SHORT COMMUNICATION

Canine mammary tumour cells exposure to sevoflurane: effects on proliferation and neuroepithelial transforming gene 1 expression.

1   **Abstract**

2   **Objective** The influence of perioperative factors, such as anaesthetic and analgesic techniques, on

3   metastatic spread following surgery for primary cancer removal is of growing interest. The present

4   study investigated the effects of sevoflurane on canine mammary tumour cell proliferation (MTT

5   colorimetric assay) and on the expression of the neuroepithelial transforming gene 1 (NET1).

6   **Study design** Prospective controlled *in vitro* trial.

7   **Study material** Primary canine tubular adenocarcinoma (CIPp) and metastatic canine tubular

8   adenocarcinoma (CIPm) cells.

9   **Methods** To perform the MTT tests, cell lines were seeded at a density of 3,000 cells per well and

10 incubated with sevoflurane (1, 2.5 or 4 mM) or only with the culture medium (control). Sevoflurane

11 was added to the cell cultures every hour to avoid changes in drug concentration. MTT assays were

12 performed after 6 hours of exposure obtaining absolute values of absorbance. The RNA isolated

13 from the lysates of the same cell lines underwent quantitative polymerase chain reaction to evaluate

14 NET1 gene expression changes compared to controls. One- and 2-way ANOVAs were used as

15 appropriate ($p < 0.05$).

16 **Results** A significant increase in cell proliferation compared to controls was observed in CIPp

17 treated with lower sevoflurane concentrations, while a significant decrease in cell proliferation was

18 found in CIPm treated with all the sevoflurane concentrations. All treatments of CIPp did not

19 induce changes in gene expression compared to controls, while a significant increase in gene

20 expression was observed in CIPm between controls and the higher sevoflurane concentration.

21 **Conclusions and clinical relevance** Sevoflurane treatments modified the cell proliferation rate in

22 both cell lines showing an increase or a decrease when applied to primary or metastatic canine

23 tubular adenocarcinoma cells, respectively. Expression of the NET1 gene increased after treatment

24 with sevoflurane 4 mM in metastatic cells. The role of sevoflurane on cancer recurrence should be

25 further investigated.

26 ***Keywords*** canine mammary tumour cells, cell proliferation, MTT assay, NET1 gene, tumour,

27 sevoflurane

Introduction

29 Malignant mammary tumours are a significant cause of morbidity and mortality in dogs,

30 representing one of the most common types of cancer and causes of cancer-related death

31 (Karayannopoulou & Lafioniatis 2016), even though the incidence of severe canine mammary

32 tumour cases has been reduced in regions that regularly perform early sterilization (Vascellari et al.

33 2016). Usually, death is the result of recurrence and metastasis (Vascellari et al. 2016). Available

34 treatments are numerous, however, surgical removal of the primary mass is still a major pillar

35 (Karayannopoulou & Lafioniatis 2016). Therefore, the possible influence of perioperative factors

36 on metastatic spread, such as anaesthetic and analgesic techniques, is of growing interest and has

37 been investigated by recent retrospective studies in human medicine (Wigmore et al. 2016).

38 *In vitro* (Ecimovic et al. 2013) and retrospective clinical trials (Wigmore et al. 2016) have shown

39 that volatile agents like sevoflurane might have a pro-tumourigenic effect and consequently

40 facilitate the development of metastasis in many solid tumours (Wigmore et al. 2016). In particular,

41 sevoflurane has been shown to increase human breast cancer cell proliferation, migration and

42 invasion *in vitro* (Ecimovic et al. 2013).

43 Volatile agents are commonly used to maintain anaesthesia of dogs undergoing mastectomy. It is

44 unknown whether sevoflurane could potentially facilitate tumour cell proliferation and migration in

45 this species. Therefore, this study was designed to evaluate, *in vitro,* sevoflurane's ability to affect

46 primary and metastatic canine mammary tumour cell proliferation using a colorimetric assay (MTT:

47 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide). In addition, sevoflurane's effects

48 on the expression of neuroepithelial transforming gene 1 (NET1), that has been associated with cell

49 migration ability (Ecimovic et al. 2014), was also investigated. We hypothesized that 6 hours of

50 sevoflurane exposure to canine mammary tumour cells would lead to an increase in cell

51 proliferation and NET1 gene expression.

