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REVIEW

Electrochemotherapy in the treatment of neoplasms in dogs and cats

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ABSTRACT. Electrochemotherapy (ECT) is a technique that combines chemotherapy with local application of specific electric pulses with the aim of increasing the permeability of the plasma membrane in a reversible way, improving the influx of chemotherapeutic drugs into the cytoplasm and potentiating their cytotoxic effects. This technique has broadened the range of possible treatments for oncological patients, either on its own or as adjuvant to surgical procedures. It is especially useful in tumors located in regions with only a small surgical safety margin, such as the limb extremities, skull, oral cavity, neck and perianal region, among others. ECT makes it feasible to perform procedures more conservatively, or even to perform otherwise infeasible procedures, by expanding the margins without removing healthy tissues. The objective of this paper is to provide a brief bibliographic review of the principles, applications and future possibilities of electrochemotherapy, helping to disseminate pertinent information about this relatively new technique for the treatment of cancer.

Key words: bleomycin, electroporation, veterinary oncology, electrochemotherapy.

MECHANISM OF ELECTROPORATION AND ELECTROCHEMOTHERAPY

Electroporation, or electropermeabilisation , consists of the exposure of cells to specific electrical pulses (1000 V/cm, 100 microseconds, 8 pulses at a 5 kHz frequency), which transiently increase the permeability of the plasma membrane, allowing access to the cytoplasm for molecules to which the membrane is normally impermeable or only slightly permeable (Mir *et al* 1996, Teissie *et al* 2005, Mir 2006). Under physiological conditions, the plasma membrane is subject to the so-called membrane resting potential, which remains balanced by pumps and ion channels, keeping the electrical potential of the membrane stable. Exposure of the cells to an external electric field produces an induced transmembrane potential difference, altering its resting potential and causing rearrangement of the molecules in the lipid bilayer, resulting in the formation of pores (Almers *et al* 2010, Kotnik *et al* 2010). Electroporation depends on the specific electrical parameters; the amplitude and duration of the electric field to which the cells are exposed are key points of the process (figure 1). At low amplitude and duration, there is no detectable effect on the plasma membrane in terms of its permeability, while at moderate amplitude and duration, electropermeabilisation occurs. In the pore model, the pores close after the end of exposure, causing the cells to remain viable. Excessive amplitudes and/or durations cause nonthermal irreversible electropermeabilisation , possibly due to nonclosure or late closure of the pores, resulting in a form of cell death called mitotic catastrophe. Mitotic catastrophe is cell death preceded by multinucleation that occurs during the metaphase (Kroemer *et al* 2009). Irreversible thermal electroporation can also occur when even higher amplitudes are applied, causing thermal damages to the cells (Cemazar *et al* 2015). An ultrastructural study showed some changes in the membranes, such as defects in the dynamic assembly of lipids and proteins after exposure to the electric field used in electroporation, i.e. proteins formed membrane agglomerates and hydrophilic pores. These defects in dynamic assembly would be responsible for the entry of molecules during electroporation (Spugnini *et al* 2007[c]).

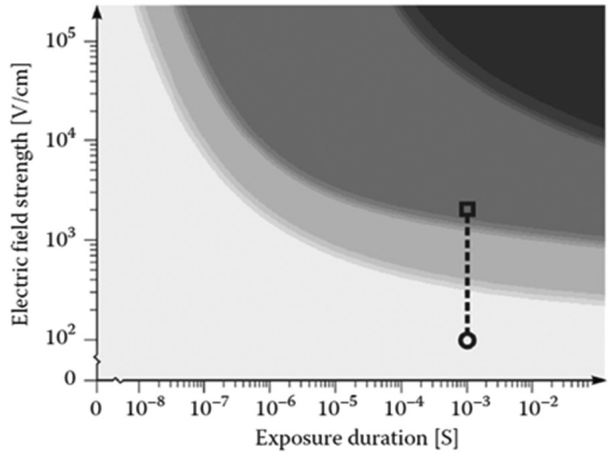

Figure 1. Electroporation and thermal effects caused by exposure of cells to electric fields. Graphics representing reversible and irreversible electroporation and thermal damage caused to cells due to the electric field strength ([V/cm]) and exposure duration ([s]) in seconds. Source: Cezamar, et al., 2015 (adapted).

