The effect of interleukin-2 on canine peripheral nerve sheath tumours after marginal surgical excision.
A double-blind randomized study.

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1. ABSTRACT

The objective of this study was to evaluate the effect on outcomes of intraoperative recombinant human interleukin-2 injection after surgical resection of peripheral nerve sheath tumours. In this double-blind trial, 40 patients due to undergo surgical excision (<5 mm margins) of presumed peripheral nerve sheath tumours were randomized to receive intraoperative injection of interleukin-2 or placebo into the wound bed. After statistical analysis we found no significant differences in any variable investigated or in median survival between the two groups. The median recurrence free interval was 874 days (range 48-2141 days), The recurrence-free interval and overall survival time were significantly longer in dogs that underwent the primary surgery by a specialist-certified surgeon compared to referring general practice veterinarians regardless of whether additional adjunct therapy was given. Overall, marginal excision of peripheral nerve sheath tumours in dogs resulted in a long survival time, but adjuvant treatment with recombinant human interleukin-2 (rhIL-2) did not provide a survival advantage.

2. INTRODUCTION

Peripheral nerve sheath tumours (PNSTs) are spindle cell tumours that arise from the connective tissue components of the peripheral nerve and which can infiltrate the subcutaneous and surrounding tissues locally [1]. They appear to be pseudo-encapsulated, are locally invasive, and grow relatively slowly. Although PNSTs rarely metastasize, local recurrence is described commonly after resection [2,3,4,5,6].

Surgical removal of PNST is the therapy of choice, but since surgical removal alone does not guarantee complete eradication of the tumour, immunotherapy as adjuvant therapy has become more popular in human medicine. It decreases the rate of recurrence and metastases in a variety of tumours, including sarcomas [7,8,9]. Interleukin-2 (IL-2) activates the immune system to enhance immune-mediated responses against the tumour, thereby diminishing its metastatic potential [10]. IL-2 is a T-cell growth factor and induces clonal expansion of antigen-specific T-cells and activation of antigen-presenting cells (APC). It also increases the production of cytokines, stimulates natural (NK) and lymphokine-activated killer cells (LAK), and modulates or promotes major histocompatibility complex (MHC) antigen expression, driving the tumour towards regression [6].

Local injection of recombinant human IL-2 (rhIL2) leads to extravasation of erythrocytes at the injection site [11], causing stagnation of blood flow and leading to tumour cell death. It also decreases the size of distant metastases by an as yet unknown mechanism [11,12,13,14].

The aim of this retrospective study was to evaluate the possible beneficial effect of local rhIL2 administration after marginal PNST removal in dogs.

3. HYPOTHESIS

H_0 = There is no significant difference between the control group and the IL-2 administered group for the risk of recurrence and survival statics.

H_1 = There is significant difference between the control group and the IL-2 administered group for the risk of recurrence and survival statics.

4. PROCEDURE

A retrospective study uses existing data that have been recorded for reasons other than research. After the power analysis in 2000 calculated a total sample size of 40 dogs, using survival data obtained from previous studies [3,6] (with an expected mean RFI difference of 20% per procedure and a variation coefficient of 15%, an α of 0.05, and a β of 0.15), I begin to analyse (October 2012) national administrative data of 40 dog patients from the Department of Clinical Sciences of Companion Animals, Utrecht University (UU), Utrecht and the Animal Medical Centre Amsterdam (AMCA), Amsterdam.

The patients were first processed in Microsoft Excel, containing the following measurements; sex, castration, age at the beginning of the study, weight, PNST localization (1. phalanges to carpus, 2 carpus to elbow, 3. elbow to shoulder, 4. cervical region, 5. phalanges to tarsus, 6. tarsus to stifle, 7. stifle to hip, 8. flank and 9. head), size of the tumour in cm^2 and cm^3, fine-needle biopsy (FNAB) and histology findings, date of presence and location of metastasis, tumour grade, operating surgeon (Jolle Kirpensteijn or Bart Sjollema), previous surgery, date of tumour presence, date of recurrence, date of death, reason of death (due or not due to tumour) and if the patient received local rhIL-2 or placebo.

Unfortunately, not every patient's file was complete or updated. Therefore, the dog's owner, veterinarian and/or surgeon had to be contacted by phone or e-mail for follow-up. Furthermore, extra literature was searched about PNSTs on NCBI Pubmed.com.

During the whole research period, at least once weekly contact was kept with the supervisor.