52

53 **Materials and methods**

54 Cell culture

55 Established canine mammary tubular adenocarcinoma cell lines derived from one individual's

56 primary (CIPp) and metastatic (CIPm) lesions were used (Uyama et al. 2006). Cells were grown in

57 Roswell Park Memorial Institute medium supplemented with 10% foetal bovine serum (Sigma-

58 Aldrich, MO, USA), 100 μg mL^{-1} penicillin (Sigma-Aldrich), 100 μg mL^{-1} streptomycin (Sigma-

59 Aldrich), 1.5 mg mL^{-1} amphotericin B (Sigma-Aldrich) and incubated for 24 hours at 37°C in a

60 humidified atmosphere with 5% carbon dioxide.

61

62 MTT assay

63 Cells were grown in 75 cm^2 standard tissue culture flask (Sarstedt Ltd, Ireland) as monolayers.

64 Media were changed every three days. Before each experiment, cells were harvested from 70%

65 confluent cultures by trypsinization and counted with an automated cell counter (Automated Cell

66 Counter TC20; Bio-Rad, Italy). Cells were seeded at a density of 3,000 cells per well for a total of

67 six experimental wells in a 96-well cell culture plate (Eppendorf Cell Culture Plate, Eppendorf

68 S.r.l., Italy). The number of 3,000 cells per well was chosen from the preliminary evaluation of a

69 time-dependent exponential cell growth curve. Cells were seeded in triplicates in a concentration

70 range from 1,000 to 10,000 cells per well and incubated for 4, 6 and 12 hours. Subsequently, the

71 proliferation index was assessed using the MTT colorimetric assay. The concentration of 3,000 cells

72 per well was found optimal to show time related cell growth. Before treatment exposure, cells were

73 incubated for 12 hours with 100 μL of normal culture medium to allow homogeneous cell adhesion.

74 A clinically available sevoflurane formulation (Sevorane; AbbVie Oy, Finland) was utilized for the

75 treatment in three different concentrations: 1, 2.5 or 4 mM (treatments: S1, S2.5 and S4,

76 respectively). Cells grown only in the culture medium were used as control. Medium containing

77 sevoflurane (S1, S2.5 and S4 concentrations) was added every hour to the culture medium to avoid

78 decreases in drug concentration over time due to evaporation (Ecimovic et al. 2013). Treatments

79 were removed after 6 hours and cell survival and proliferation assessed with an MTT colorimetric

80 assay according to Tada et al. (1986). Briefly, 20 µL of MTT were diluted in phosphate-buffered

81 saline to reach a concentration of 5 mg mL^{-1} and a pH of 7.5. The solution was added to each well

82 and incubated for 4 hours at 37°C. Subsequently, 0.1 mL of 10% sodium dodecyl sulfate (Sigma-

83 Aldrich) diluted in a solution of 0.01 M HCl was added to each well and incubated overnight.

84 Absolute absorbance was then measured with a spectrophotometer (Microplate Model 680, Bio-

85 Rad) on an ELISA plate reader with a wavelength of 590 nm. Values lower and higher than controls

86 indicated reduction and increase in cell proliferation after treatment, respectively.

87 Quantitative PCR

88 Three hundred thousand cells were seeded in triplicates in p6 culture plates (Eppendorf Cell Culture

89 Plate; Eppendorf S.r.l.). After 6 hours of incubation to permit cell attachment, they were treated

90 with 1 or 4 mM of sevoflurane (treatments: S1 and S4, respectively) for 6 hours. Similarly to the

91 MTT assay and to compensate for the evaporation tendency of this agent, the same concentrations

92 of sevoflurane were added to the cell cultures every hour. Cells cultured without treatment were

93 used as controls. In order to isolate total ribonucleic acid (RNA), culture media were removed at the

94 end of the treatment and 0.5 mL of a ready-to-use reagent designed to isolate high quality total

95 RNA (TRIzol, Sigma-Aldrich, Dublin, Ireland) was added to each well to lyse the cells, according

96 to manufacturer`s instructions. Once a microscopic examination revealed cells to be lysed, the cell

97 lysate was transferred to a 1.5 mL microfuge tube. Thereafter, 200 µL of chloroform were added.

