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REVIEW

APPLICATIONS OF ELECTROPORATION IN HUMAN AND VETERINARY ONCOLOGY

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ABSTRACT: Electroporation (EP) is a methodology that favors the transport of different molecules in various *in vitro* and in *vivo* settings, by the application of electric pulses displaying suitable amplitude and waveforms. EP has been widely implemented in veterinary oncology and one of the most well recognized applications is electrochemotherapy (ECT), that is presently adopted as a first-line cure for numerous neoplastic pathologies. Other applications in veterinary oncology include irreversible electroporation and EP based cancer vaccines. In human oncology, EP is still mostly restricted to the cure of cutaneous tumors, and to palliation of cutaneous and visceral metastases of malignant tumors. This review article summarizes the state of the art of EP in veterinary and human oncology, reporting the most important outcomes gotten to date.

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Impact statement: The use of electric pulses to facilitate the transport of anticancer molecules in *in vitro* and *in vivo* models is an extensively acknowledged procedure, that is identified as electroporation (EP). This article reviews the state of art of distinct EP applications, such as electrochemotherapy (ECT), irreversible electroporation and EP based cancer vaccines in veterinary and human oncology.

Key words: *electrochemotherapy; electroimmunotherapy; irreversible electroporation; cancer vaccine.*

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INTRODUCTION

Electroporation (EP) is a method to favor the transmembrane flow of different molecules in cells by means of electric fields having stable parameters (1). This technology is able to alter membrane permeability finally leading to two possible consequences: 1) termination of the phenomenon of boosted permeability and reestablishment of the original conditions, 2) failure of the cell to return to the original conditions, thus eliciting the apoptotic or necrotic pathways with consequent osmotic death. The first circumstance is termed reversible electroporation, while the second is irreversible electroporation (2). EP-based oncologic therapies embrace: irreversible electroporation (IRE), gene electrotransfer (GET), electrochemotherapy (ECT), calcium electroporation (Ca-EP), and therapeutic vesicles loading (*i.e.*, liposomes, exosomes *etc*.) (EP-TVL). Unfortunately, these therapeutic strategies are still in a largely experimental phase and their use is limited to a few tumor pathologies. This article aims to describe the state of the art of these methodologies, the current and future applications and the most important lines of research on the topic. The physical properties of the electric pulses, that is shape, number and duration, are the utmost key factors influencing membrane electroporation (1). EP initially favors the formation and expansion of pores. These pores in an in-

itial phase are defined as transient electropores. They subsequently stabilize, assuming the characteristics of long-lasting electropores (3-5). While larger molecules necessarily use transient electropores, small molecules can use both types of pores with a superior amount moved in the course of the long-lasting period. The types of electrical waves that have proven most effective in influencing cell permeability in both *in vitro* and *in vivo* systems are electric square and biphasic pulses while exponentially decaying pulses are less efficacious (1).

THE VARIOUS PROCEDURES OF ELECTROPORATION

IRE is a methodology in which EP is applied by means of 60-100 high voltage (1.5-3 kV) bursts of 80-100 µs to induce osmotic cell death. The cellular mechanism responsible for this osmotic death consists in an activation of reactive oxygen species due to a hyperaccumulation of intracellular calcium resulting from an altered transmembrane ion flow. The cell death triggered by IRE happens without considerable thermal warming or heat-induced tissue damage (6).

The GET methodology is based on the creation of micropores capable of making genetic particles, such as plasmids, cross the cell membrane to induce the expression of genes responsible for the induction of the immune system or the death of the target cell (9).

ECT is a method in which the administration of chemotherapy drugs locally or systemically is accompanied by the application of high voltage electric fields on the tumor tissue to promote the permeabilization of tumor cells to anti-tumor drugs (10). Several different electric protocols have been proposed. In **Figure 1A**, **B**, **C** the most adopted electric fields for ECT are depicted. The most used cytotoxic chemotherapeutics are bleomycin, which is equally effective intravenously or intra-tumoral, and cisplatin, which has a greater impact when administered locally. They permeate the plasma membrane very slowly, but following the application of the electrical pulse, when the drug reaches maximum concentration, transport across the membrane increases considerably (11). Quite a lot of studies have proved the clinical strength of this treatment in terms of higher tumor control, with generally narrow toxicity both in human and veterinary medicine (10).

Calcium EP is a methodology in which the application of high voltage electric fields is used to favor the entry of high concentrations of calcium ions inside the neoplastic cells, thus favoring the activation of reactive oxidative species and, consequently, cell death by apotoptosis (12-14).

