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Efficacy and safety of electrochemotherapy combined with peritumoral IL-12 gene electrotransfer of canine mast cell tumours

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Abstract

Electrochemotherapy combined with peritumoral interleukin-12 (IL-12) gene electrotransfer was used for treatment of mast cell tumours in 18 client-owned dogs. Local tumour control, recurrence rate, as well as safety of combined therapy were evaluated. One month after the therapy, no side effects were recorded and good local tumour control was observed with high complete responses rate which even increased during the observation period to 72%. IL-12 gene electrotransfer resulted in 78% of patients with detectable serum IFN-γ and/or IL-12 levels. In the treated tumours vascular changes as well as minimal T-lymphocytes infiltration was observed. After 1 week, the plasmid DNA was not detected intra- or peritumorally and no horizontal gene transfer was observed. In summary, our study demonstrates high antitumour efficacy of electrochemotherapy combined with IL-12 electrotransfer, which also prevented recurrences or distant metastases, as well as its safety and feasibility in treatment of canine mast cell tumours.

Keywords
dogs, efficacy, electrochemotherapy, interleukin-12, mast cell tumour, safety

Introduction

Electrochemotherapy is now an established treatment of cutaneous and subcutaneous tumours in human oncology.[1] This therapy is also used in veterinary oncology for treatment of tumours in dogs, cats and horses.[2–5] It is a local ablative treatment were cytotoxicity of either bleomycin or cisplatin is increased by the use of applied electric pulses to the tumours.[1] The response rate of electrochemotherapy is very high, reaching up to 100% of complete response in basal cell carcinoma in human oncology and 100% of complete response in sarcoids in horses.[3,6] Pronounced antitumour effectiveness of electrochemotherapy is because of the several mechanisms, among which is also activation of the immune response. It is due to the tumour antigen shedding into surrounding tissue and blood, caused by electrochemotherapy-induced immunogenic cell death. Immunogenic cell death is induced by both chemotherapeutic drugs currently used in clinical electrochemotherapy, bleomycin and cisplatin.[7–9] The action of both drugs on DNA molecules results in induction of endoplasmic reticulum stress leading to the immunogenic cell death that activates immune cell response, which helps in complete eradication of clonogenic tumours cells. However it is not enough for prevention of the tumour growth after challenge and does not prevent the growth of distant tumours.[10,11] Therefore, in order to increase the systemic antitumour effect, electrochemotherapy was combined with different immune therapies, particularly gene electrotransfer of IL-12.[12–15] Electrochemotherapy with IL-12 gene electrotransfer was used for

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treatment of several tumour models in mice[16–18] and was also tested in spontaneous tumours in dogs, mainly for the treatment of aggressive carcinomas and sarcomas. A clinical response to the combined treatment was observed, with minimal side effects regardless of the tumour type.[19,20]

In all of the above mentioned studies therapeutic IL-12 gene was included into plasmid DNA delivered into target cells by electrotransfer. Although, no adverse events related to the therapeutic protein was reported in these studies, none of the studies evaluated the safety of plasmid DNA itself. Although, the plasmid DNA is regarded as safer compared with viral vectors, in recent years, several concerns have been raised regarding plasmid DNA, such as integration into cellular DNA, autoimmunity and the presence of antibiotic resistance gene. The latter being the most worrying, as antibiotic resistance gene can be transferred to other bacteria by horizontal gene transfer. Therefore, the regulatory agencies, FDA (US Food and Drug Administration) and EMA (European Medicines Agency) recommend totally avoiding the use of antibiotic resistance genes as a part of plasmid vectors or the use of ones that are not commonly used to treat human infections. Currently, only the kanamycin resistance gene meets the requirements posed by regulatory agencies.[21,22]

As we recently proposed, electrochemotherapy can be used as *in situ* vaccination that can be boosted by immunogene therapy resulting not only in increased local tumour control but having also protective effect on local recurrence and distant metastases.[11] Therefore, we conducted a clinical study in spontaneous mast cell tumours in dogs by the use of electrochemotherapy in combination with immune therapy with IL-12, and in addition to that for the first time we evaluated the safety of the therapy regarding the side effects of the therapy and horizontal gene transfer.

Materials and methods

Animals and tumours

Between January and December 2010 the studied animals were referred to the Veterinary Faculty in Ljubljana for evaluation of cutaneous or subcutaneous tumour nodules. Eighteen subjects (8 males and 10 females of 10 different breeds, with age ranging from 5 to 15 years; Table 1) corresponded to inclusion criteria (at least one cytologically or histologically confirmed mast cell tumour in different anatomical locations (Table 1), good general health status of the animal with the basic haematology and biochemistry profile within reference limits and normal renal and cardiovascular function) for the clinical study and were thus included in the study. Animals included in the study were the ones whose owners refused any other type of standard treatment at the time of inclusion due to cost, availability, invasiveness of surgical procedure (e.g. involving amputations of limbs or extensive reconstructive surgery) or other subjective reasons. Prior to inclusion, written consent for participation in the clinical study for each animal was obtained from their owners. The study was approved by the Ethical Committee at the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (approval No. 323-451/2004-9). Before the treatment, staging in all patients was performed according to modified World Health Organization (WHO) staging criteria with physical examination, examination of thoracic radiographs, abdominal ultrasonography and basic bloodwork. Basic bloodwork consisted of a complete blood count with differential white blood cell count, which was performed using an automated laser haematology analyzer with species-specific software (Advia 120, Siemens, Munich, Germany). The automated chemistry analyzer RX-Daytona (Randox, Crumlin, UK) was used for determination of the following biochemical parameters: urea, creatinine, serum alkaline phosphatase (SAP) and alanine aminotransferase (ALT). Of 10 tumours 18 were submitted to biopsy before the combined therapy and all treated areas were sampled again 4 weeks later.