98 The mixture was gently shaken, left at room temperature (25°C) for 15 minutes and centrifuged at

99 13,000 revolutions per minute (RPM) for 15 minutes at 4°C. The upper aqueous layer was

100 transposed into another 1.5 mL tube carefully without touching the genomic and protein-containing

101 interphase. A total of 0.5 mL of ice-cold isopropanol was added to the aqueous phase, the tube

102 gently shaken and left to stand on ice for 10 minutes before being centrifuged at 13,000 RPM for

103 another 10 minutes at 4°C. The supernatant was removed and 1 mL of sterile ethanol (75%) was

104 added to wash the pellet by gently centrifuging (7,500 RPM for 5 minutes). After ethanol removal,

105 the pellet was let to air-dry for 5 minutes before being re-suspended in 50 μL of nuclease-free water

106 by heating it at 60°C for 15 minutes. Total RNA was quantified with an automated electrophoresis

107 system (Experion Electrophoresis System; Bio-Rad,) and complementary deoxyribonucleic acid

108 (cDNA) was synthesized from 1 μg of total RNA using a reverse transcription kit (QuantiTect

109 Reverse Transcription kit; Qiagen, Italy). According to manufacturer's instructions, 1 μg of total

110 RNA was incubated with 2 μL of DNAse buffer treatment (gDNA Wipeout Buffer; Qiagen,) and

111 RNAse free water to reach a total volume of 14 μL for 2 minutes at 42°C and left for 10 minutes on

112 ice. Thereafter, 1 μL of reverse transcriptase (Quantiscript Reverse Transcriptase; Qiagen), 4 μL of

113 a dedicated buffer (Quantiscript RT Buffer 5X; Qiagen,) and 1 μL of a dedicated primer mix (RT

114 Primer mix; Qiagen,) were added and incubated for 15 minutes at 42°C following 3 minutes at

115 95°C to inactivate the reverse transcriptase. One μL of cDNA was used for quantitative polymerase

116 chain reaction (qPCR) to evaluate the relative amount of specific NET1 gene transcript. One μL of

117 cDNA was subjected to qPCR with a dedicated detection chemistry system (IQ SYBR Green

118 Supermix; Bio-Rad) and an optical software system (IQ5 Optical System Software; Bio-Rad,). The

119 sequences of primers used were: canine glyceraldehyde-3-phosphate dehydrogenase (GAPDH, gene

120 bank entry AB038240.1) forward 5'-GGCACAGTCAAGGCTGAGAAC-3', canine GAPDH

121 reverse 5'-CCAGCATCACCCCATTTGAT-3', canine NET1 (Gene bank entry XM_54427.5)

122 forward 5'-CATCAAGAGGACGATCCGGG-3', and canine NET1 reverse 5'-

123 ATTGCTTGGCTCCTCTTGCT-3'. The reaction conditions were: reverse transcription, 3 minutes

124 at 95°C (1 cycle) followed by denaturation for 30 seconds at 95°C and annealing for 30 seconds at

125 60°C (35 cycles). Glyceraldehyde-3-phosphate dehydrogenase expression levels were used to

126 normalize NET1 gene expression. Gene expression was calculated using a relative quantification

127 assay corresponding to the comparative cycle threshold (Ct) method: the amount of target gene,

128 normalized to the endogenous housekeeping gene (GAPDH) and relative to the calibrator (control

129 sample), was then transformed by $2\Delta\Delta Ct$ (one fold increase), where $\Delta\Delta Ct = \Delta Ct \text{ (sample)} - \Delta Ct$

130 (control) and ΔCt is the Ct of the target gene subtracted from the Ct of the housekeeping gene.

131 Values for ΔCt were obtained in triplicate for each sample.

132 Statistical analysis

133 One overall mean value of ΔCt was used for statistical analysis per biological sample (i.e. mean

134 values of the technical triplicates). After log-transformation, residuals were approximately normal

135 and variances approximately equal in all groups (visual inspection). One- and 2-way ANOVA tests

136 were used to analyze the data. In the 2-way ANOVA test, the target variables were the mean values

137 of absorbance at 6 hours of treatment, while the treatment (the three sevoflurane concentrations and

138 the control) and the cell-type (primary and metastatic cells) were used as explanatory variables. In

139 the one-way ANOVAs, the target variables were mRNA expressions of the NET1 gene and the

140 treatment (two sevoflurane concentrations, S1 and S4, and the control) were the explanatory

141 variables. In both sets of ANOVAs, pairwise differences between treatments and controls were

142 tested for significance; a value of $p < 0.05$ was considered statistically significant. All statistical

143 analyses were performed with an open-source statistical software package (R-studio, version 3.2.0;

144 www.r-project.org, MA, USA). Data are presented as mean ± standard error and ranges for the %

145 increases or decreases of cell proliferation rate.

