A QUANTITATIVE EVALUATION OF DNA DAMAGE FROM IRRADIATION OF FELINE ORAL SQUAMOUS CELL CARCINOMA CELLS

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by
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A candidate for the degree of Master of Science,

And hereby certify that, in their opinion, it is worthy of acceptance.

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# TABLE OF CONTENTS

ACKNOWLEDGEMENTS.................................................................................................................. ii

LIST OF FIGURES............................................................................................................................ iv

ABSTRACT.......................................................................................................................................... v

Chapter

1. INTRODUCTION.............................................................................................................................. 1
   Tumor biology................................................................................................................................. 1
   Treatment and outcomes............................................................................................................... 3
      Surgery......................................................................................................................................... 3
      Chemotherapy.......................................................................................................................... 3
      Radiation therapy..................................................................................................................... 8
   Using single gel electrophoresis to detect DNA damage and repair........................................ 13
      Protocol and measurement...................................................................................................... 13
      Relationship between cellular radiosensitivity................................................................. 14
      Application............................................................................................................................. 15

2. SINGLE GEL ELECTROPHORESIS USING COMET ASSAY.................................................. 16
   Methods and materials........................................................................................................... 16
   Results......................................................................................................................................... 20
   Discussion............................................................................................................................. 20

3. CONCLUSIONS AND FUTURE DIRECTIONS........................................................................... 22

4. APPENDIX...................................................................................................................................... 23

5. BIBLIOGRAPHY............................................................................................................................ 38
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>23</td>
</tr>
<tr>
<td>Stitched 2-well comet slide image</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>24</td>
</tr>
<tr>
<td>Total comet primary and secondary cellular analysis object masks</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>25</td>
</tr>
<tr>
<td>Comet areas included in comet tail moment calculation</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>26</td>
</tr>
<tr>
<td>SCCF1 percent DNA in the tail</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>27</td>
</tr>
<tr>
<td>SCCF2 percent DNA in the tail</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>28</td>
</tr>
<tr>
<td>SCCF3 percent DNA in the tail</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>29</td>
</tr>
<tr>
<td>SCCF1 comet tail moment</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>30</td>
</tr>
<tr>
<td>SCCF2 comet tail moment</td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>31</td>
</tr>
<tr>
<td>SCCF3 comet tail moment</td>
<td></td>
</tr>
</tbody>
</table>

Table

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>32</td>
</tr>
<tr>
<td>Imaging parameters</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>33</td>
</tr>
<tr>
<td>Image processing parameters</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>34</td>
</tr>
<tr>
<td>Comet head cellular analysis parameters</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>35</td>
</tr>
<tr>
<td>Total comet cellular analysis parameters</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>36</td>
</tr>
<tr>
<td>Percent DNA in the tail analysis</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>37</td>
</tr>
<tr>
<td>Comet tail moment analysis</td>
<td></td>
</tr>
</tbody>
</table>
ABSTRACT

Feline oral squamous cell carcinoma is a locally invasive neoplasm that continues to carry a poor prognosis despite advances in multimodality oncologic treatment. Location, completeness of surgical excision or complete response to radiation therapy at day 30 have been shown to be predictive of survival. Tolerable treatment with radiation and concurrent chemotherapy has been described, but the ideal radiation protocol has not been determined.

Though risk factors and prognostic indicators have been identified, there is no test to predict outcome prior to treatment. Three feline oral squamous cell lines (SCCF1, SCCF2, SCCF3) were used to investigate the potential of the alkaline comet assay to predict radiosensitivity.

The three cell lines were irradiated with 6 MV photons and initial DNA damage using percent DNA in the comet tail and comet tail moment were plotted against all doses (0 - 9 Gy) for each cell line. There was a significant difference between the means of SCCF1 cells that received 0 Gy and 9 Gy (p=0.019), as well as 3 Gy and 9 Gy (p=0.022) when assessing the DNA in the tail. The difference between the means for comet tail moment was significant for SCCF1 at 0 Gy and 9 Gy, which corroborates part of the previous finding.

Based on other experiments that evaluated clonogenic and comet assays together, correlation between the greatest mean tail moment and lowest surviving fraction indicate radiosensitivity, not cytotoxicity. The clinical feasibility of performing the comet assay on FOSCC tumor biopsies to elucidate radioresponsiveness prior to initiating treatment remains questionable for this specific disease. Performing the comet assay to evaluate DNA repair capabilities is recommended at various time intervals after irradiation to optimize fractionation protocols. Validation of DNA damage from double-stranded breaks could be compared using a chemiluminescent assay to detect levels of mouse anti-human monoclonal antibody for γ-H2AX.
CHAPTER 1: INTRODUCTION

FELINE ORAL SQUAMOUS CELL CARCINOMA TUMOR BIOLOGY

Although oral tumors are less frequently encountered in cats compared to dogs, most of these neoplasms are malignant (90%) and of those, squamous cell carcinoma predominates (61.2%).\textsuperscript{1-4} Feline oral squamous cell carcinoma is an aggressive epithelial neoplasm that frequently invades bone and can affect the gingival and mucosal surfaces of the maxilla, mandible, tongue/sublingual area, and cheek.\textsuperscript{5} Though there is a mild metastatic risk to regional lymph nodes (31%) and lungs (10%), this does not significantly impact survival as most patients succumb to local disease.\textsuperscript{5} Clinical signs of cats with oral squamous cell carcinoma can vary based on location and extent of disease, but include difficulty prehending food, hyporexia-anorexia, lethargy, weight loss, oral pain, ptyalism, halitosis, loosening of teeth, and decreased grooming.\textsuperscript{4} This cancer carries a poor prognosis with a one-year survival rate of less than 10%, often because tumors are at an advanced at diagnosis; however, advances in multimodality treatment have shown an improvement in median survival times with some cats experiencing durable remissions one to six years beyond initial tumor treatment.\textsuperscript{6-10}

Apart from the stochastic risk of genetic mutations that can lead to the development of cancer, risk factors for oral squamous cell carcinoma (SCC) in cats have been identified. Multivariate relative risk analyses performed on questionnaires sent to the owners of 112 cats with histopathologically confirmed oral squamous cell carcinoma showed a significantly increased risk of oral SCC in cats that wore flea collars (RR 5.3), ate canned food (RR 3.6), or more specifically regular consumption of canned tuna fish (RR 4.7).\textsuperscript{11} Exposure to pesticides is a known carcinogen in people and it was postulated that the proximity of flea collars to the oral cavity of the cat may play a role.\textsuperscript{11} In humans there is a parallel to poor dental hygiene and the development of oral cancer, though this study did not show a relationship between periodontal disease and SCC in cats.\textsuperscript{11} There was a nonsignificant 2-fold increase in risk (RR 2.3) of SCC in cats that were exposed to household environmental tobacco smoke (ETS).\textsuperscript{11} Additionally, alterations in expression of tumor suppressor gene protein, p53, were 4.5 times more likely to be observed in feline oral SCC biopsy samples in cats that were exposed to ETS based on CM-1
antibody immunohistochemistry. This finding, though not statistically significant, suggested that p53 dysregulation (overexpression) may be involved in the development of feline oral SCC, though there are likely other associated pathways leading to tumorigenesis.