Plasmid isolation

The pORF-hIL-12 plasmid (InvivoGen, Toulouse, France), encoding human IL-12, was selected based on our previous positive results.[23] The plasmid was prepared using the Qiagen Endo-Free kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions and diluted to a concentration of 1 mg mL^{-1}. Purified plasmid DNA was subjected to quality control and quantity determinations,

Table 1. Patients characteristics, treatment parameters and response to treatment

Pt No	Breed	Sex	Age (years)	Location of tumour nodule	Tumour grade (Patnaik)	Clinical stage (WHO)	ECT (No. of sessions, type, dose and route of chemotherapeutic)	GET (No. of sessions and dose of intradermal pIL-12)	Size of tumour before therapy (cm^3)	Response to therapy at 1 month	Response to therapy at the end of OP	OP (months)	Detection of serum hIL-12 elevation	Detection of serum cIFN-γ elevation
1	Mixed breed	M	7	Hind leg	I	III	1 × CDDP 2 mg i.tu.	1 × 1 mg	0.16	SD	SD	59	N/D	N/D
2	Shar Pei	M	7	Prepuce	N/D	I	1 × CDDP 3 mg i.tu.	1 × 1 mg	0.19	CR	CR	42	R	R
3	Labrador Retriever	M	9	Hind leg	N/D	I	1 × CDDP 3 mg i.tu.	1 × 1 mg	0.32	CR	CR	49	R	R
4	German Boxer	F	6	Hind leg	I	I	1 × CDDP 3 mg i.tu.	1 × 1 mg	0.40	CR	CR	6	R	NR
5	Golden Retriever	M	7	Tail	II	I	2 × CDDP 2 mg i.tu.	2 × 1 mg	0.47	CR	CR	49	R	NR
6	Mixed breed	F	5	Lateral thorax	II	I	1 × CDDP 2 mg i.tu.	1 × 1 mg	0.68	CR	CR	38	NR	NR
7	Beauceron	F	5	Ventral abdomen	N/D	I	1 × CDDP 2 mg i.tu.	1 × 1 mg	0.86	CR	CR	57	R	NR
8	German Boxer	F	10	Hind leg	II	II (Inn meth)	1 × CDDP 2 mg i.tu.	1 × 1mg	1.18	CR	CR	25	R	R
9	Greater Swiss Mountain Dog	M	7	Hind leg	N/D	II (Inn meth)	1 × CDDP 2 mg i.tu.	1 × 1 mg	2.10	PR	PR	15	R	R
10	Golden Retriever	F	7	Fore leg	N/D	I	1 × CDDP 2 mg i.tu.	1 × 1 mg	2.20	CR	CR	48	R	R
11	Dalmatian	F	15	Head	II	II (Inn meth)	2 × CDDP 2 mg i.tu.	2 × 1 mg	2.26	CR	PD	4	R	R
12	Mixed breed	F	5	Head	II	I	1 × CDDP 3 mg i.tu.	1 × 1 mg	2.35	CR	CR	58	R	R
13	Mixed breed	F	7	Head	N/D	I	2 × CDDP 3 mg i.tu.	1 × 1 mg	12.71	CR	CR	55	R	R
14	Jack Russel Terrier	M	5	Hind leg	N/D	III	1 × CDDP 1 mg i.tu.	1 × 1.5 mg	14.24	SD	PD	6	NR	NR
15	American Staffordshire terrier	M	5	Fore leg	II	I	1 × BLM 7.5 mg i.v.	1 × 2 mg	2,10	CR	CR	33	N/D	N/D
16	Greater Swiss Mountain Dog	F	5	Head (dorsal nose)	I	I	1 × CDDP 3 mg i.tu., 1 × BLM 3 mg i.tu.	1 × 2 mg	2,15	PR	CR	36	R	R
17	German Boxer	M	6	Ventral abdomen	II	I	1 × CDDP 3 mg i.tu. + 1 × BLM 3 mg i.tu.	1 × 1 mg	3,14	PR	PR	43	R	R
18	Greater Swiss Mountain Dog	F	8	Tail	II	I	1 × CDDP 5 mg i.tu. + 1 × BLM 12 mg i.v.	2 × 2 mg	16,88	PR	CR	37	R	R

BLM, bleomycin; CDDP, cisplatin; ECT, electrochemotherapy; F, female; GET, gene electrotransfer; i.tu., intratumoural, i.v., intravenous; M, male; N/D, not determined; NR, non-responder; OP, observation period, R, responder.