146

147 **Results**

148 Mean ± standard errors absorbance values are displayed in Figures 1a and 1b. A statistically

149 significant increase in cell proliferation rate compared to controls was observed in CIPp treated

150 with S1 and S2.5 (Fig. 1a) of 23% and 13%, respectively. Conversely, a significant decrease in cell

151 proliferation rate was found in CIPm treated with all the tested concentrations of sevoflurane (Fig.

152 1b; S1= -33%, S2.5= -41% and S4= -62%).

153

154   Both, S1 and S4 did not induce any significant change in NET1 gene expression in CIPp cells

155   compared to controls (Fig. 1c). A significant increase in gene expression was observed only in

156   CIPm between controls and the cells treated with S4 (Fig. 1d).

157

158   **Discussion**

159   In the present study, a commercially available sevoflurane formulation effectively modified cellular

160   proliferation in both cell lines in a divergent manner, increasing cell proliferation in CIPp but

161   decreasing it in CIPm. Interestingly, NET1 gene expression was significantly increased only in

162   CIPm cells treated with the higher concentration of sevoflurane.

163   Both tests, MTT and qPCR, have been extensively used in *in-vitro* cancer research (van Meerloo et

164   al. 2011; Ecimovic et al. 2014). The MTT test is frequently used for the evaluation of the number of

165   viable cells. The test measures the conversion of MTT into purple-coloured formazan crystals,

166   which are induced by living cells' redox activity. A cellular redox activity decrease indicates

167   reduced cell viability or decreased cell number while a cellular redox increase indicates cell

168   viability or cell number increase. For the case of cancer cells, an increase or decrease in cell number

169   count can be interpreted as an increase or decrease of the proliferation rate of the studied cells (van

170   Meerloo et al. 2011). Therefore, in the current study, it may be inferred that sevoflurane prevents

171   the proliferation of CIPm but enhances the proliferation of CIPp.

172   Present study findings are not completely in line with what is available in the scientific literature.

173   Ecimovic and colleagues (2013) showed that 6 hours of sevoflurane exposure at the concentrations

174   of 2, 3 and 4 mM increased cell proliferation by 50 - 63% and by 50 - 67% in metastatic human

175   breast adenocarcinoma cells that were oestrogen receptor positive (MCF7 cell line) or negative

176   (MDA-MB-231 cell line), respectively. Controversially, a sevoflurane (2 mM) anti-proliferative

177   effect was shown in C6 glioma cells (O'Leary et al. 2000). It should be noticed that, apart from

178   being different tissue cells, the C6 glioma cells were not in a tumour transformation state, thus

179   possibly reflecting the role of cell type and cell evolutional phase, rather than other factors like

180   concentration and contact time, on the ability to respond to drug exposure.

181   To the authors' knowledge, this is the first study to investigate the effects of sevoflurane on NET1

182   gene expression. The NET1 gene is a RhoA specific guanine nucleotide exchange factor that

183   enables tumour cells to invade and migrate (Ecimovic et al. 2014). The NET1 plays an important

184   role in cytoskeletal reorganization, N-cadherin expression and RhoA activation (Ecimovic et al.

185   2014). Therefore, an increased NET1 expression has been associated with malignant cellular

186   behaviours (Leyden et al. 2006). Consistently, NET1 was described as being overexpressed in

187   highly invasive cancer types such as human breast and gastric adenocarcinomas (Leyden et al.

188   2006). Expression of NET1 seems to be affected by some medications used in the perioperative

189   period (Ecimovic et al. 2014). In the present study, NET1 expression was only increased in CIPm

190   after being exposed to the higher evaluated concentration of sevoflurane (i.e. 4 mM). This could be

191   interpreted as sevoflurane enhancing the migration ability of CIPm.

192   Exposure time and concentrations used in the present study were chosen based on what has been

193   reported in human medicine, in order to make reasonable comparisons between studies (Ecimovic

194   et al. 2013). Sevoflurane concentrations chosen by previous authors were made after evaluating

195   sevoflurane plasma concentrations observed in people undergoing elective cardiac surgery and

196   receiving sevoflurane 1.8% inspiratory volume. Considering that the sevoflurane minimal alveolar

197   concentration in dogs is similar to that reported in people, it was assumed that concentrations

198   between 1 and 4 mM applied to the cell cultures would resemble the plasma concentrations of dogs

199   anesthetized with sevoflurane in clinical practice.