To attempt to understand the biology of feline oral squamous cell carcinoma (FOSCC), immunohistochemical staining on tumor tissues showed about 70% of samples had altered patterns of epidermal growth factor receptor (EGFR) expression. EGFR is a transmembrane protein tyrosine kinase receptor involved in cellular signaling and EGFR overexpression is implication in tumor pathogenesis and progression. Furthermore, cats with EGFR-positive cutaneous SCC tumors had a significantly worse outcome, which was independent of other factors like tumor differentiation and mitotic activity. Though EGFR is a poor prognostic indicator, it may become a druggable target in the future with EGFR inhibitors. A different study evaluating expression of cancer-associated fibroblasts (CAFs) within FOSCC tumor tissues found that 74.5% of samples were CAF-positive. CAF cells express α-smooth muscle actin are a component of the epithelial tumor stroma, which also promotes many of the hallmarks of cancer. Interestingly part of the inclusion criteria was that cats could only receive palliative care, so that the survival of patients with CAF-positive tumors was confirmed to be significantly shorter than those without CAF expression (35 vs. 48 days).
TREATMENT AND OUTCOMES

Surgery

Surgical excision of feline oral squamous cell carcinoma is often considered not feasible, especially with advanced disease at diagnosis. Resectable tumors were previously considered to be either small in size or located rostrally. Anatomic limitations of the feline head make obtaining surgical margins of greater than 1 cm a challenge; moreover, even if clean margins are obtained, tumor recurrence remains a risk.\textsuperscript{14-16} A case series of seven cats with oral squamous cell carcinoma that had a mandibulectomy procedure followed by external beam radiation therapy reported a median survival time of 14 months, which was much improved from the median survival of six months with surgery alone.\textsuperscript{16-18} Another study found cats with mandibular squamous cell carcinoma had a significantly shorter survival time (median 217 days), compared to other oral tumor types, though most cats had a good functional outcome by regaining the ability to eat.\textsuperscript{14} Boston et al. retrospectively analyzed the records for eight cats, seven of which had squamous cell carcinoma, that had a radical mandibulectomy and aggressive post-operative analgesia and enteral support.\textsuperscript{15} The estimated mean survival time was 712 days and six of the cats regained the ability to eat.\textsuperscript{15} Most recently, the Veterinary Society of Surgical Oncology conducted a multi-institutional retrospective study of outcome and complications in 60 cats that were treated with a maxillectomy.\textsuperscript{7} Even though 56.7\% of cats experience post-operative complications, this number was considered acceptable when considering the adverse reactions to other treatments for feline oral tumors.\textsuperscript{7} Far fewer cats necessitated feeding tube placement than what was required with mandibulectomies, and all cats resumed voluntary feeding.\textsuperscript{7} Most notable was the improved 1- and 2-year survival rates of 83\% for cats with squamous cell carcinoma, which may be multifactorial resulting from better local control, adjuvant chemotherapy or radiation therapy, or selection bias.\textsuperscript{7}

Chemotherapy

Chemotherapy administered alone in the gross disease setting to treat feline oral squamous cell carcinoma is minimally effective. Multimodal treatments for human head and neck
carcinomas are recommended in attempt to achieve local tumor control and improve survival. Detailed below are summaries of reported chemotherapeutics administered in combination with radiation therapy to treat feline oral squamous cell carcinoma.

**Gemcitabine**

Gemcitabine is a deoxycytidine analog that exhibits S-phase specific cytotoxicity and has a strong synergy with radiation.\(^{19}\) The first study in treating feline oral squamous cell carcinoma assessed the tolerability of concurrent twice weekly coarse-fractionated radiation therapy (6 fx x 6 Gy) and gemcitabine (25 mg/m\(^2\)) in eight cats. There was an overall response rate of 75% with no observed acute radiation effects, though one cat developed a grade I neutropenia.\(^1\) Of note, only half of the cats received all six fractions of radiation and all six doses of gemcitabine. Three cats died of complications related to treatment and the median remission duration was 42.5 days with a median overall survival of 111.5 days.\(^1\) Gemcitabine was suspected to have a transient biological effect, though there was no benefit on survival.\(^1\)

To evaluate the radiosensitization potential of gemcitabine, LeBlanc et al. treated ten cats with oral squamous cell carcinoma and fifteen dogs with sinonasal carcinoma; however, these patients received a definitive-intent radiation therapy protocol and a twice weekly radiosensitizing dose of gemcitabine, which was half of the reported cytotoxic dose for each species.\(^{20}\) Regarding feline toxicity from treatment, only six cats were able to receive the prescribed radiation dose of 57 Gy as the others were treated to 53 Gy due to severity of normal tissue toxicity. Based on the acute radiation morbidity scoring system established by the Veterinary Radiation Therapy Oncology Group (VRTOG), there were a range of local tissue toxicities observed with grade 3 to the mucous membranes (n=3), grade 2 to the skin, eyes, and mucous membranes (n=9), and grade 1 to the skin and eyes (n=5).\(^{20,21}\) The average gemcitabine dose was 21 mg/m\(^2\) (15-25 mg/m\(^2\)) which was given to the cats over a range of three to six doses.\(^{20}\) There were four dose reductions or delays due to hematologic toxicity, consisting of two grade 3-4 and ten grade 1-2 neutropenias, and two dose reductions or delays from quality of life concerns.\(^{20}\) Though the treatments were not as uniform as planned, the median time of tumor control was 90 days with seven cats succumbing to progressive local disease.\(^{20}\) This protocol produced significant local
toxicity above what is generally expected with radiation therapy alone and there was a surprising
degree of myelosuppression, which was attributed to the radiosensitization and dosing schedule
of gemcitabine.20