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performed by agarose gel electrophoresis and by means of spectrophotometry.

Treatment protocol

The therapy was performed on patients in general anaesthesia. Dogs were premedicated with intravenous application of midazolam (Midazolam Torrex, Chiesi, 0.2 mg kg^{-1} bw) and general anaesthesia was induced using propofol (Diprivan, Zeneca; 3–6 mg kg^{-1} bw) and maintained with sevoflurane (Sevorane, AbbVie, Campoverde di Aprillia, Italy). During anaesthesia, animals received Hartmann's solution (B. Braun Melsungen AG, Melsungen, Germany) at the rate of 5 mL kg^{-1} h^{-1}. Before the therapy the hair on and around tumour nodules was carefully removed, avoiding any unnecessary manipulation of tumours, which can result in degranulation of mast cells thus provoking local or systemic histamine effects. Each nodule was measured in three perpendicular directions (a, b, c). Volume of tumour was calculated using the following formula: $V = a \times b \times c \times \pi/6$. The therapy consisted of two phases. In the first phase, electrochemotherapy was performed using direct intratumoural application of cisplatin (*cis*-diamminedichloroplatinum II, Cisplatyl; Aventis, Paris, France) or bleomycin (Blenoxane, Bristol-Myers, New York, USA). Cisplatin was dissolved in distilled water at a concentration of 2 mg mL^{-1} and was given intratumourally at a dose of ~1 mg cm^{-1}. Bleomycin was dissolved at a concentration of 3 mg mL^{-1} and was applied intratumourally at the same dose as cisplatin (1 mg cm^{-1}) or intravenously in a dose 0.3 mg kg^{-1}. Cisplatin was always the first drug of choice regardless of the size of the tumours. In case of no or minimal antitumour response at 4 weeks, cisplatin was replaced with bleomycin. Intravenous injection of bleomycin was performed in two dogs. In one dog due to the large size of the tumour to ameliorate the distribution of the drug and in the second dogs due to the location (above the *v. cephalica antebrachii*). One to two minutes after intratumoural injection of the drug, eight electric pulses were delivered (each of 100 μs duration and amplitude to electric distance ratio of 1300 V cm^{-1} and frequency of repetition 5 kHz with electric pulses generator Cliniporator™ (IGEA s.r.l., Carpi, Italy), using two parallel stainless steel plate electrodes with 6 mm distance between them or four needle row electrodes with 4 mm distance depending on the size of the tumour. In the case of intravenously injected bleomycin, electric pulses, using the same parameters, were applied 10 min later. Electric pulses were first delivered at the tumour margin in order to reduce the blood flow to the tumour and then continued in concentric circles to the centre of the tumour nodule to minimize the potential release of histamine and other vasoactive substances from tumour cells.

Immediately after the completion of the first phase of the therapy, the second phase of therapeutic procedure, IL-12 gene electrotransfer was applied. The IL-12 plasmid was injected intradermally in equidistant locations around tumour nodule on two locations. Based on previous experience, the dose of 1–2 mg per patient was set (Table 1).[23] Immediately following injection of plasmid, electric pulses were delivered using the same electric pulse generator and electrodes as mentioned above, but using different electrical parameters. One high voltage pulse was delivered (amplitude to electric distance ratio 1200 V cm^{-1}, duration 100 μs), immediately followed by one low voltage pulse (amplitude to electric distance ratio 140 V cm^{-1}, duration 400 ms).[24] Of 18 patients 12 received single combinational therapy, in 3 patients, the procedure was repeated after 1 month. In 3 of 18 patients, only additional electrochemotherapy was performed 1 month after the first treatment (Table 1).

At the end of the procedure, all patients received a single intravenous application of analgesic carprofen (Rimadyl, Pfizer, New York, USA) at the dose 4 mg kg^{-1} bw. When patients completely recovered from anaesthesia, they were released from the hospital. Prior to release into the home environment, animals received Elizabethan collars in order to prevent any wound licking. Furthermore, treated tumour nodules were protected with a suitable dressing to prevent any possible contact with humans or other animals with the treated area.

Evaluation of treatment outcome and sample collections

Local and systemic treatment outcome and possible side effects were evaluated at regular follow-up examinations 7, 14 and 28 days after therapy, then

monthly until 6 months after the procedure and thereafter every 6 months until the end of the observation period (10 May 2015). At each time point, clinical examination of the patient was performed, including measurements of tumour nodules. Furthermore, before and up to 3 months after therapy, blood samples for determination of cytokine's level in serum were collected. For determination of IL-12 and IFN-γ in patients' sera enzyme-linked immunosorbent assay (ELISA) kits (Human IL-12 Quantikine ELISA Kit and Canine IFN-γ Quantikine ELISA Kit, respectively, both R&D Systems, Minneapolis, MN, USA) were employed. In addition, skin swabs of the treated area for the detection of the presence of residual plasmid DNA were taken. Culturable aerobic bacteria were isolated from skin swabs taken from different regions of the dog, as well as from the treated regions to determine the possible horizontal gene transfer (also 2 days after therapy).