After completing the Microsoft Excel file including all the above-mentioned variables in December 2012, the statistical analysis was performed using SPSS Statistics 20.0. The following statistical methods were utilized in the study; frequency distributions were calculated and categorical data were compared using Chi-square analysis. Fisher's exact test was used when sample sizes were small, i.e. if more than 25% of the samples were smaller than 5. Normally distributed, continuous and interval categorical data were

analysed using an analysis of variance (ANOVA). Logarithmic transformation was performed on variables that were not normally distributed. A hazard ratio (HR) of different variables on MFI, RFI, and OS was calculated using multivariate Cox proportional hazards analysis. The Kaplan-Meier product limit method was used to estimate median RFI, MFI and OS*. Group comparisons were made using the mantel-Cox log rank test. Statistical significance was defined as $P<0.05$. Dogs that had died from causes to their PNST's or which were still alive at the time of follow-up and without signs of either recurrence or metastases were considered censored. If a patient died without follow-up, the case was classified as lost to follow-up (LTF) and the last physical and diagnostic examination was used as end point. If a physical examination was performed but no diagnostic investigations, only RFI and OS were recorded; MFI was then coded as LTF.

$P < 0.05$ was considered significant.

During the last months, the paper was send to BMC Veterinary Research and reviewed by others. At first, major revisions had to be corrected. The most important revision was to be made about the statistics. After correcting the statistical analyses the paper was send again to the editor and returned, now with minor revisions. Because of these revisions the research project was prolonged. The study was finally finished and published in the BMC Veterinary Research journal on the 8th of August 2013.

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*Overall survival time (OS) was defined as the interval between the date of surgery and death due to the disease or the date on which the dog was last known to be alive. Metastasis free (MFI) and recurrence free (RFI) interval was defined as the interval between the date of the surgery and the date of either metastases or recurrence, or if there were no signs of recurrence or metastases, as the interval between surgery and the time on which the dog was last known to be alive.

5. THE STUDY

5.1. POPULATION

In the period between 2000 and 2003, 40 dogs (any breed, age, or sex) with a clinical diagnosis of PNST were referred to two referral practices in the Netherlands. The inclusion criterion was presumed PNST that could not be removed with adequate 3-cm margins. Dogs with metastases or multiple tumours were excluded.

5.2 MATERIALS AND METHODS

All dogs were screened for metastases during the study, using right lateral, left lateral, dorsoventral or ventrodorsal radiographs of the lungs. A fine-needle aspiration biopsy (FNAB) was taken from all tumours. All tumours were removed with margins of 5 mm or less and submitted for histological examination by a certified pathologist (MK) to determine the type and grade of the tumour and the completeness of resection.

Recombinant human IL-2 (rhIL2; specific activity 18×10^6 IU/mg; Proleukin® a gift from Chiron, Amsterdam, the Netherlands) was reconstituted to 1 mg/ml with distilled water. Polygeline was added to increase the stability of interleukin. The placebo contained distilled water with polygeline. Directly after surgical removal of the tumour, rhIL2 (1 ml containing 4.5 million IU) or placebo was injected evenly into the wound bed (0.01–0.05 ml per site) in an at random double-blinded fashion [15,16]. Surgery was performed by one of two surgeons (BS and JK). Blinding was broken after data had been accumulated and analysed.

Directly after surgery, the wounds were evaluated and all dogs were routinely screened for physical signs of regrowth and radiographic signs of metastasis at 1, 3, 6, and 12 months after surgery and every 12 months thereafter.

5.3 RESULTS

Marginal excision (<5 mm) was the only surgical option for all tumours. The dogs were of different breeds and the median weight was 31 kg (range 12-51 kg). There were 23 male dogs and 17 female dogs. The median age of the dogs at referral was 9 years (range 3-14). Nineteen dogs had previously undergone tumour resection.

Four tumours were located on the forelimb in the area from the phalanges until the carpus, 15 from the carpus until the elbow, 7 from the elbow until the shoulder, 5 from the tarsus until the stifle, 4 from the stifle until the hip, 4 on the head, and 1 on the flank (Table 1). Most tumours were located at the antebrachium (38%) or the brachium (18%). Fine-needle aspiration biopsies (FNABs) were used to diagnose 13 mesenchymal proliferations and 15 mesenchymal tumours. There were 29 neurofibrosarcomas, 4 fibrosarcomas, 2 myxosarcomas, and 5 haemangiopericytomas. Tumour grade was determined to be low (grade 1) in 8 cases, medium (grade 2) in 20 cases, and high (grade 3) in 12 cases. The median tumour volume was 38.5 cm^3 (range 1-2890 cm^3). All tumours had dirty surgical margins (tumour reached or extended into the surgical margin).