**Mitoxantrone**

Mitoxantrone is an antitumor antibiotic that intercalates DNA by inhibiting topoisomerase II
and has strong synergy with radiation.19, 22 Eleven cats with oral squamous cell carcinoma were
treated with mitoxantrone (2.5-6.0 mg/m², every 21 days) at the start of a three-week radiation
therapy protocol consisting of ten to fifteen fractions to a total dose of 44-65 Gy.22 Oral mucositis
was the only described acute radiation toxicosis and there were no mitoxantrone-induced toxicoses
(median 2 doses, range 1-10).22 Eight cats achieved a complete remission that lasted for a median
time of 170 days and one cat had a partial response of 80 days; survival times were not
documented.22 This treatment was more effective than prior reports of either treatment alone and
the lack of myelosuppression warrants further studies in mitoxantrone dose escalation.22

**Carboplatin**

Carboplatin is an alkylating agent that causes excessive DNA cross-linking and has no
known synergy with radiation.19 Fidel et al. previously determined an accelerated radiation therapy
protocol comprised of fourteen fractions of 3.5 Gy given twice daily over nine days (49 Gy total)
was well tolerated with an acceptable level of toxicity to the skin and mucous membranes.23 The
addition of carboplatin (90-100 mg/m²) given 1 hour before the first fraction and again halfway
through treatment (day 4.5) was hypothesized to increase treatment efficacy.24 Thirty one cats
completed the combination therapy with minimal deviations in protocol leading to a median survival
of 163 days.24 Acute radiation toxicity was observed to the skin (VRTOG grade 1-2, n=27) and
mucous membranes (VRTOG grade 2-3, n=28).21, 24 Late radiation toxicity discovered over 100
days post-treatment affected two cats with bone necrosis (n=2, VRTOG grade 3) and lip fibrosis
(n=1, VRTOG 2).21, 24 Most cats (n=25) needed enteral support with placement of a feeding tube
and supportive care with antibiotics (n=27) and nonsteroidal anti-inflammatory drugs (n=18), which
made discerning chemotherapy side effects difficult, though there were no renal or hematologic
toxicoses seen.\textsuperscript{24} Seven cats received adjuvant carboplatin at 180 mg/m\textsuperscript{2} and six cats had neoadjuvant or adjuvant surgery.\textsuperscript{24}

\textbf{Combination bleomycin, thalidomide, piroxicam}

Bleomycin is an alkylating agent that intercalates DNA which prevents thymidine incorporation into DNA, and also forms complexes with iron that lead to free radical formation when combined with oxygen.\textsuperscript{8, 19} Thalidomide is an immunomodulator that has anti-angiogenic activity based on the inhibition of vascular endothelial growth factor (VEGF).\textsuperscript{8, 25} Piroxicam is a nonsteroidal anti-inflammatory with nonspecific COX-inhibition. Marconato et al. evaluated of six cats with unresectable head and neck squamous cell carcinoma treated with neoadjuvant medical therapy consisting of weekly bleomycin (10 UI/m\textsuperscript{2}), daily thalidomide (2 mg/kg), and every other day piroxicam (0.3 mg/kg), followed by surgery and adjuvant radiation therapy or radiation therapy and adjuvant surgery, pending the tumor’s response to medical treatment.\textsuperscript{8} An accelerated hypofractionated radiation protocol comprised of twice daily 4.8 Gy fractions for five consecutive days was delivered to all six cats.\textsuperscript{8} After local treatment, two cats developed a second cancer, though both primary tumors had responded (one CR, one PR).\textsuperscript{8} Four cats continued medical treatment with a total of ten doses of bleomycin administered. One cat died of metastatic disease, but the remaining three cats had complete responses and were alive at the end of the study at 362, 458, and 759 days.\textsuperscript{8} No hematologic or renal toxicities occurred and four cats developed mucositis (VRTOG grade 1-2), three of which necessitated temporary feeding tubes.\textsuperscript{8, 21} Despite the aggressive multimodal approach to treatment, these cats in this pilot study maintained a good quality of life during therapy and had resolution of clinical signs.\textsuperscript{8}

Following the promising results of six cats, a retrospective case series was published assessing 51 cats with macroscopic oral squamous cell carcinoma that received medical treatment with the same regime as detailed above using bleomycin, thalidomide, and piroxicam as sole treatment (n=24) or in addition to accelerated hypofractionated radiation therapy (48 Gy in ten fractions over five days; n=27).\textsuperscript{26} Cats only treated medically had a median progression free interval of 30 days and median overall survival of 38 days.\textsuperscript{26} Cats that had both therapies had a significantly improved outcome with a progression free interval of 179 day and median overall survival of 138
days; however, development of severe acute radiation side effects correlated to a significantly shortened survival. All of the medically treated cats developed hematologic toxicities and all 27 of the irradiated cats had radiation side effects. The median overall survival time decreased with increasing VRTOG grade, with grade 1 (n=11) at 344 days, grade 2 (n=8) at 89 days, and grade 3 (n=8) at 84 days. Six cats were euthanized as a sequel of their acute effects. The poor outcome and severity and occurrence rate of acute toxicity made this protocol unsafe for further use.

**Toceranib phosphate**

Toceranib phosphate is a small molecule inhibitor that inhibits several receptor tyrosine kinases, such as kit, vascular endothelial growth factor, platelet-derived growth factor, colony stimulating factor-1, and FMS-like tyrosine kinase 3 that is suspected to have direct antitumor effects and inhibit angiogenesis. Toceranib phosphate in combination with other treatment modalities in 35 cats with oral squamous cell carcinoma was retrospectively evaluated. A median dose of 2.75 mg/kg of toceranib phosphate was given on a Monday, Wednesday, Friday schedule for a median treatment duration of 77 days. Nine cats received palliative-intent hypofractionated radiation therapy at varied protocols and prescriptions. Three cats failed treatment with toceranib phosphate and received either carboplatin or mitoxantrone and nineteen cats were treated concurrently with a nonsteroidal anti-inflammatory drug. Six out of the seven toxicities were attributed to toceranib phosphate, though all were mild (anorexia, vomiting) apart from a hepatopathy that prompted discontinuation. Toceranib phosphate appeared to be well tolerated by most cats; survival and response was not measured.