Local response to therapy was evaluated by measurements of tumours' sizes. Response to the treatment was determined 4 weeks after the last treatment and at the end of the observation period as a complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD) according to "Response evaluation criteria in solid tumours" (RECIST). Possible side effects were determined according to Veterinary Cooperative Oncology Group toxicity scale (VCOG-CTCAE) at each follow-up examination.[25]

Histopathology and immunohistochemistry

Biopsies were used to confirm cytologic diagnosis to classify mast cell tumour[26] and to characterize tissue changes, presence of inflammatory infiltrates and vascular modifications at different post-treatment times. The surgical specimens were fixed in a 10% formalin solution before being embedded in paraffin wax and routinely processed and stained using haematoxilin and eosin and Gomori's modified trichrome stain.

Measurement of plasmid DNA

In the skin swabs taken before and at regular intervals after the therapy, the presence of plasmid IL-12 was determined. For plasmid DNA isolation, 2 mL of phosphate buffered saline (Gibco, Waltham, MA, USA) was added to the swabs and plasmid DNA was isolated using PureLink HiPurePlasmid Miniprep Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The residual concentration of the therapeutic plasmid in the skin swabs was measured by quantitative polymerase chain reaction (qPCR).

Assay for amplification was designed in a way to be specific only for IL-12 plasmid. Serial dilutions of purified plasmid DNA were used as standards for absolute quantification of residual concentration of the IL-12 plasmid. Amplification was carried out using forward primer (5′-GGAATTCTTT TGCTTTTTACCCTCGA-3′), reverse primer (5′G CATTCTAGTTGTGGTTTGTCCAAA-3′) and probe (5′FAM-CTAGCTCGACATGATAAGAT-BHQ-3′). The specificity of the assay was tested using DNA isolated from *Escherichia coli* (ATCC 25922) and human microvascular cell line HMEC-1 with PureLinkTM Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Detection of GADPH gene was used as a control of DNA isolation and PCR inhibition.[27]

All PCR mixtures were conducted in a total volume of 20 μL and consisted of 10 μL TaqMan Gene-Expression Master Mix (Applied Biosystems, Carlsbad, CA, USA), 1 μL of the assay and 9 μL of 3-times diluted isolated DNA. The amplification process was carried out on 7300 Real Time PCR (Applied Biosystems), with the following conditions: 50 °C for 2 min, 95 °C for 10 min and 45 cycles at 95 °C for 15 s, 60 °C for 60 s.

Calculation of plasmid copy number per unit of volume was performed as previously described.[28,29] The molecular weight of the plasmid was calculated using web-based application The Sequence Manipulation Suite.[30] Based on this data the plasmid concentration was calculated and presented as nanogram per millilitre.

Isolation and identification of skin bacteria before and after therapy

Culturable aerobic bacteria from the patient's skin before and after the therapy application were isolated in order to determine whether the ampicillin resistance gene from plasmid pORF-hIL12

could be horizontally transferrred. All strains isolated after therapy were screened for the presence of the pORF-hIL-12 resistance gene. In addition, selected isolates from different genera were used as recipients for *in vitro* transformations with plasmid pORF-hIL-12 under laboratory conditions.

The first sampling was performed before therapy in order to identify potential recipient bacteria of plasmid genes. After basic shaving, the application area was wiped using sterile swabs soaked in 0.9% saline solution. Swab samples were streaked on LB and BAB agar plates, supplemented with 5% bovine blood, and incubated aerobically at 30 and 37 °C up for a week. Strains were selected for further identification on the basis of their colony morphology and Gram stain. Genomic DNA was isolated from whole bacterial cells by boiling according to Le Bouguenec et al.[31] The 16S rRNA genes were PCR amplified with oligonucleotide primers fD1 and 1392r as described previously.[32] The PCR products were analyzed by electrophoresis on 1% (w/v) agarose gel and subsequently purified with the QIA Gel Extraction Kit (Qiagen). The purified PCR products were sequenced on our request at Macrogen Korea using the fD1 and 1392r sequencing primers. Bacterial identification of the species and/or genus level was performed by using the RDP classifier software available at the ribosomal database homepage http://rdp.cme.msu.edu/classifier/classifier.jsp.[33] Isolation and identification of bacteria 2 and 7 days after therapy was the same, except that the samples were additionally streaked on LB agar plates supplemented with 100 mg L^{-1} of ampicillin (LBAmp).