ELECTROCHEMOTHERAPY

Electrochemotherapy (ECT) is a technique that combines chemotherapy with the application of such specific

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electrical pulses, allowing the entry of nonpermeating antineoplastic drugs (or those with low permeant potential) into cells (Mir 2006). The waveforms used in electrochemotherapy are square-wave mono- or biphasic pulses. The most common electric parameters used in electrochemotherapy are 8 pulses, 100 µs length, 1-5 kHz frequency and 1000 V/cm (for needle parallel electrodes) or 1300 V/cm (plate electrodes) of electric field intensity[1] (Spugnini *et al* 2007[a], Pavlin *et al* 2009, Tozon *et al* 2016, Maglietti *et al* 2016, Pierini *et al* 2016, Suzuki *et al* 2018, Lowe *et al* 2017). For this technique, bleomycin and cisplatin are the main drugs used (Sersa *et al* 2008, Escoffre and Rols 2012). Bleomycin is widely used in electrochemotherapy due to its high intrinsic cytotoxic potential and cell selectivity caused by the mechanism of mitotic cell death. Once inside the cell, bleomycin acts as an endonuclease, with the potential to promote single- and double-stranded ruptures in DNA, predominantly affecting cells in the process of proliferation (Orlowski *et al* 1988, MIR *et al* 1988, Mir 2006).

Although bleomycin can enter permeabilised cells using plasma membrane proteins, its capacity is highly restricted by the plasma membrane, which explains its limited action in these cells. This is because only some molecules are effectively internalised and reach the DNA (Mir *et al* 1996). Electropermeabilisation is a way of overcoming such barriers; *in vitro* studies report that electropermeabilisation potentiates the cytotoxic effect of the drugs by up to thousands of times (Poddevin 1991, Mir *et al* 1996, Gehl 2003, Orlowski *et al* 2016). ECT increases the absorption of antineoplastic drugs, which will remain internalised in the cell after membrane permeability is restored, maximizing their cytotoxicity. Another property of ECT is that its action is restricted to tissues exposed to the electrical pulses, reducing the risk of chemotherapeutic side effects (Spugnini and Porrello 2003, Gehl 2003, Miklavcic *et al* 2014, Tafuto *et al* 2015). Bleomycin is a drug that induces two mechanisms of cell death, depending on the number of molecules internalised after electropermeabilisation. At low concentrations of bleomycin, the cell dies after three doubling times (called mitotic death); at high concentrations, cell death occurs in a manner analogous to apoptosis (called mimetic apoptosis) (Tounekti *et al* 1993).

In order to reach the electroporation threshold, several aspects must be considered, such as physical factors (cell size and shape), biological features (cytoskeleton structure) and adequate electrical parameters. Hypothetically, however, it is possible to perform electroporation independently of the type of cell or tissue (Rols and Teissie 1992, Teissie *et al* 2005, Cemazar *et al* 2015, Sersa *et al* 2015).

Two factors must be taken into consideration for the efficiency of ECT. First, the chemotherapeutic drug can be applied either intratumorally, in which case immediate electropermeabilisation must be performed, or intravenously, with an interval of approximately 8 minutes until electropermeabilisation. The waiting time for the intravenous route allows the antineoplastic drug to reach its pharmacological peak in the tumor, remaining for approximately 20 to 25 minutes at an effective concentration according to the principles of the technique. The second factor is that the entire tumor, including any infiltration into adjacent tissue, must be exposed to a sufficient electric field to promote reversible electropermeabilisation, this being obtained by correct choice and placement of electrodes and application of electrical pulses with suitable parameters (Marty *et al* 2006, Domenge *et al* 1996). In the case of tumors with larger proportions due to abnormal vascularization, hindering proper distribution of the drug, both intravenous and intratumoral administration can be considered (Maglietti *et al* 2016).

Due to the stochastic nature of the process, access to the tumor through electrode placement and efficient distribution of the electric field could be more important factors than the chemosensitivity of the neoplasia to the drug (Gehl 2003, Mir 2006, Cemazar *et al* 2015).

In addition to increased membrane permeability, other mechanisms are described in relation to ECT, such as the effect of vascular lock and involvement of the immune response. According to Sersa *et al* (2008), electrochemotherapy promotes a process of vascular dysregulation throughout two distinct mechanisms. One of them is that the electrical pulses cause transient cytoskeleton damage and swelling of the vascular endothelium, which leads to the death of some tumor and vascular cells. Another is the endothelial cytotoxicity promoted by ECT. Both mechanisms reduce local blood flow, decreasing the oxygenation of the tumor, leading to hypoxia and subsequent necrosis of tumor cells (Spugnini *et al* 2006). Additionally, some studies have used intravital microscopy to confirm vasoconstriction induced by ECT in normal and tumoral blood vessels. Vascular lock (or vascular sequestration), induced transiently by electroporation alone and for a prolonged time by electrochemotherapy, keeps the antineoplastic accumulated and prevents it from rapidly exiting the tumor due to reduced blood flow (Jarm *et al* 2010).