There was no significant difference between the control group and the IL-2 group in sex, age, weight, tumour localization, left versus the right side, radiographic appearance of the tumour, results of fine-needle biopsy, location of metastasis, and tumour grade (Table 1). There were also no between-group differences after exclusion of dogs with myxosarcomas and haemangiopericytomas, which are not classified as true PNSTs. The side effects of therapy were minimal and could not be distinguished from the normal side effects observed after skin surgery (minor redness, haemorrhage, swelling and tenderness of the wound). There were no significant between-group differences in side effects (data not shown).

Overall, the median RFI was 874 days (range 48-2141 days), the median MFI was 1884 days (range 407-2141 days) and the median OS was not reached (range 197-2141 days). There was no significant difference in OS between the treatment groups (Figure 1) or by tumour group or grade. The rate of recurrence was 45% (9/20) in the rhIL2 group and 35% (7/20) in the placebo group. Six dogs developed metastatic disease, 5 in the lungs and 1 in

the lymph nodes; all dogs had previously undergone surgery. Metastases developed in 3 dogs (15%) that had received placebo and in 3 dogs that had received rhIL2 and the incidence of metastases did not differ significantly between treatment groups. Of the 6 dogs with metastases, 2 were from grade 1 (n=8), 3 from grade 2 (n=20) and 1 from grade 3 tumors (n=12). There was no significant difference in incidence of metastases between the three groups.

The only significant difference in OS (P=0.006) was found for dogs that had previously undergone surgery compared with those that had their first surgery at the referral clinics (UU or AMCA; Figure 2). Using multivariate analysis, the hazard ratio (HR) for recurrence was higher in dogs that had previously undergone surgery at the time of inclusion compared to dogs that had not had prior surgery (p<0.001, HR 10.7, 95% CI 2.3-21.2; Figure 3). The only other factor that had a significant HR was the (body?) weight (P=0.02, HR=1.08, 95% CI 1.01-1.16). There was no significant difference in occurrence of metastases between dogs that had undergone previous surgery and the ones that had not, using multivariate analysis.

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6. DISCUSSION

This study describes the effect on surgical outcomes of local injection of rhIL2 or placebo into the wound bed after surgical excision of PNST-like tumours. Treatment with rhIL2 did not significantly influence the MFI, RFI, or OS. In contrast, it has been reported to have a beneficial effect in various types of tumours in humans [7,8] and animals [17,18,19,20,21,22,23].

Incomplete tumour resection increases patient morbidity, treatment costs, risk of further recurrence and ultimately decreases survival time [24,25,26,27]. We similarly found a much lower recurrence rate in dogs that had not previously undergone surgery. This difference can be explained in two ways: the veterinarians who removed the primary tumour might have used even smaller tumour margins than the specialist-certified surgeon or the previously removed tumour might have been more aggressive and more likely to recur. However, there was no significant difference in grade of the removed PNST between the dogs with or without previous surgery. Nor did we find

survival or recurrence rates to differ by tumour grade, but this might be a reflection of the relatively low number of cases.

The rate of recurrence was similar in the two treatment groups, 45% (9/20; rhIL2 group) versus 35% (7/20; placebo group). The overall recurrence rate (40%) was higher than the 15% reported in a comparable study of 41 dogs, in which all dogs that had surgery for recurrence had been referred after inadequate primary tumour resection [28]. This difference might be because we had a longer follow-up of minimally 5 years.

Most PNSTs in dogs have low metastatic rates. In this study, 15% of the PNSTs (6/40) had metastasized to lungs or lymph nodes, and rhIL-2 therapy did not influence the rate of metastasis. One study of IL-2 used in an adjuvant setting as local inhalation therapy for carcinoma reported that 2 of 7 treated dogs achieved full remission for more than a year (29%) [29]. Other canine STS studies have reported lower metastasis rates than the rate reported here [26,27,28]. The higher metastatic rate in our study might have been due to the longer follow-up, a more vigilant surveying system, or the presence of dormant cells in the tumour margins. The results of this study indicate that aggressive primary surgery is advisable for these tumours.