In vitro transformation of pORF-hIL-12

In all transformations 0.12 μg of pORF-hIL-12 plasmid DNA was introduced into electrocompetent bacterial cells of selected isolates belonging to different genera by electroporation. Transformants were plated onto LB and LB supplemented with ampicillin (LBAmp) and incubated for 24–72 h at 30 or 37 °C, dependent of the recipient strain. Where available plasmids specific for the selected genera were used as a positive control. Electrocompetent cells and subsequent electroporation of *Staphylococcus* isolates were carried out according to the protocol of Augustin and Gotz.[34] Plasmid pUB110 Kan^R was used as a control. Control transformation mixture was plated on LB agar plates supplemented with 40 mg L^{-1} of kanamycin (LBKan). Electroporation of isolates from the genus *Bacillus* was carried out as described by Xue et al.[35] Transformants with control plasmid pED302 Kan^R were selected on LBKan plates. Transformations of *Rhodococcus* sp., *Arthrobacter* sp., *Streptomyces* sp., *Kocuria* sp. and *Micrococcus* sp. isloates were performed according to Sekizaki et al.[36], Zhang et al.[37], Pigac and Schrempf[38] and Matsumura et al.[39], respectively. Electrotransformation of *E. coli* and *Acinetobacter* sp. was carried out as described by Ausubel[40] and the Transformation Protocol for the Multiporator/Electroporator 2510 (Eppendorf AG, Hamburg, Germany). Plasmids JAC100 bla_{NDM} (selection media supplemented with cefotaxime – 2 mg L^{-1}) and pUC19 AmpR (selection media LBAmp) were used as a positive control for *Acinetobacter* sp. and *E. coli*, respectively.

Screening for the presence of Amp^R gene from pORF-hIL-12

Transformants isolated from LB or LBAmp agar plates and selected strains isolated after therapy from skin were tested for the presence of the pORF-hIL-12 sequences by PCR using primers MaF1 (5′-CGAGGTCCCTCCAAACCGTTGTCA-3′) and MaR (5′-CGTATCGTAGTTATCTACACGAC-3′) encompassing part of the Amp^R gene, pMB1Ori and SV40 pAn (nucleotide position 2305–3725). A 25 μL reaction mixture contained 5 μL of total genomic DNA prepared by boiling or using the DNeasy Blood Tissue Kit (Qiagen), 12.5 μL PCR Master Mix (Thermo Scientific), 1 μL (10 pg) of each primer and 5.5 μL of sterile nuclease free water. The PCR mixture was first denatured at 95°C for 5 min and then subjected to 30 cycles as follows: denaturation at 94 °C for 30 s, primer annealing at 60 °C for 30 s, and elongation at 72 °C for 2 min. The final elongation step was 7 min at 72 °C. PCR products were analyzed by electrophoresis on 0.9% (w/v) agarose gel.

Data analysis

The data were analyzed and presented using SigmaPlot software (Systat Software, London, UK).

Results

Treatment outcome and systemic release of cytokines

Diagnosis of mast cell tumours was made through means of fine needle aspiration biopsy of tumour nodules, as well as of local lymph nodes. In 11 of 18 patients punch biopsies were also performed before the treatment and this allowed the tumour grading: 3 neoplasms were grade I tumours and 8 were grade II. The median volume of treated tumours was 2.1 cm^3 (ranging from 0.16 to 16.88 cm^3). At the end of the observation period (median 40 months), CR was achieved in 13 of 18 (72%) of the treated mast cell tumours with another two nodules achieving PR, bringing OR of combined therapy of mast cell tumours in our study to an overall rate of 83%. Long-term (5 years) stable disease was achieved in one patient with multiple tumour nodules (patient no. 1 – Table 1). Response to the therapy correlated with the size of the tumour, because CR was achieved in all eight modules with volume < 2 cm^3. The response rate in bigger tumours was lower (CR 60% and OR 80% in the group of tumours with volume > 2 cm^3). The change of tumour volume at 4 weeks after the first treatment is presented in Figure 1A. With additional electrochemotherapy (patient no. 13) or additional combinational treatment (patient no. 18) we were able to achieve CR in tumours as big as 12 and 16 cm^3, respectively (Figure 1, Table 1). Beside tumour volume, another important predictive factor for the outcome of therapy was the clinical stage of the disease, because both PD cases (2/18 or 11%) were patients (patients nos 11 and 14) with higher clinical stage either II or III), one of them already having metastases in regional lymph nodes. However, short-term response in both of these two patients was encouraging (one CR and one SD) and both progressed only later in the course of observation. On the contrary histological grading did not influence the outcome of the disease in treating animals. All patients, which responded to therapy, remained in good clinical condition throughout the observation period, without any additional clinical signs of the disease. The only two exceptions were patients with PD, which were euthanized between 4 and 6 months after therapy (Table 1) due to progression of clinical signs. Another three patients (nos 4, 8 and 9), were euthanized before the end of observation period due to the causes unrelated to mast cell tumours [renal failure, development of another primary tumour (prostate carcinoma), heart failure].

The elevation of human IL-12 in serum was observed in 14 of 16 examined patients (88% of responders) at different time points after therapy. The highest concentration (15 $pg\,mL^{-1}$) was achieved in patient no. 18, who received the highest dose of plasmid DNA, 2 weeks after the procedure (data not shown). In the majority of patients, human IL-12 was detected up to 1 month after the procedure, except in three (patients nos 5, 13 and 18), where longer expression of human IL-12 was observed (3 months). Interestingly, patients nos 13 and 18 were those, in which CR was achieved despite having large volume tumours. Canine IFN-γ was detected in slightly lower percentage of treated patients (11/16 i.e. 69% of responders), but the concentrations of this cytokine were much higher than of human IL-12. The highest detected concentration was 134.4 $pg\,ml^{-1}$ in patient no. 3, 4 weeks after therapy. Both cytokines reached peak concentrations 4 weeks after therapy (7.1 $pg\,mL^{-1}$ for IL-12 and 83.8 $pg\,mL^{-1}$ for IFN-γ), with a decline in the majority of patients afterwards.