200   The *in vitro* nature of the present study presents some limitations. Firstly, the mechanisms studied

201   are only small pieces of the big puzzle of cancer propagation. Indeed, mechanisms that influence

202   cancer recurrence are extremely numerous and complex and it cannot be excluded that sevoflurane

203   influences cancer cells migration and proliferation by other means such as the modulation of the

204   immune system or the up-regulation of hypoxia-inducible stress factors. In addition, the

205 concomitant effects of other agents given in the routine clinical practice could potentially interfere

206 with sevoflurane effects on cancer cells. For instance, it was shown that serum from patients with

207 breast cancer who received general anaesthesia in the form of sevoflurane and systemic opioids

208 applied to MDA-MB-231 breast cancer cells increased both proliferation and migration of cancer

209 cells compared with serum of patients receiving propofol infusions and paravertebral blocks

210 (Deegan et al. 2009). Finally, it is difficult to extrapolate *in vitro* results to *in vivo* conditions.

211 Interestingly, a large retrospective study evaluating long-term survival of lung cancer patients

212 undergoing volatile or intravenous anaesthesia for elective surgery showed a statistically and

213 clinically significant survival time reduction in patients receiving inhalational anaesthetics

214 including sevoflurane (Wigmore et al. 2016).

215 In conclusion, sevoflurane treatments modified cell proliferation rate in both cell lines showing an

216 increase or a decrease when applied on CIPp or CIPm cells, respectively, compared to cell growth

217 in the sole cell culture medium. The expression of NET1 gene increased only after treatment with

218 sevoflurane 4 mM in metastatic cells. Further studies are much needed for a better understanding of

219 the role of sevoflurane on canine mammary cancer cells.

220

221   **References**

222

223   Deegan CA, Murray D, Doran P, et al (2009) Effect of anaesthetic technique on oestrogen receptor-

224   negative breast cancer cell function *in vitro*. Br J Anaesth 103, 685-690.

225   Ecimovic P, Mchugh B, Murray D et al (2013) Effects of Sevoflurane on Breast Cancer Cell

226   Function In Vitro. Anticancer Res 33(10), 4255-4260.

227   Ecimovic P, Murray D, Doran PP, Buggy DJ (2014) Propofol and bupivacaine in breast cancer cell

228   function *in vitro* − Role of the *NET1* gene. Anticancer Res 34, 1321-1332.

229   Karayannopoulou M, Lafioniatis S (2016) Recent advances on canine mammary cancer

230   chemotherapy: A review of studies from 2000 to date. Revue Méd Vét 167(7-8), 192-200.

231   Leyden J, Murray D, Moss A, et al (2006) Net1 and Myeov : computationally identified mediators

232   of gastric cancer. Br J Cancer 94(8), 1204-1212.

233   O'Leary G, Bacon CL, Odumeru O, et al (2000) Antiproliferative actions of inhalational

234   anesthetics: comparisons to the valproate teratogen. Int J Dev Neurosci 18(1), 39-45.

235   Tada H, Shiho O, Kuroshima K, et al (1986) An improved colorimetric assay for interleukin 2. J

236   Immunol Methods 93(2), 157-165.

237   Uyama R, Nakagawa T, Hong SH, et al (2006) Establishment of four pairs of canine mammary

238   tumour cell lines derived from primary and metastatic origin and their E-cadherin

239   expression. Vet Comp Oncol 4(2), 104-113.

240   Van Meerloo J, Kaspers GJL, Cloos J (2011) Cell Sensitivity Assays: The MTT Assay. Methods

241   Mol Biol 731, 237-345.

242   Vascellari M, Capello K, Carminato A et al (2016) Incidence of mammary tumors in the canine

243   population living in the Veneto region (Northeastern Italy): Risk factors and similarities to

244   human breast cancer. Prev Vet Med 126, 183-189.

245   Wigmore TJ, Mohammed K, Jhanji S (2016) Long-term Survival for Patients Undergoing Volatile

246   versus IV Anesthesia for Cancer Surgery. Anesthesiol 124, 69-79.

Figure Legend

Figure 1 Changes in absorbance values in primary (a; CIPp) and metastatic (b; CIPm) canine mammary tubular adenocarcinoma cells receiving different concentrations (S1: 1 mM; S2.5: 2.5 mM; S4: 4 mM) of a commercially available sevoflurane formulation when compared to control cells (C). Fold changes in NET1 gene expression in primary (c; CIPp) and metastatic (d; CIPm) canine mammary tubular adenocarcinoma cells receiving different concentrations of a commercially available sevoflurane formulation when compared to control cells (***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$)