PNSTs on extremities often cannot be excised with wide margins and amputation of the limb is a suggested treatment alternative. Marginal excision of low-grade (G_1) STSs from the extremities of 35 dogs resulted in only 4 recurrences [30]. Similarly, a study showed a grade-dependent recurrence after marginal excision in 7% (3/41) of grade 1 (G_1) tumours, 34% (14/41) of grade 2 (G_2) tumours, and 75% (3/4) of grade 3 (G_3) tumours [25]. Radical resection of STSs on extremities by limb amputation should therefore be considered as a last resort for recurrent and high-grade tumours, taking into account that the risk of metastases is higher in cases that had a recurrence [30,31]. The study reported here, had a longer follow up and a grade variation between groups that was identical but the comparison with historic data prevents any major conclusions when survival outcome is compared to the above-mentioned studies.

The study had several limitations. Although the entry criteria were strict and the double-blinded study had a well-executed follow-up regimen, a major limitation remains the compliance of owners. Owner compliance is never

100% and some owners lived too far away to bring their dogs to follow-up evaluations. In these cases, we consulted the referring veterinarian for follow-up information, but we have no way of knowing whether he/she had recently examined the patient. The variation in tumour size, location, and grade can also affect outcomes, especially in small case cohorts. In total, 4.5 million IU of rhIL2 was administered, but the area of distribution varied with the wound size, which limits extrapolation of the exact local dose of rhIL2. The size of the wound should have been measured prior to injection of rhIL2 or placebo to allow a better calculation of the exact dose per square cm^2. Power analysis indicated that 40 dogs would be needed to detect a statistically significant difference between treatments. But this is still a small number when multiple observations and variables are used, such as the grade and histologic diagnosis. The use of two instead of one referral institute may have influenced the data although there were no significant differences between the two institutes for the variables examined (data not shown).

7. CONCLUSION

The long-term prognosis of PNSTs was generally good, even after marginal resection. This study showed that recurrences can occur years after the primary surgery, that recurrences are much more common after prior surgery, that metastases can occur in both lymph nodes and lungs, and that intralesional IL-2 does not confer a survival advantage in dogs with PNSTs. Future studies should use a stringent follow-up evaluation schedule as used in this study with minimally a 5-year follow-up to allow proper analysis of survival statistics. Although marginal excision of PNST (with or without adjuvant treatment with rhIL2) resulted in a long survival in a subset of dogs, presurgical incisional biopsy, wide excision, and/or adjuvant radiotherapy are advisable to prevent recurrence and possible metastases.

8. ACKNOWLEDGEMENTS

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10. APPENDIX

Table 1: Statistical data of dogs with peripheral nerve sheath tumours receiving recombinant human interleukin-2 (rhIL2) or a placebo.

	rhIL2	Placebo	Significance
Sex	12M: 8F	10M: 10F	NS
Localisation	Front leg 10 Rear leg 6 Axial 2	Front leg 14 Rear leg 3 Axial 3	NS
Left vs right	L 9: R11	L14: R6	NS
Grade	Grade 1: 2 Grade 2: 11 Grade 3: 7	Grade 1: 6 Grade 2: 9 Grade 3: 5	NS
Previous sx	yes: no 10:10	yes: no 9:11	NS
Size*	45.6 $\pm$ 16.0	19.8 $\pm$ 4.0	NS
Age*	9.3 $\pm$ 0.55	8.9 $\pm$ 0.60	NS
Weight*	33.1 $\pm$ 1.7	29.2 $\pm$ 2.1	NS

NS= no significant difference between the two groups; vs = versus; sx = surgery; M= male; F= female; L= left; R= right; axial= head or trunk; * described as mean $\pm$ standard error