Histology of the tumours

Histologic investigations performed on tissue samples collected before the treatment confirmed the cytological diagnosis of mast cell tumours. Punch biopsies performed before the treatment allowed the tumour grading: three neoplasms were grade I tumours and eight were grade II. Four weeks after combined therapy, the neoplastic tissue was drastically reduced and confined to small areas delimited by a well differentiated connective tissue. In these areas microvessel density was reduced and lymphocyte infiltrates were present and mainly located around blood vessels. Three months post treatment a further reduction in the microvessel density was evident accompanied by the reduction of lymphocytic infiltration.

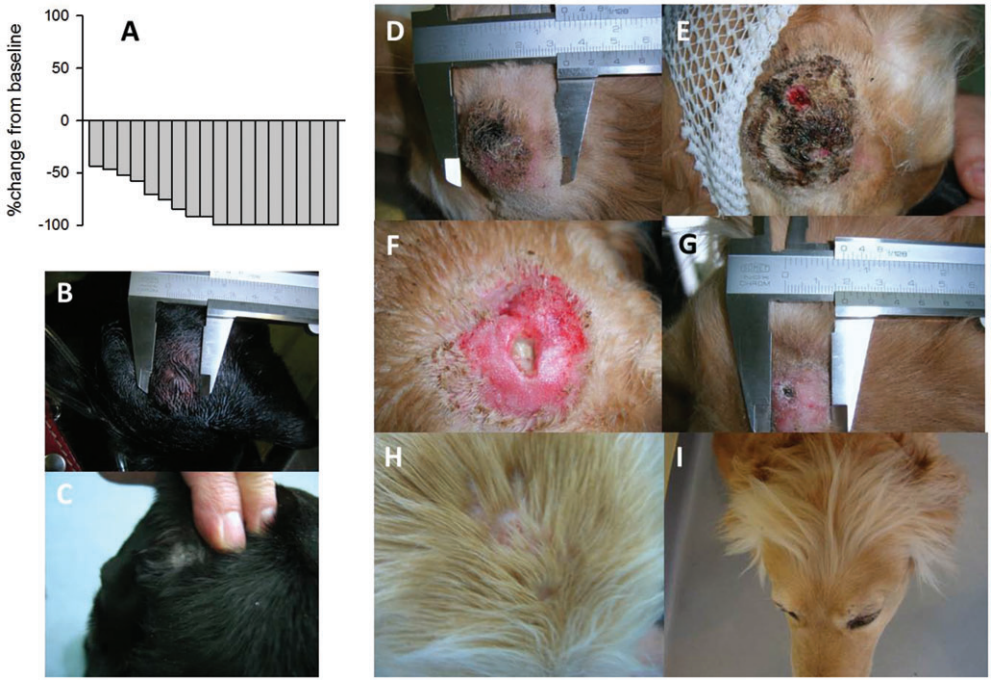

Figure 1. (A) Waterfall plot depicting change in tumour volume 4 weeks after the first treatment in all treated dogs. (B) Patient #12: maytocytoma on the base of the ear, single treatment with electrochemotherapy with cisplatin (1 mg) and IL-12 electrotransfer (1 mg). Size of tumour 2 × 1.5 × 1.5 cm at the beggining of treatment (B); after 4 months (C); (D) Patient #13: mastocytoma on the head, treatment 2× electrochemotehrapy with cisplatin i.t. + Il-12 electrogene therapy with 1 mg of plasmid. Size of tumour 3 × 3 × 2.7 cm at the beggining of treatment (D), 1 week after (E), 2 weeks after (F), 4 weeks after (G), 3 months after (H), 3.5 years after (I).

Side effects

In order to evaluate possible systemic effects of the therapy, Veterinary Cooperative Oncology Group toxicity scale (VCOG-CTCAE) at each posttreatment examination was followed. According to regular clinical examination and selected bloodwork at different time points after the procedure, no significant side effects, either on local or systemic level, were detected, even in the patients with detectable systemic release of both cytokines. All examined blood parameters remained in the reference range.

Detection of plasmid DNA

The presence of IL-12 plasmid was followed up to 4 weeks after the therapy. Immediately after therapy the maximal concentration of IL-12 plasmid detected at the site of injection was 40 ng of plasmid mL^{-1}. At the next examination, 1 week after therapy, the maximal concentration dropped to 0.13 ng mL^{-1}, at 2 weeks the concentration was 0.06 ng mL^{-1} and at 4 weeks, except in one patient having 0.01 ng mL^{-1}, all the rest of the patients were negative, meaning that the IL-12 plasmid could not be detected.