Involvement of the immune system after electrochemotherapy is important for eliminating tumoral cells. Due to the heterogenicity of tumor cells (affecting the orientation, size and distribution of chemotherapy in the tumor), not all cells are eliminated by electrochemotherapy because some have not been effectively electroporated or internalized sufficient amounts of the chemotherapeutic drug (Miklavcic *et al* 2014, Sersa *et al* 1997). As with other physical methods, such as radiotherapy, when the fraction of remaining cells is low enough, they can be eliminated by the immune system. A similar situation was observed

[1] Tellado M, Maglietti F, Olaiz N, Michinski S, Marshall G. 2014. Electroquimioterapia como herramienta terapéutica en melanoma oral en caninos. Available at: http://vetoncologia.com/wp-content/uploads/2014/09/melanoma-oral-canino.pdf

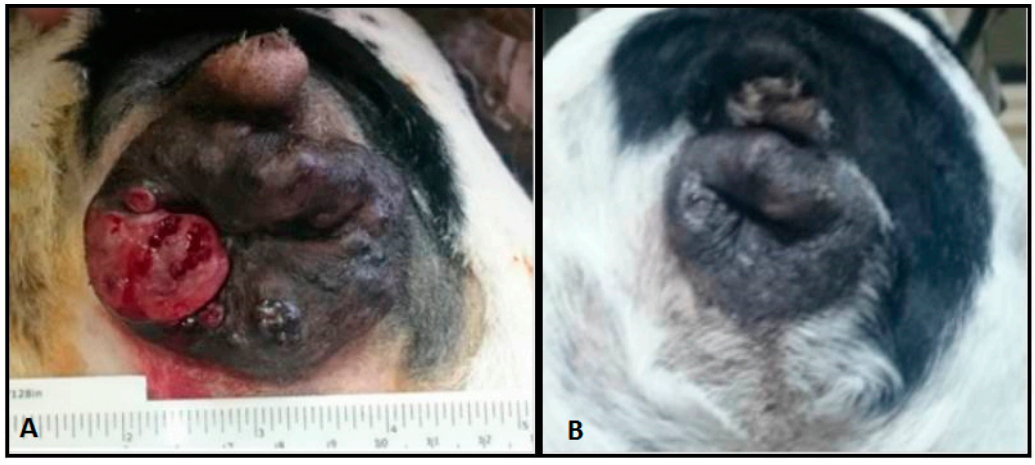

Figure 2. Anal hepatoid gland carcinoma in a dog treated with one session of electrochemotherapy (endovenous bleomycin 20 mg/m^2). Before ECT (A), 2 months after ECT (B).

in immunodeficient organisms, which had a significantly lower cure rate than immunocompetent individuals after electrochemotherapy treatment (Sersa *et al* 1997). This might occur due to exposure of immunogenic molecules, such as calreticulin, and the release of neoplastic antigens from the cells destroyed by the ECT, which can activate immune cells against the tumor (Calvet *et al* 2014, Gerlini *et al* 2013, Gerlini *et al* 2012, Miklavcic *et al* 2012).

CLINICAL RESULTS OF ELECTROCHEMOTHERAPY USE

Many studies have reported favorable results in different cancer types traditionally known for limited or no treatment option or for poor response to standard therapy (table 1). Additionally, ECT could be an option for unresectable tumors or those in areas with anatomical limitations that make them amenable to this treatment. For ECT evaluation, many oncologists use the criteria for solid tumors proposed by Veterinary comparative oncology group (VCOG) (Nguyen *et al* 2013): Compete response (CR) is defined as disappearance of all target lesions and pathologic LNs measuring < 10 mm along the short axis. Partial response (PR) consists of at least 30% reduction in the sum of the diameters of the target lesions. Progressive disease (PD) is defined as either the appearance of one or more new lesions or at least a 20% increase in the sum of the diameters of the lesions, taking as a reference the minimal sum on examination. Additionally, the sum must show an increase of 5 mm. For stable disease (SD), the evaluation must include tumors with less than 30% reduction (PR) or a 20% increase (PD) in the sum of the diameters of the target tumors, compared to the smallest sum of diameters during monitoring. Additionally, many studies include an overall response (CR+PR) to evaluate the study group.