Figure 1: Kaplan-Meier survival curve of overall survival (OS) showing a non-significant difference between dogs with PNSTs treated with rhIL2 (dotted line) versus placebo (continuous line).
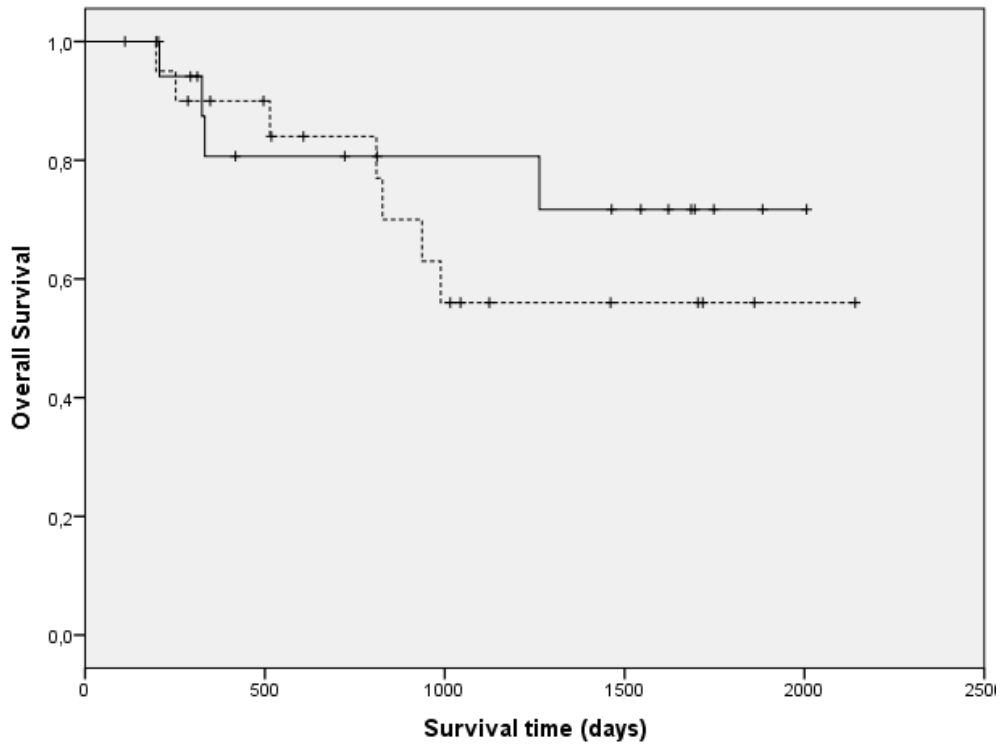
Figure 2: Kaplan-Meier survival curve of overall survival (OS) showing a significant difference (P<0.001) between dogs with PNSTs that had undergone a previous surgery (dotted line) versus the ones that did not (continuous line).
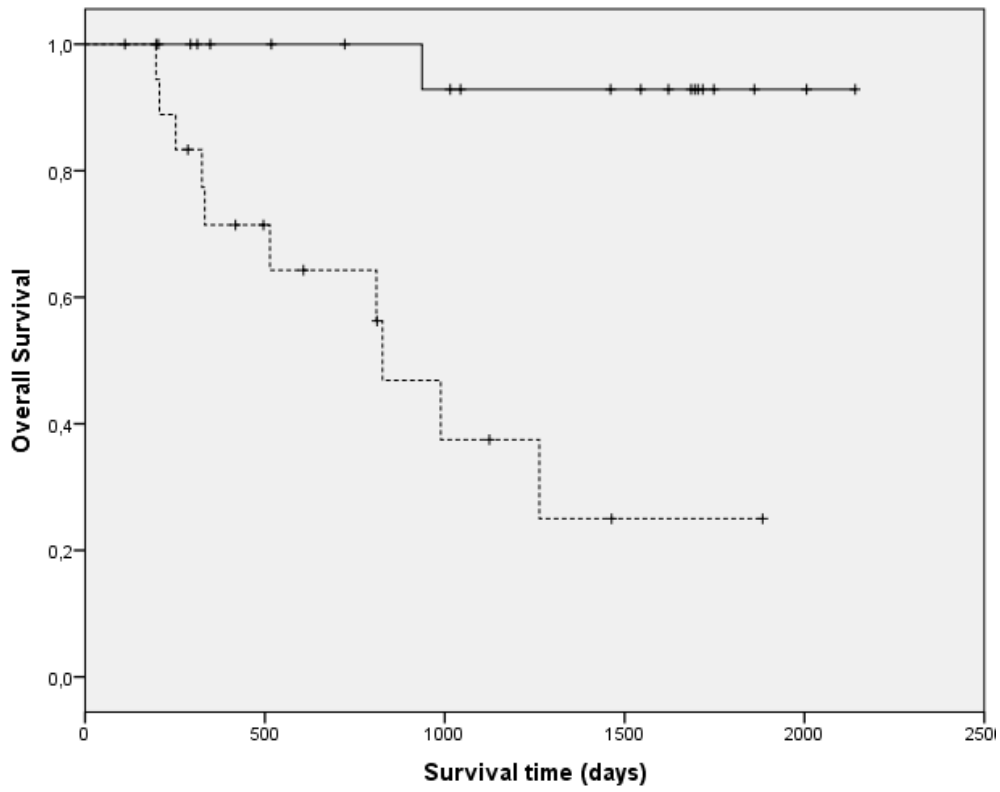
Figure 3: Kaplan-Meier survival curve of recurrence free interval (RFI) showing a significant difference (p<0.001) between dogs with PNSTs that had undergone a previous surgery (dotted line) versus the ones that did not (continuous line).