Horizontal gene transfer

Altogether 69 strains belonging to 17 different bacterial genera were isolated from skin before therapy and 53 strains from 17 genera after the therapy (Table 2). Whereas the majority of isolates before therapy belonged to *Bacillus* sp., *Microbacterium* sp., *Macrococcus* sp. and *Staphylococcus* sp., isolates from the genera *Staphylococcus*, *Acinetobacter*, *Bacillus* and *Pseudomonas* were more prevalent after the therapy. Strains from rather different genera were isolated from individual patients and from the same patient before and after the therapy. After *in vitro* transformation of the therapeutic plasmid into selected isolates no transformants containing part of the screened Amp^R region of IL-12 plasmid were detected by PCR. The only exception was an

E. coli strain isolated from the skin of one patient. Further, none of the ampicillin resistant and ampicillin susceptible strains isolated after the therapy contained sequences from the therapeutical IL-12 plasmid.

Discussion

The results of our clinical study demonstrated that electrochemotherapy combined with peritumoral IL-12 electrotransfer was effectively reducing tumour burden, leading to 83% of OR, with 72% of the long term CR. The therapy was more effective in small tumours ($<2\,cm^3$), but even very large tumours ($>10\,cm^3$) were cured by combined therapy. Furthermore, all patients with OR were without the recurrence during the observation period (median 40 months). Even though the number of treated dogs was rather small, the clinical staging of tumours before the treatment seems to influence the outcome of the disease, in fact the two patients with clinical stage III had a SD. On the contrary, the tumour grading seems not to be predictable of the outcome of the disease after the therapy. Levels of cytokines (human IL-12 and canine IFN-γ) were not predictive of the response to therapy. Based on histopathological and immunohistochemical examination of tumours after 4 weeks the response to the therapy was mainly mediated by vascular effects of electrochemotherapy and IL-12 gene electrotransfer. No local or systemic side effects were observed. Absence of the IL-12 plasmid in the treated area 4 weeks after the therapy and in the isolated bacterial strains as well as the inability of the *in vitro* transformation into the isolated strains confirms, for the first time, that combination of electrochemotherapy with IL-12 electrogene therapy is also a safe therapy, without potential to spread antibiotic resistance.

Electrochemotherapy of cutaneous tumour of different histological types is a well-established treatment in both human and veterinary oncology. For certain histiotypes of human tumours, the CR rate is above 90% (basal cell carcinoma).[41] Although it was recently demonstrated that electrochemotherapy induces immunological cell death, the therapy on its own is not able to induce a systemic immunity, thus not having the abscopal effect.[9] Therefore, to add a systemic component to local electrochemotherapy treatment, it was combined on a preclinical level with several immunological approaches, such as the addition of IL-2 secreting cells, recombinant TNF-α and also IL-12 gene electrotransfer, which proved to be very effective.[12–14,16–18,42] Even on its own, IL-12 electrogene therapy was successful in the eradication of melanoma and sarcoma tumours in mice.[15,43] Intratumoural application of IL-12 gene electrotransfer resulted in 90% of CR in SA-1 sarcomas, peritumoral delivery to skin in 16% of CR and intramuscular in 28%.[15,44] In our previous study, intramuscular delivery of human IL-12 to 6 dog's patients with advanced disease, which were pre or concomitantly treated either by surgery of chemotherapy was evaluated. Human IL-12, was selected according to available data indicating that canine and human IL-12 share approximately 90% homology based on amino acid sequence analysis.[45] In addition, it was shown that human IL-12 activates *in vitro* the proliferation of canine peripheral blood mononuclear cells and that human IL-12 can trigger a number of immune responses in canine mononuclear cells.[46] The therapy with human IL-12 gene therapy resulted in prolonged survival of the patients, even for more than 4 years in grade III MCT.[47] In addition, antitumour effect of intratumoural IL-12 electrogene therapy alone was already evaluated in canine mast cell tumours.[23] In the clinical study 8 dogs with 11 mast cell tumours were treated with direct intratumour application of IL-12 gene electrotransfer. Contrary to preclinical observation, the results of a clinical study showed a smaller effect as only 36% of CR were observed[23], while electrochemotherapy on its own resulted in 70% complete responses of tumours.[48] The results of our current study support the combined therapy, because the CR rate was increased to 72% of all treated tumours and even to 100% in tumours smaller than $2\,cm^3$. Recently, a clinical study on several different types of dogs' tumours (squamous cell carcinoma, acanthomatous ameloblastoma, plasmocytoma and sarcoma) was performed evaluating the combined therapy of electrochemotherapy with bleomycin or gemcitabine and plasmid IL-12. The results of the study demonstrated the effectiveness of this therapy, however, with lower percentage of

Table 2. Identified bacterial isolates from the dog's skin before and after therapy

Identified bacterial genera, isolated from dog skin before therapy	No. of identified isolates of the same genus	Identified bacterial genera, isolated from dog skin after therapy	No. of identified isolates of the same genus
Acinetobacter	1	*Acinetobacter*	9
Arthrobacter	1	*Agromyces*	1
Bacillus	18	*Bacillus*	8
Enterococcus	1	*Brachybacterium*	2
Kocuria	3	*Escherichia*	1
Macrococcus	9	*Janibacter*	1
Microbacterium	10	*Kocuria*	1
Micrococcus	7	*Macrococcus*	3
Oceanobacillus	1	*Microbacterium*	1
Paenibacillus	1	*Micrococcus*	3
Planococcus	1	*Mycobacterium*	1
Pseudomonas	1	*Oceanobacillus*	1
Rhodococcus	3	*Pseudomonas*	5
Rothia	2	*Rhizobium*	2
Sporosarcina	1	*Rhodococcus*	2
Staphylococcus	8	*Rothia*	2
Streptomyces	1	*Staphylococcus*	10