In canines, favorable results are observed in skin tumors, especially mast cell and perianal tumors (figure 2). In the case of mast cell tumors, using the histological grade established by Patnaik *et al* (1984), two studies administering intratumoral cisplatin achieved complete remission in 62.6% of grade I and III tumors (25 cases) and in 78% (37 cases), including 7 grade I, 24 grade II and 6 grade III cases, with an average of 1,218 days until recurrence (Kodre *et al* 2009, Spugnini *et al* 2011). Another study using endovenous bleomycin in mast cell tumors reported complete objective remission when analysing 80 dogs; histological grade details were not included (Tozon *et al* 2016). However, ECT showed the greatest effectiveness in tumors < 2 cm. Lowe *et al* (2017) used a similar treatment, evaluating four groups and concluding that 51 dogs had 100% objective response and a recurrence time of 1,500 days. For perianal tumors, the objective response rate using intratumoral bleomycin or cisplatin was 92% when evaluated at 34 months (Tozon *et al* 2005) and 94% when evaluated at 14 months, with complete remission in 84.2% (Tozon *et al* 2010).

In cats, ECT has emerged as an effective treatment for squamous cell carcinomas (SCC), especially when the tumor invasion suggests aggressive treatments (figure 3). Several studies have reported that ECT can reach 100% objective response (73% complete remission + 27% partial response), with a median survival time of 452 days, for all stages of SCC considered together (Pierini *et al* 2016). Previous studies in SCC found an objective response rate of 87.5% (17 masses), with a recurrence rate of 22% observed in 2 to 8 years (Tozon *et al* 2014). Spugnini *et al* (2015) determined an 81% objective response rate in 21 cats with SCC. This study used a control group and compared the results with the ECT group. The median recurrence time was 30.5 month for the ECT group and 3.9 months for the control group.

In soft tissue sarcoma (STS) treatment using intratumoral bleomycin and cisplatin, a sample of 22 dogs had an objective response rate of 95% (Spugnini *et al*

Table 1. Comparative results of different tumors using electrochemotherapy.

Specie	Diagnosis	Cases (n)	Drug	Results	Reference
Dog	STS	22	Bleo (it)	CR: 90%	Spugnini *et al* 2007
Dog	Perianal tumor	12 (26 tumors)	Cis (it) Bleo (it)	OR. 92%	Tozon *et al* 2005
Dog	Perianal tumor	21 (66 tumors)	Cis (it) Bleo (it)	OR: 94%	Tozon *et al* 2010
Dog	MCT	51	Bleo (ev)	CR: 93-64%	Lowe *et al* 2016
Dog	MCT	25	Cis (L)	CR: 62,5%	Kodre *et al* 2009
Dog	MCT	37	Cis (it)	CR :78%	Spugnini *et al* 2011
Dog	CTVT	3	Bleo (it)	CR: 100%	Spugnini *et al* 2008
Dog	Nasal tumors	11	Bleo (ev)	CR: 27% OR: 91%	Maglietti *et al* 2017
Dog	MT	34	Bleo (it)	CR: 88,7%	Silveira *et al* 2010
Feline	STS	39	Bleo (it)	R: 4-19 months	Spugnini *et al* 2007
Feline	STS	64	CIs (it)	R: 666 days R: 180 days control group	Spugnini *et al* 2011
Feline	SCC	16 (17 tumors)	Bleo (ev)	CR:75%	Tozon *et al* 2014
Feline	SCC	12 (16 tumors)	Bleo (ev)	CR: 73%	Lowe *et al* 2016
Feline	SCC	26 cats	Bleo (iv)	CR: 81 %	Spugnini *et al* 2015
Feline/Dog	Lymphoma	2 dogs 4 felines	Bleo (it)	CR: 100%	
Feline/Dog	MT	140 dogs 36 cats	Bleo (iv)	R: 33%	Lowe 2016
Feline/Dog	MCT	80 dogs 20 cats	Cis (it), Bleo (it/ev)	CR: 100 % <2cms	Tozon *et al* 2016

SCC: Squamous cells carcinoma, MCT: Mast Cells Tumor, STS: Soft tissue sarcoma, CTVT: Canine transmissible venereal tumor, MT: Multiple tumors, Bleo: Bleomicina, CIS: Cisplatin, it: intratumoral, ev: endovenous.

Figure 3. Nasal squamous cell carcinoma in a cat treated with one session of electrochemotherapy (endovenous bleomycin 20 mg/m^2). Before ECT (A), 2 months after ECT (B).