The benefit of toceranib phosphate and toxicity was evaluated in a population of 23 cats compared to a control group (n=23), in the absence of radiation therapy in a total of 46 cats with macroscopic oral squamous cell carcinoma. There was an overall response rate of 56.5% to toceranib phosphate (median starting dose of 2.52 mg/kg every 48 hours) with or without a concurrent nonsteroidal anti-inflammatory drug. Cats that were treated had a significantly longer median survival time of 123 days, compared to 45 days for cats that did not receive toceranib phosphate. Of the 13 cats that responded, the median overall survival time was 201.5 days, but
the ten cats that did not respond had a significantly shorter survival of 73 days.\textsuperscript{27} Adverse events of toceranib phosphate included low grade gastrointestinal signs, lethargy, grade I-II anemia or neutropenia, progressive azotemia, and hepatotoxicity.\textsuperscript{27} Further pharmacokinetic data in cats treated with only toceranib phosphate is warranted prior to considering it as a part of a multimodal therapy.\textsuperscript{27}

Radiation therapy

External beam radiation therapy

Hypofractionated

Palliative-intent radiation therapy protocols are often prescribed based on the owner’s treatment goals for their pet with the aim of alleviating pain caused by a tumor and potentially improving function. Seven cats with oral squamous cell carcinoma were treated with coarse fractions of 8 Gy given on days 0, 7, and 21.\textsuperscript{2} Some cats were undergoing concurrent treatment with mitoxantrone (6 mg/m\textsuperscript{2} every 21 days, n=2) and piroxicam (0.3 mg/kg daily, n=3).\textsuperscript{2} Three cats received one or two fractions of radiation before cessation of treatment and humane euthanasia was elected due to reduced quality of life from acute radiation side effects or tumor progression (stomatitis, halitosis, mucositis, anorexia, depression).\textsuperscript{2} The median overall survival was 60 days and although this result represented a small sample size, there was a lack of palliation among patients.\textsuperscript{2}

To retrospectively evaluate quality of life with a palliative-intent radiation protocol, the medical records of 54 cats with oral squamous cell carcinoma treated with three to four fractions to a total of 24-40 Gy over four to five weeks were reviewed.\textsuperscript{28} 32 cats received concurrent chemotherapy with a carboplatin (89-225 mg/m\textsuperscript{2} every 21 days) with or without alternating gemcitabine (50-80 mg/m\textsuperscript{2}).\textsuperscript{28} Acute radiation side effects occurred in 9 cats (VRTOG grade 1 skin, grade 2 mucositis) and three cats developed tongue fibrosis (VRTOG 3), though the majority of cats had no side effects.\textsuperscript{21, 28} Five cats did not complete treatment due to disease progression and of the remaining 49 cats, the median overall survival was 92 days.\textsuperscript{28} Interestingly, there was no clear benefit of concurrent chemotherapy, as absence of
Chemotherapy was associated with a better outcome with a median survival of 113 days compared to 80 days (carboplatin only 84 days, carboplatin/gemcitabine 71 days). A subjective improvement in quality of life was noted by the owners of 32 cats.

Hypofractionated accelerated

In 2007 researchers at Washington State University first described an accelerated protocol consisting of 14 fractions of 3.5 Gy given twice daily over nine days to treat feline oral squamous cell carcinoma in an attempt to reduce the impact of repopulation on radioresistance. Nine cats were treated, primarily with electrons, to 49 Gy, with concurrent supportive care (analgesia, NSAIDs, antibiotics, feeding tube placement), and no chemotherapeutic agents were administered. All cats had a subjective improvement in quality of life, despite occurrence of acute radiation side effects to the mucous membranes (VRTOG grade 2 (n=2), grade 3 (n=7)).

All tumors responded to therapy with three cats achieving a complete remission and six cats had a partial response with a greater than 50% reduction in tumor volume. Although a good response to treatment was seen, the overall median survival was still poor at 86 days.

Another protocol that delivered ten once daily fractions of 4.8 Gy to 21 cats with oral squamous cell carcinoma was assessed for efficacy and toxicity. Neoadjuvant surgery was performed in six cats and three cats received a dose of carboplatin (240 mg/m²) with no response. Over the protocol duration of twelve days, 17 macroscopic tumors and four microscopic tumors were irradiated. All cats had a temporary feeding tube placed, completed the prescribed protocol, and developed VRTOG grade 2 mucositis. Late side effects apart from leukotrichia and alopecia were noted in two cats with a complete remission: one cat developing a nonhealing sublingual ulcer and another had an oronasal fistula. The median progression-free survival was 105 days with a median overall survival of 174 days. Based on RECIST criteria, 16 out of 17 cats responded with seven complete remissions, five partial responses, and four cats with stable disease. Six cats survived longer than one year and four of cats developed metastatic disease to the regional lymph nodes, brain, lung, and/or skin. This protocol was well tolerated with an acceptable side effect profile, but the short duration of response remained, indicating room for improvement with multimodal therapy.
Stereotactic Radiation Therapy

Limited outcome data using stereotactic radiation therapy in the treatment of feline oral squamous cell carcinoma exists. A prospective clinical trial from Colorado State University was aimed at evaluating prognostic factors in 20 cats treated with stereotactic radiation therapy and the outcomes were well described.\textsuperscript{31} Two cats received 3 fractions of 10 Gy before the protocol was adjusted to one fraction of 20 Gy due to risk of repeat anesthetic episodes for patients with comorbidities.\textsuperscript{31} The median progression-free interval was 87 days and the median overall survival was 106 days.\textsuperscript{31} Nine cats developed mild acute radiation side effects (VRTOG grade 1 eye, skin, and/or mucous membranes), yet more concerning were the late radiation side effects encountered, including six mandibular fractures, laryngeal fibrosis in three cats, and one cat with an oronasal fistula.\textsuperscript{31} With conventionally fractionated radiation therapy, there is a 3\% risk of late side effects and if they occur it is generally many months to years later; conversely, the late side effects with this stereotactic protocol generally happened within 3 months. An overall response rate of 38.5\% was seen based on advanced imaging of 13 cats performed 30-days after stereotactic radiation therapy completion.\textsuperscript{31} This protocol appeared to be more palliative-intent and did not provide a survival benefit despite the increased expense and risk of late effects.\textsuperscript{31}