CRs compared with our study.[19] Several reasons for the observed differences in antitumour effectiveness between the two studies could be foreseen. Firstly, different tumour types were treated, which can respond differently due to the intrinsic properties; secondly the treatment protocol between the studies was different, which can also influence the treatment outcome. Namely, in the study of Cutrera et al.[19], the plasmid DNA was mixed with bleomycin or gemcitabine. Especially, in the case of bleomycin, which acts as an endonuclease, one can expect that the plasmid DNA will be damaged before injection into the tissue and thus leading to lower levels of the therapeutic protein, IL-12.[49] Nonetheless, also the parameters of electric pulses differed, which is a well known factor that affects the level of increased permeability of the cells' membrane and thus intake of the molecules (chemotherapeutic drug or plasmid DNA).[50] Furthermore, the size of tumours was a predictor of tumour response in our study; tumours smaller than 2 cm^3 respond better than bigger tumours, while in the study of Cutrera et al. the treatment was equally effective regardless of the tumour volume. Similarly, size was also a predictor of response in electrochemotherapy of human tumours, and the cutoff was the same as observed in our study (2 cm^3), as demonstrated by meta-analysis.[51]

Intratumoural plasmid IL-12 gene electrotransfer into malignant melanoma in humans, resulted in the abscopal effect on distant untreated tumours. In our clinical study, the patients that were included in the study, had only one tumour nodule present at the time of inclusion into the study. Therefore, we could not follow the abscopal effect, but we followed the effect on the recurrence and the development of metastases. In literature, after surgical treatment with histologically clean margins grade II tumours very rarely recur.[52]

In addition, up to 22% of grade II MCT metastasize and cause death in up to 56% of affected patients due to either local treatment failure or metastatic disease.[53] In our study, six MCT grade II tumours without metastases were included. The dogs were cured by combined treatment and were also without any detectable metastases until the end of the observation period. Progression of the disease was seen only in two patients, both of them were having metastatic disease present at the time of treatment and one also with very big tumour (patient no. 14; 14.24 cm^3). In addition, based on our previous data on electrochemotherapy alone and surgical excision of MCTs[23,48], the combined treatment also results in better long-term control. Namely, all the dogs that achieved PR, were without the progression of the disease until

the end of the observation period (median 40 months), while 50–70% recurrence rate was determined at 22 months after surgery and after electrochemotherapy.[48] Thus, we can speculate that for local tumour regression, treatment with electrochemotherapy is enough, however, long-term tumour control and prevention of metastases could be attributed to the development of antitumour immunity mediated by immune action of IL-12, which has already been demonstrated in preclinical studies.[11,15,44] In addition, the histological analysis, which was performed 4 weeks after the therapy, showed mainly vascular action of the therapy and also minimal infiltration of T lymphocytes. Further immune-oriented studies determining the profile of immune cells in blood are needed to complete demonstrate the vaccination effect of the therapy.

We paid attention to two possible groups of adverse effects in the treated population. Mechanical manipulation of MCTs can lead to degranulation of mast cells, causing histamine release from granules, which is one of the major concerns during biopsy, surgical manipulation, etc. In the present and previous studies, we did not detect any such side effects.[48] The second set of possible adverse reactions could arise from the toxic effects of systemically circulating IL-12. However, a range of haematological and biochemical parameters indicative of renal function did not show any significant alterations.

Importantly, according to our best knowledge, our study is the first where the safety issues regarding the use of plasmid DNA were assessed. Namely, the persistence of the plasmid DNA at the site of injection was monitored, as well as possible horizontal gene transfer of antibiotic resistance gene present in the plasmid to commensal bacteria present at the dogs' skin. Our results undoubtedly showed that, although little amount of the plasmid DNA can be detected up to 2 weeks after the therapy, this plasmid cannot be horizontally transferred into the culturable bacteria found on the treated patients. When the strains were made competent by *in vitro* methods, the IL-12 plasmid was transferred to the *E. coli* only. This result was in accordance with our expectations since *E. coli* is the host strain for manipulation and amplification of pORF-hIL-12. Although *E. coli* might frequently colonize the skin due to licking of the genital region, little attention is paid, because the species is thought to be naturally not competent.[54] However, some authors argue that *E. coli* could be competent in some environments [55,56] or on specific media.[37] Thus, although, we could not detect the IL-12 plasmid in *E. coli* in our study, the recommendation for further studies would be to use the plasmid devoid of antibiotic resistance gene or if this is not an option, the owners should be especially careful for the first two weeks and the dogs should strictly wear Elisabethian collars.

In conclusion, our study demonstrates significant antitumour response of electrochemotherapy and IL-12 electrotransfer, which resulted also in prevention of local recurrences and distant metastases. Importantly, safety regarding the use of plasmid DNA was also demonstrated.

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Conflict of interest

The authors declare no conflict of interest.

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