2007[a]). On the other hand, two studies of feline STS in a total of 103 cats had overall responses rates of 70% and 54%, with recurrence times of 666 days and 570 days, respectively (Spugnini *et al* 2011[g], Spugnini *et al* 2007[b]). In both studies, tumors with smaller size had better responses. The evaluation of ECT in tumors with poor response to surgery or chemotherapy is very positive. Twelve dogs with melanoma treated with ECT using bleomycin IV reached an OR of 83.6% with a complete remission rate of 41.4%[2]. Maglietti *et al* (2017) used a single needle electrode for eleven dogs with intranasal tumors, achieving complete response in 27% and partial response in 67%. In this case, the survival time of the ECT group was significantly higher than that of the control group, with mean survival times of 16.86 months and 5.3 months, respectively. On the other hand, dogs with tumors that were chemotherapy resistant to standard protocols had been treated with ECT. In this case, three dogs with transmissible venereal tumors with a resistance to vincristine were treated with intratumoral bleomycin, achieving complete remission (Spugnini *et al* 2007). Similar results were reached in two dogs and four cats with cutaneous lymphoma (Spugnini *et al* 2008). Finally, Lowe (2016) treated different spontaneous tumors and achieved objective response in 66% of 176 patients.

Several points can be listed that support the applicability of the ECT, making it an exceptional tool in the treatment of skin and subcutaneous solid tumors. For example:

- High remission rate: complete remissions were obtained in 58.4% of tumors, independent of histological origin, and partial responses in 24.7% of nodules treated after a single session, according to the meta-analysis provided by Mali *et al* (2013) involving 1894 tumors;
- It not only treats the tumor but also acts on the adjacent margins, thus eliminating possible infiltrated tumor cells (Miklavcic *et al* 2012);
- It presents an increase in the response rate when reapplied at weekly intervals, on tumors that did not have complete remission with single application (Campana *et al* 2009);
- It stimulates the immune response against possible remaining cells after ECT (Miklavcic *et al* 2012);
- It is effective in areas previously treated by means of surgery or radiotherapy, and even to tumors that are refractory to chemotherapy (Miklavcic *et al* 2012);
- It does not cause immediate or delayed toxic effects to patients (Miklavcic *et al* 2012);
- It has a good cost-benefit relation regarding the technology and chemotherapy used, not requiring large investments (Marty *et al* 2006).

[2] Electroquimioterapia como herramienta terapéutica en melanoma oral en caninos. www.vetoncologia.com/wp-content/uploads/2014/09/melanoma-oral-canino.pdf. Retrieved:04 August 2018.

The technique has been developed to enable the use of ECT in tumors and internal cavities (Miklavcic *et al* 2012), and to imaging exams such as positron emission tomography, ultrasonography and magnetic resonance imaging, making it possible to locate such tumors and even position the electrodes in internal organs (Miklavcic *et al* 2010, Pavliha *et al* 2013). The exact localization of the tumor and the use of special needle electrodes allow the application of ECT in nonsuperficial tumors such as metastases of melanomas, sarcomas, bone tumors and even brain tumors (figure 2), or its use in the surgical treatment of internal organs or percutaneous treatment of the limbs. In these cases, the retraction of the electrodes could cause hemorrhage; however, the effect of vascular blocking would minimise this situation (Miklavcic *et al* 2010, Jarm *et al* 2010, Cemazar *et al* 2015). Techniques to avoid interferences on cardiac electrical activity are also being developed. The planning of the electric field is critical to the effectiveness of ECT and must be correlated with the site of application due to the heterogeneity of tissue conductivities, which may result in an irregular distribution of the electric field, and care must be taken not to affect adjacent organs (Domenge *et al* 1996, Deodhar *et al* 2011, Mali *et al* 2008, Suzuki *et al* 2018). Electrochemotherapy is under development. Currently, electroporation devices are being improved, and special types of electrodes for use in specific areas are being created as well as synchronisation techniques timing electrical pulses to the electrocardiogram for regions close to the heart. The use of guided laparoscopy is also being developed in addition to techniques for real-time monitoring of electroporation and distribution of the electric field, which allow improved control of the electrode positioning and an effective treatment of tumors even in areas of difficult access (Kranjc *et al* 2011, Mahmood *et al* 2011, Miklavcic *et al* 2012, Cemazar *et al* 2015).

Although electrochemotherapy has become a well-established treatment for malignancies of skin and nonskin origin, standard evaluations and higher-quality clinical data are needed. Therefore, a standardisation of terminology and reporting criteria is necessary to facilitate effective communication among researchers and appropriate comparison between different treatment technologies (Campana *et al* 2016).

CONCLUSION

ECT is a technique that increases the range of possible treatments for cancer patients, making it possible to perform more conservative procedures and even surgical procedures that had previously been technically unfeasible. The use of electrochemotherapy along with other therapies has shown encouraging results in dogs and cats with different types of cancer. Further research on this technique promises to contribute major advancements in the treatment of cancer.

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