Intratumoral etanidazole and hypofractionated radiation therapy

Etanidazole is a nitroimidazole that has a high electron affinity and undergoes a reduction process in the absence of oxygen that leads to permanent DNA damage.\textsuperscript{19} Though etanidazole is less neurotoxic than misonidazole, the clinical benefit of both drugs is questionable when compared to radiation therapy alone.\textsuperscript{19, 32} Eleven cats with macroscopic oral squamous cell carcinoma were treated with twelve fractions of 4.3 Gy delivered on a Monday, Wednesday, Friday schedule.\textsuperscript{32} Intratumoral injections of etanidazole (350 mg/ml, 0.1 ml every 0.5 cm) were administered either on alternating fractions or at each fraction of radiation therapy, at least 30 minutes before irradiation.\textsuperscript{32} All eleven cats experienced a partial response to treatment with a median tumor volume regression of 70\%.\textsuperscript{32} During therapy, adverse events included self-limiting fever and leukopenia.\textsuperscript{32} Radiation toxicities encountered were anorexia caused by tongue necrosis and osteonecrosis (n=5), which, in addition to tumor recurrence, lead to euthanasia.
(median overall survival 116 days). The late effects and lack of improved outcome compared to radiation therapy alone warrant further investigation before considering radiosensitization with intratumoral etanidazole.

**Brachytherapy with holmium-166 microspheres**

A microbrachytherapy pilot study using holmium-166 microspheres that emit β-radiation (half-life 26.8 hours, average energy 1.84 MeV, mean penetrance of 3.2 mm, 10-30 μm diameter) was evaluated for safety and efficacy in thirteen cats with oral squamous cell carcinoma. Microsphere treatment groups were divided based on amount of absorbed dose, high (>800 Gy), intermediate (400 Gy), and low (200 Gy), with a median absorbed dose of 546 Gy. Ten cats had sublingual tumors and five of these had laser ablation performed either before or after microbrachytherapy. Two cats died during or immediately after treatment and another two cats died within one week of treatment. Six cats with small tumor volumes responded to treatment (3 complete remission, 3 partial response), which correlated to a greater median survival of 296 days, though the overall survival of all cats was 113 days. Other adverse events included gingival ulceration related to ¹⁶⁶Ho treatment and rostral tongue necrosis following laser surgery in two cats. Though this minimally invasive, likely one-off microbrachytherapy treatment may have provided benefit to a small subset of cats, the survival benefit compared to the risk of immediate death failed to be elucidated.

**Plesiotherapy with strontium-90**

A two-patient case report describing the use of strontium-90 plesiotherapy that emits β-radiation (half-life 28.8 years, average ⁹⁰Y energy 2.27 MeV, superficial penetrance up to 3 mm, 8 mm active diameter) was used to treat feline oral squamous cell carcinoma. One cat received a total of five fractions of plesiotherapy to treat three lesions on the hard palate over a period of 21 months. During the follow-up period, a squamous cell carcinoma was discovered on the left caudal mandible and was surgically resected via a left mandibulectomy. This cat received four doses of adjuvant mitoxantrone in addition to piroxicam (doses not reported) and survived a total of 5 years and 9 months following initial diagnosis, ultimately succumbing to a spinal injury. The other cat had a sublingual mass in the frenulum that was treated with two fractions delivered seven
weeks apart (100 Gy to six fields, then 100 Gy to 3 fields). The response to each treatment was short-lived with the first remission lasting six weeks and the second was eight weeks before disease progression lead to euthanasia 7 months after diagnosis. Plesiotherapy remains an attractive option with minimal side effects for the few patients that may present with superficial squamous cell carcinoma.
Protocol and measurement

The single-cell gel electrophoresis, or comet assay, is a sensitive and relatively easy test to measure deoxyribonucleic acid (DNA) damage and repair at the cellular level. Cells are eluded into a single-cell suspension, embedded into agarose, lysed, and then undergo electrophoresis. During electrophoresis, an electrical current is applied to the cells submerged in an alkaline solution and the negatively charged damaged DNA fragments move out of the nucleus toward the anode, which creates a comet tail structure. Undamaged DNA remains in the nucleus and forms the head of the comet. Comets can be visualized after application of a DNA-binding dye under fluorescent microscopy and scored using automated image analysis software or visual scoring. Computer image analysis is preferred to the subjective classification of visual scoring as it uses pixel-based computation to provide a range of objective quantified variables from each comet.

Percent DNA in the tail and comet tail moment are common measurements analyzed from comet assay data. The circularity of the entire comet and comet head were measured by creating primary and subpopulation parameters for the Agilent BioTek Gen5 data analysis software. The equation used was:

\[
\text{Percent DNA in tail} = \left[1 - \left(\frac{\text{total comet circularity}}{\text{comet head circularity}}\right)\right] \times 100
\]

The above fraction is the proportion of DNA in the comet head, and by subtracting this from 1, the remaining amount times 100 is the percentage of DNA within the comet tail. Increased genotoxicity was hypothesized with increasing radiation dose, which was apparent as the percent DNA in the tail increased.

Comet tail moment calculations used the same comet circularity parameters previously described in addition to size measurements (length). The tail moment length was the addition of half of the comet tail length to half of the comet head length. Tail moment length was multiplied by the percent DNA in tail, then divided by 100 to remove the percentage from the expression. The equation used was:
Comet tail moment = percent DNA in tail x \([(\text{total comet length} - \text{comet head length})/2) + (\text{comet head length}/2)] / 100

Tail moment values increased with more DNA damage, so if there was little damage the values would near zero.\(^{41}\)

