutic challenge. However, whereas epithelial tumors are relatively homogenous and less difficult to maintain in vitro, sarcomatous tumors might be predicted to present a greater challenge because of their cellular heterogeneity, as well as extracellular matrix characteristics (i.e., fibrous nature). Although hemangiosarcomas (HSAs) are not STSs, a retroperitoneal HSA (tumor 8) was included in our study because preoperative aspiration of the mass indicated it to be a spindle cell tumor. Although histopathology confirmed the tumor to be an HSA, its histopathologic
characteristics were similar to those of an STS, supporting its inclusion in the study.

WCM was chosen for incubation because of its availability and broad use in tissue slice systems.15,16,20,32–34 After the first four tumors were processed, it was decided to extend the incubation period to 24 hours (when sample size was sufficient) and to incubate a second population of slices in HIM for comparison to those in WCM. HIM differs from WCM with the addition of dexamethasone, insulin, and pyruvate, each of which may aid in glucose metabolism.

These pilot data did not demonstrate either WCM or HIM to be superior for incubation of sarcomatous tumors, an unsurprising finding based on the limited number of tumors studied. However, it seems that additional viability indices, the measurement of ATP at extended time periods, and, potentially, different media for sarcomatous tissue should be investigated. Additionally, potassium may not be a good indicator of viability and is the least sensitive of the three viability indices we investigated (histopathology, ATP, and potassium).28

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Pyknosis</th>
<th>Nucleoli</th>
<th>Mitotic Index</th>
<th>Necrosis (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Median</td>
<td>Low</td>
<td>High</td>
<td>Mean %</td>
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<tr>
<td>WCM</td>
<td>1</td>
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<td>2</td>
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<td></td>
<td>2</td>
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<td>0</td>
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<td>7</td>
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<td></td>
<td>8</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Overall (1–9)</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>57</td>
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<tr>
<td>Overall (6–9)</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>48.5</td>
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<tr>
<td>HIM</td>
<td>6</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1</td>
<td>0</td>
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<td></td>
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<td>0</td>
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<tr>
<td></td>
<td>9</td>
<td>1.4</td>
<td>0.64</td>
<td>5</td>
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<tr>
<td>Overall</td>
<td>1.35</td>
<td>0</td>
<td>5</td>
<td>26.1</td>
</tr>
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</table>

HIM = Hepatocyte Incubation Medium, WCM = Waymouth culture medium.

*Nucleoli is presented as a proportion of slices/tumor that were scored as a 1 or as having more than 20% of cells containing nucleoli.

Sem calculated rather than SD for overall population statistics.
While several of the indices measured in the current study underwent significant change during the incubation period, interpretation is difficult because the power of all tests was suboptimal (<0.8). We suspect this is due to the great variability within sarcomatous tumors and the low case number. We attempted to maximize the power of the study by comparing intertumoral variability over time; however, the inconstancy within each tumor overcame these efforts. We concluded that more slices per time period are needed to minimize the impact of the heterogeneity seen in sarcomas.

Homogenization before freezing was only able to be performed in the first 4 tumors. Attempts at homogenization before freezing samples were unrewarding for tumors 5 and 6, presumably due to their high fibrous tissue content. In general, slice homogenization of other tissues can be performed after thawing, just before ATP analysis. Pre-freeze homogenization efforts were therefore aborted for tumors 7 through 9. However, homogenization after thawing was unsuccessful for these tissues; therefore, ATP data are not available for these tumors. ATP appears to be an indicator of health in tumors 1 through 4 incubated in WCM, supporting its continued use as an outcome measure in future studies.

Environmental conditions of incubation may influence tissue survival. We chose a temperature and gas mixture commonly reported in tissue slice studies. It is possible that our tumor slices required a different oxygen tension for gas and nutrient penetration. Toutain et al. demonstrated that a rotating organ culture system with high oxygen tension (5% carbon dioxide, 95% oxygen) such as that used in this study was optimal for long-term (72 hours) incubation of liver slices. Certain tumors have been documented to adapt to hypoxic environments via release of hypoxia inducible factor 1α. The level of hypoxia may cause cells to lose their ability to respond to a change in oxygen tension, thus affecting their viability in this system. Toutain et al also demonstrated that...
incubation in a medium devoid of substrates for mitochondrial oxidative phosphorylation (e.g., WCM) may limit the rate of ATP production. Our use of WCM may have contributed to the decrease in ATP concentrations that occurred at 12 hours, despite the apparent increase in ATP typical of recovering slices that occurred between 2 and 8 hours (Figure 2). Nutrient replenishment (i.e., a change in medium) may have prevented this decline. Alternatively, the decline in ATP may have reflected progressive cell death, as is suggested by the significant drop without recovery in potassium concentrations. Unfortunately, tumors 6 through 9 incubated in either HIM or WCM were too rigid to homogenize, and ATP concentrations could not be obtained for comparison.

The significant drop in potassium concentration indicates cell membrane disruption and correlates poorly with the maintenance of ATP concentrations over 8 hours. This implies that although cell membrane integrity was compromised, mitochondria remained intact, suggesting apoptosis as opposed to necrosis. Either type of death could be facilitated by an inadequate environment or have been already programmed to occur within the tumor.

Mitotic index is a known indicator of viability; however, it may only accurately reflect viability of tumors with a relatively high initial mitotic index. Additionally, mitotic index is more difficult to determine in sarcomatous tissue (relative to more homogenous tissues) because of the large extracellular matrix content, suggesting that perhaps apoptotic index would be more appropriate for these tumors. Other measurements of viability, such as live/dead assays, incorporation of [3H]thymidine, and caspase-3 measurement, have been used with tissue slices and may be helpful in future studies with sarcomatous slices.

The original histopathology samples were scored and compared with time 0 sample scores to evaluate the effect of refrigerating the tissues overnight before slicing. Nucleoli and mitotic index were both significantly decreased ($P = .01$ and $.03$, respectively) at time 0 compared with the original histopathologic samples, indicating a loss of tissue viability during
storage before processing. The purpose of keeping harvested tissues intact before processing is to allow them to “rest.” However, the size of the tissue samples (4 to 8 cm in diameter) may have precluded nutrient diffusion into cells in the center of the sample. Ideally, samples should be processed immediately after harvest from the patient. Potential disadvantages to the use of tissue slice methodology in the clinical setting include the size of incubated samples (8 mm × ~200 µm), which may not fully represent a heterogenous tumor; the need for expert technical skills; and cost.22,29 Heterogeneity might be better represented if multiple cores are collected from different areas of a tumor and slices from each core are averaged. Although the periphery of certain tumors may be preferred to avoid a necrotic center, the heterogeneity of sarcomas allows for necrotic areas throughout a tumor, placing less emphasis on avoiding the center while coring. For our study, several tumors were not large enough to avoid the center, which may or may not have contributed to the marked variability in necrosis for some tumors (e.g., tumor 9).

Extracting multiple cores to obtain the number of slices needed to accurately represent tumor heterogeneity necessitates a relatively large tumor. For smaller tumors, selected incubation time points may need to be eliminated. The initial 2 to 4 hours of incubation are a “recovery period” for slices, as indicated by a transient drop in viability indices, and data from this timeframe, therefore, are not likely to contribute to final results.38,39 Regardless of tumor size, incubation is ideally performed in replicates in an attempt to overcome the influence of heterogeneity and other inherent variables. In the clinical setting, slices must also be allotted for evaluation of multiple chemotherapeutics at various time points. In this pilot study, tumors 1 and 3 were not large enough to provide slices beyond those needed for a 4-hour study. Smaller (e.g., 5 mm) slices might be in-
dicated for smaller tumors (≤2 × 3 cm) so that heterogeneity can be adequately assessed. Among the limitations of this study is tumor consistency. In our study, approximately 50% of tumors collected had to be rejected because their consistency was either too soft or hard for slicing. Ideally, tumors suitable for this technique would have a moderately firm, homogeneous consistency, similar to that of liver tissue.

Another consideration to the clinical use of this methodology is cost. Currently, the cost of evaluating these tumors is approximately $150 to $175 per tumor. The price depends greatly on the medium; V7 is made in 1-L batches, must be used within 7 days of compounding, and is relatively expensive. On the other hand, when considering the average cost of chemotherapy for one patient, the cost of determining tumor sensitivity is easily justified.

**CONCLUSION**

Based on our results, ATP concentrations seem appropriate for detecting viability in sarcomatous tissue. The utility of the histopathologic indices evaluated in this pilot study remains obscure. Additionally, while previous studies have been performed in triplicate to account for inherent tissue variability, this does not seem adequate for the heterogeneity seen within sarcomas. Further studies are indicated to identify and validate histopathologic viability indices and the number of replicates needed to accurately represent sarcomatous tissue.

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**REFERENCES**