The comet assay can be performed under neutral or alkaline conditions, which pertains to the pH of the electrophoresis solutions. Alkaline solutions are rich in hydroxide ions that better denature smaller amounts of DNA damage, like single-stranded breaks (SSBs), base damage, and DNA cross-links as well as double-stranded breaks (DSBs).\(^{37,42}\) Conversely, neutral solutions are typically used to quantify only DSBs.\(^{35,36}\) There is debate claiming both solutions pick up both kinds of DNA breaks as histones are removed in the lysis step, which allows for the unwinding of broken DNA.\(^{35,38}\) An explanation for the difference in homogeneity of comet appearance could be whether the DNA matrix organization was maintained (neutral) or detached (alkaline) during electrophoresis.\(^{38}\) More recent approaches to increase the sensitivity for specific lesions use the comet assay combined with a lesion-specific endonucleases, that are able to discern an array of lesions, such as purines from pyrimidines, pyrimidine dimers, and DNA repair intermediates.\(^{38,43}\)

**Relationship between cellular radiosensitivity**

The comet assay can be performed at different time intervals to assess for the amount of DNA damage and subsequent repair.\(^{40}\) Mechanisms to repair DNA damage can occur quickly over 10-20 minutes for single-strand breaks and more slowly, up to four hours after treatment, for double-strand breaks.\(^{42}\) DNA that was mis-joined or not re-joined (residual DNA damage) may lead to chromosomal aberrations, mitotic catastrophe, and eventual cell death.\(^{42,44}\) Previous studies looked at radiosensitivity using the clonogenic survival assay that measured reproductive death induced by radiation. The amount of residual DSBs after a minimum of four hours after irradiation was the greatest parameter of clonogenic radiosensitivity.\(^{42,44,45}\) This was directly corelated to the damage observed in the alkaline comet assay. To preserve time compared to the clonogenic assay, the comet assay can be valuable in predicting the radiosensitivity of a cell.\(^{42,44}\)
**Application**

There have been a multitude of applications of the comet assay since its introduction in 1988. From evaluation of human and animal cells to plants and even food, the comet assay is a rapid and relatively simple means to assess DNA damage and repair. Leukocyte damage from exposure to dietary free radicals, chemotherapeutics, and radiation in a variety of species has been assessed. The radiosensitivity of different cancer cell lines has been evaluated by combining the comet assay with clonogenic survival. There have been implications of the comet assay to predict the prognosis and an individual’s response to therapy, though this single time-point sample may only give insight into a moment of the complexity that is carcinogenesis.
CHAPTER 2: SINGLE GEL ELECTROPHORESIS USING COMET ASSAY

Methods and materials

Materials

Assay and experimental components

High glucose DMEM (Gibco #11965092)

Heat inactivated fetal bovine serum (FBS) (Gibco #16140071)

Penicillin-streptomycin antibiotics (Gibco # 15140122)

Dulbecco's phosphate-buffered saline (DPBS), Ca** and Mg** free (Gibco #14190144)

TrypLE™ Express Enzyme (1X) (Gibco #12604021)

CometAssay LMagarose (part number 4250-050-02, Trevigen Inc. Gaithersburg, MD)

Tissue culture dish, TC-treated, 10 cm (Corning #430167)

2-well CometSlides (part number 4250-004-03, Trevigen Inc. Gaithersburg, MD)

Sodium hydroxide pellets (Fisher #BP359)

Lysis Buffer (part number 4250-050-01, Trevigen Inc. Gaithersburg, MD)

CometAssay Electrophoresis System (part number 4250-050-ES, Trevigen Inc. Gaithersburg, MD)

SYBR Gold Nucleic Acid Gel Stain, diluted to 1X (part number S-11494, ThermoFisher, Carlsbad, CA)

Countess 3 cell counter (Invitrogen)

Agilent BioTek Lionheart FX automated microscope

Agilent BioTek Gen5 data analysis software

Agilent BioTek Lionheart FX automated microscope

The Agilent BioTek Lionheart FX is a modular multimode microplate reader combined with automated digital microscopy. Filter- and monochromator-based microplate reading is available, and the microscopy module provides up to 60x magnification in fluorescence, brightfield, color brightfield, and phase contrast. With special emphasis on live-cell assays, Lionheart FX features temperature control to 65°C, CO2/O2 gas control, and dual injectors for
kinetic assays. The instrument was used to image the stained DNA on the CometSlides using the GFP imaging channel. A slide adapter (part number 1220548) was used to hold the 2-well CometSlides.

**Agilent BioTek Gen5 data analysis software**

Integrated Gen5 software controls Lionheart FX for automated digital microscopy and fluorescence. Image acquisition is automated including sample translation, focusing, and exposure control. Cellular analysis allows independent analysis of the entire comet and comet head.

**Methods**

**Cells and cell culture**

Three established FOSCC cell lines, SCCF1, SCCF2, and SCCF3, were kindly provided by Dr. Angela McCleary-Wheeler for this study, through the generous gift of Dr. Joseph Wakshlag (Cornell University). Each cell line was tested for mycoplasma expression by quantitative polymerase chain reaction (qPCR) before experimentation. Cells were grown in complete medium containing high glucose DMEM, 10% heat inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin (10,000 U/ml), and incubated at 37°C in 5% CO₂. To maintain high viability, cells were trypsinized and split every 3 days at 1:4 (SCCF1), 1:10 (SCCF2), or 1:40 (SCCF3) ratios.

**Ionizing radiation**

FOSCC cell lines were grown in 10 cm plates and used for experiments once they reached 80% to 90% confluence. Each cell line contained a control that was transported but did not receive treatment with radiation (mock-irradiated). Irradiated cells received 3, 6, or 9 Gy. Irradiation was performed with 6 MV photons from a linear accelerator (Integrity, Elekta AB, Stockholm, Sweden). Irradiation occurred from 180° with a field size of 30 cm × 30 cm at a dose rate of 600 MU/minute (approximately 1 cGy/MU). There was a source-surface distance (SSD) of 110 cm and cell plates rested on a 1 cm tissue equivalent preformed gel sheet (Superflab, Eckert & Ziegler BEBIG GmbH, Berlin, Germany).
Standard alkaline comet assay

The standard alkaline comet assay was performed using three established FOSCC cell lines that were either the control cells or irradiated with 3, 6, or 9 Gy. Cells were trypsinized, collected using complete medium, and counted using the Countess 3 cell counter. Cells were then spun at 500g for 5 minutes and the pellet was resuspended at a concentration of $1 \times 10^5$ cells/mL. The cells were embedded in low melting agarose, and immobilized on 2-well specially treated slides to promote adherence. The slides were then placed at 4°C for 45 minutes to allow agarose polymerization. Subsequently, the slides were placed in lysis buffer for 30-60 minutes at 4°C in the dark, followed by a 20-minute room temperature equilibration in an alkaline unwinding solution. Alkaline electrophoresis was run at 21 volts for 30 minutes at 4°C using the CometAssay Electrophoresis System unit. Slides were neutralized by washing twice with excess dH$_2$O for 10 minutes at room temperature and immersed in 70% ethanol for 5 minutes at room temperature. The agarose was dried (37°C for 30-45 minutes) before staining the DNA with diluted SYBR Gold. Dry images were analyzed with Lionheart FX and integrated Gen5 Data Analysis Software. This experiment was performed on each cell line in triplicate.

Statistical analysis

Data were coded in Microsoft Excel (2018, Microsoft, Redmond, Washington) and analyzed with SigmaPlot for Windows (Version 14.0, 2017, Systat Software, San Jose, California). Differences in the percent DNA in tail and comet tail moment between the cell lines were tested for statistical significance using one-way analysis of variance test, as the quantitative continuous data was non-parametric. To determine if a treatment dose was significant, pair-wise comparisons were made using the Tukey Test. The means, standard error of the means, and standard deviations were illustrated using SigmaPlot. Results of statistical analyses with a P-value <0.05 were considered statistically significant.

2-well CometSlide imaging and analysis

Accurate automated comet imaging was first validated using the CometAssay alkaline control cells containing known levels of DNA damage, processed using the standard alkaline comet assay on 2-well CometSlide slide format. All slide wells were automatically imaged using
previously determined offsets, eliminating manual determination of slide well locations. As the agarose on the slides was previously dried down, the slides were placed in the slide adapter after staining with the stained wells facing down, in a typical slide configuration. Using a 5 × 4 image montage, a 3,427 × 3,345 μm area was captured from each well of the slides, yielding typical comet counts of 400 to 500 per well. Exposure parameters were set such that fluorescence values per pixel were within the quantifiable range of the CCD camera. Autofocus on all individual images in the montage was also performed. The combination allowed the signal to be accurately captured from comets containing no DNA damage, with the fluorescence emanating solely from the comet head and from comets containing high DNA damage, where fluorescence was distributed between the comet head and tail.

**Percent DNA in tail calculation**

Two separate, automated cellular analyses calculated “Percent DNA in the Tail”, considering the change in comet circularity when DNA was damaged by radiation. The circularity of the entire comet and comet head were measured by creating primary and subpopulation parameters for the Agilent BioTek Gen5 data analysis software. The equation used was:

\[
\text{Percent DNA in tail} = \left[1 - \left(\text{total comet circularity}/\text{comet head circularity}\right)\right] \times 100
\]

The above fraction is the proportion of DNA in the comet head, and by subtracting this from 1, the remaining amount time 100 is the percentage of DNA within the comet tail. Percent DNA in the tail was calculated for duplicate 2-well slides, using each of the three feline oral squamous cell lines (SCCF1-3). The percent DNA in tail values increased appropriately with increasing radiation dose.

**Comet tail moment calculation**

Comet tail moment calculations used the same comet circularity parameters previously described in addition to size measurements (length). The tail moment length was the addition of half of the comet tail length to half of the comet head length. Tail moment length was multiplied by the percent DNA in tail, then divided by 100 to remove the percentage from the expression. The equation used was:
Comet tail moment = percent DNA in tail x \[[((\text{total comet length} - \text{comet head length})/2) + (\text{comet head length}/2)) / 100\]

Tail moment values increased with more DNA damage, so if there was little damage the values would near zero.\(^\text{41}\)

**Results**

The comet assay percent DNA in tail and comet tail moment calculation results for SCCF1, SCCF2, and SCCF3 were assessed immediately following x-ray irradiation at 0, 3, 6, and 9 Gy. The means for each cell line were expected to have a linear dose response relationship; however, this was not appreciated. The SCCF2 cell line showed the least amount of DNA damage at each radiation dose in both percent DNA in tail and comet tail moment. SCCF1 showed a significant difference between the mean values among treatments with 9 Gy and the control (0 Gy) and 9 Gy and 3 Gy on evaluation of percent DNA in the tail (p=0.019 and p=0.022). The difference between 9 Gy and the control was also noted on analysis of the comet tail moment for SCCF1 cells (p=0.049).

**Discussion**

Assessment of DNA damage in cancer cells using the comet assay has been applied very little in veterinary medicine. Most studies focus on a causative agent and its effects on lymphocytes via the comet assay. Only a few reports describe the results of irradiation in vitro or in/ex vivo for canine tumors, including soft tissue sarcoma, melanoma, squamous cell carcinoma, fibrosarcoma, and hemangiopericytoma.\(^{44, 48}\) This is the first study evaluating DNA damage of any feline neoplasm. Squamous cell carcinoma was chosen based on cell availability and desire to perform a basic science project involving radiation.

*In vitro* analysis of DNA damage occurred immediately following irradiation. Cell cultures were placed on ice and returned to the tissue culture room for preparation. A control sample for each cell line was transported and handled the same as the treated cultures, without irradiation. This was presumed to be a baseline for iatrogenic cell injury, but the results were equivocal with some controls having greater DNA damage than treated cells, particularly SCCF3. Only one cell line (SCCF1) had significant differences between treatment groups of 0 Gy and 9 Gy. This linear,
dose-dependent trend of increasing DNA damage was hypothesized to occur with all cell lines, pending their inherent radiosensitivity, and produce a similar slope. The experiment was performed in triplicate on three separate occasions so as to validate comparable results, but there were large discrepancies between cell lines and experimental days.

The limitations of this study were plentiful and primarily a result of naivety to the comet assay technique and Gen 5 analysis software. Delays in cell shipment and *Mycoplasma spp.* testing resulted in a later than desired start to a benchtop experiment, which made subsequent experimental components that may have offered greater analysis time prohibitive. Determining image preprocessing and cellular analysis parameters created an unanticipated obstacle to experimental progression. The initial goal was to perform the comet assay at different time intervals after irradiation to assess the repair of DNA damage.

The Trevigen CometAssay control cells were prepared and aliquoted based on the provided instructions; however, this was the first successful comet assay experiment in the lab. As such, there was a learning curve when performing the alkaline comet assay protocol. Some sample loss occurred when trying to troubleshoot the low-melt agarose congealing after molten and in a 37°C water bath. The cells and agarose must first be adhered to the CometSlide before electrophoresis and samples washed off during lysis and unwinding steps. Any image analysis data for samples that were not present were removed from statistical analysis to eliminate type I errors.
CHAPTER 3: CONCLUSIONS AND FUTURE DIRECTIONS

Radiation therapy is a therapeutic treatment for companion animals and humans with an ever-evolving application from inflammatory conditions to neoplastic disease processes. Just as next-generation sequencing has revolutionized precision medicine, customization of therapy based on a tumor’s chemo- or radio-sensitivity and repair capacity can be performed using tools like the comet assay. The assay can be performed over an afternoon compared to the weeks required to perform clonogenic assays and it is presumptively less expensive. Further studies are necessary to validate results with a range of tumor types before clinical implementation of this tool occurs. Investigation into other cellular factors influencing radiosensitivity and cell-cycle checkpoints is warranted.

Establishing the α/β dose for more canine and feline cancers would enhance our radiobiological understanding and perceived tumor response to different radiation therapy protocols. The increased occurrence of late radiation side effects with hypofractionated or stereotactic radiation therapy protocols observed in a relatively short follow-up time may not change the recommended smaller fraction size in the treatment of feline oral squamous cell carcinoma. Maintenance of good quality life, acceptable normal tissue toxicity, while still effectively treating cancer is a fine balance. The most durable responses to this tumor type were with surgical excision alone that was seen with smaller tumors detected earlier, which emphasizes the importance of local tumor control. A few cats with oral squamous cell carcinoma will develop metastatic disease but in the initial treatment phase, aggressive multimodal treatment is recommended to prevent succumbing to tumor recurrence or progression.
Figure 1: Stitched image of SCCF1 control cells (not irradiated).
Figure 2: Comet primary (above) and secondary (below) cellular analysis object masks.
Figure 3: Comet areas included in comet tail moment calculation.
Figure 4: Box and whisker plot of SCCF1 percent DNA in the tail treated at different doses of radiation. There was a significant difference between cells treated with 9 Gy and 0 Gy, as well as 9 Gy and 3 Gy.
Figure 5: Box and whisker plot of SCCF2 percent DNA in the tail treated at different doses of radiation. No significant difference was detected between treatment groups.
Figure 6: Box and whisker plot of SCCF3 percent DNA in the tail treated at different doses of radiation. No significant difference was detected between treatment groups.
Figure 7: Box and whisker plot of SCCF1 comet tail moment treated at different doses of radiation. There was a significant difference between cells treated with 9 Gy and 0 Gy. 

* p=0.049
Figure 8: Box and whisker plot of SCCF2 comet tail moment treated at different doses of radiation. There was a significant difference between cells treated with 6 Gy and 0 Gy.
Figure 9: Box and whisker plot of SCCF3 comet tail moment treated at different doses of radiation. No significant difference was detected between treatment groups.
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<thead>
<tr>
<th>Imaging parameters</th>
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<tbody>
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<td>Fluorophore</td>
<td>GFP 469, 525</td>
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Table 1: Lionheart FX automated comet imaging parameters
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<td>Background</td>
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<tr>
<td>Background flattening</td>
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<td>Auto</td>
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<td>Image smoothing strength</td>
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<table>
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<tr>
<td>Point Spread Function (PSF)</td>
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<td>Kernel radius</td>
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<th>Image Stitching</th>
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<tr>
<td>Montage size</td>
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<td>Fusion method</td>
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<tr>
<td>Crop stitched image to remove black rectangles on the border</td>
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</table>

| Fill gaps between montage tiles with local background color | Unchecked |
| Downsized final image | Checked, reduced image to 35.72 % |

Table 2: Lionheart FX image processing parameters
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<th>Primary Cellular Analysis Parameters</th>
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</thead>
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<tr>
<td><strong>Threshold</strong></td>
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<td><strong>Background</strong></td>
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<tr>
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<tr>
<td><strong>Minimum object size</strong></td>
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Table 3: Gen5 comet head cellular analysis parameters
Secondary Cellular Analysis Parameters

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<td>Distance from Primary mask</td>
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<tr>
<td>Threshold</td>
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<td>Smooth</td>
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Table 4: Gen5 total comet cellular analysis parameters
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<th>Calculated metric</th>
<th>Description</th>
<th>Data Reduction Designation</th>
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</thead>
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<tr>
<td><strong>Primary mask</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Integral [GFP]</td>
<td>Integrated GFP fluorescence within the primary mask (comet head)</td>
<td>$M_1$</td>
</tr>
<tr>
<td><strong>Secondary mask</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Integral_2[GFP]</td>
<td>Integrated GFP fluorescence within the secondary mask (total comet)</td>
<td>$M_2$</td>
</tr>
<tr>
<td>Percent DNA in tail</td>
<td></td>
<td>$(1 - \frac{M_1}{M_2}) \times 100$</td>
</tr>
</tbody>
</table>

Table 5: Gen5 percent DNA in the tail analysis
<table>
<thead>
<tr>
<th>Calculated metric</th>
<th>Description</th>
<th>Data Reduction Designation</th>
</tr>
</thead>
<tbody>
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<td><strong>Primary mask</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Integral [GFP]</td>
<td>Integrated GFP fluorescence within the primary mask (comet head)</td>
<td>$M_1$</td>
</tr>
<tr>
<td>Area</td>
<td>Area of the primary mask (comet head)</td>
<td>$M_3$</td>
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<tr>
<td>Size</td>
<td>Size of the primary mask (comet head)</td>
<td>$M_5$</td>
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<tr>
<td><strong>Secondary mask</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Integral_2[GFP]</td>
<td>Integrated GFP fluorescence within the secondary mask (total comet)</td>
<td>$M_2$</td>
</tr>
<tr>
<td>Area_2</td>
<td>Area of the secondary mask (total comet)</td>
<td>$M_4$</td>
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<td>Tail moment value</td>
<td>$\left{ \left( 1 - \frac{M_1}{M_2} \right) \times 100 \right} \times \left( \left( \frac{M_3}{M_5} \right) / 2 \right) + \left( \left( \frac{M_4 - M_3}{M_5} \right) / 2 \right) / 100$</td>
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Table 6: Gen5 comet tail moment analysis
BIBLIOGRAPHY