In EP-TVL the target of the electroporation is no longer the tumor cell, but the nanovesicles, which in this way can be loaded with pharmacologically active molecules and used as carriers for the transport of these molecules within the cells (15).

APPLICATIONS OF EP METHODOLOGIES IN HUMAN AND VETERINARY ONCOLOGY

IRE

IRE has been successfully used in human oncology in recent years as a therapeutic approach for advanced and inoperable metastatic tumors of different organs, including prostate (16, 17), pancreas (18, 19), liver (20, 21) and breast (22). Several preclinical studies have, however, highlighted some adverse side effects such as muscle contractions and cardiac arrhythmias which must be taken into consideration and managed through electrocardiogram (ECG) synchronization and the use of neuromuscular blockers.

In veterinary oncology, the most relevant studies on

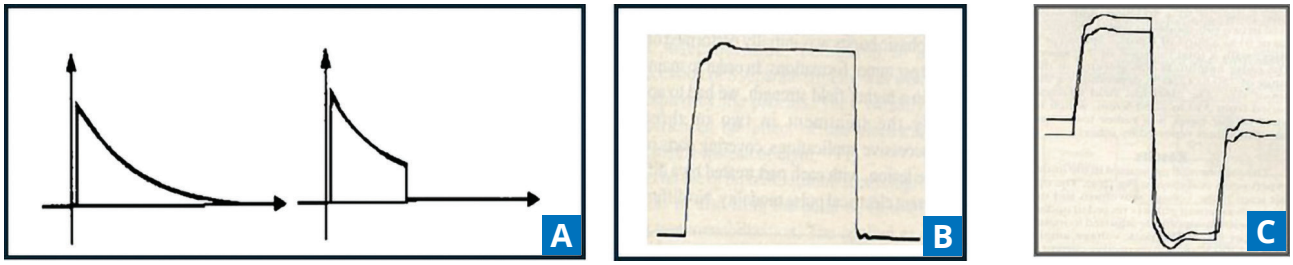

Figure 1. *Different waveforms used in ECT.* ***(A)*** *Exponentially decaying;* ***(B)*** *square shaped;* ***(C)*** *biphasic shaped.*

the use of IRE concern some brain tumors that are difficult to surgically approach (23, 24). Another tumor type in which IRE has been successfully used in pets is transitional carcinoma of the urethra, which represents another example of cancer in which surgical procedures are difficult to implement (25). Finally, transcutaneous IRE has been adopted to treat unresectable liver tumors in a canine model (26). From a careful analysis of the literature, it is clear that this methodology shows undoubted advantages in the treatment of some tumor conditions for which classical surgery cannot be used. Nonetheless, it requires particular skills of the operator both with regards to the correct insertion of the needles and for the choice of the optimal electrical parameters to guarantee therapeutic success and avoid significant side effects. Therefore, it is compulsory to define a standard operative procedure and to have specialized referral centers, to approach this technology within a multidisciplinary team.

GET

The experimental observation on which this methodology is based is that muscle cells can integrate and express foreign DNA *in vivo*, even for long periods (27). The procedures that can be used to convey ectopic DNA are divided into viral and non-viral (28, 29). Viral methods are based on the use of viral vectors (such as genetically modified adenoviruses, lentiviruses, adeno-associated viruses, and retroviruses) which guarantee high efficiency (30). Nonetheless, it must be considered that viral vectors can cause side effects, even of great importance including cytotoxicity, mutagenesis and limitations in the extents of the transgene to be inserted (31). On the other hand, non-viral methods certainly show fewer side effects, are less expensive and allow the use of large ectopic DNA sequences (32). Unfortunately, the efficiency of these non-viral methods is certainly lower, and this has greatly limited their use in the clinical field (33, 34).

There are several factors that contribute to the poor efficiency of non-viral vectors, but the use of electric fields certainly improves performance (35-38).
Several protocols have been proposed in which wave type, voltage, wavelength and wave train characteristics play an important role in enhancing the expression of plasmid DNA in muscle tissue (39, 40).
Figure 2A shows a schematic representation of a syringe for the injection for GET of plasmid DNA in which the needle electrodes for the vehiculation of the electric filed are inserted in the syringe.

ECT

In this field of application, the differences between veterinary and human oncology are very evident. ECT in veterinary oncology is today a therapeutic scheme widely used as first line or in adjuvant, neoadjuvant and during surgery (intraoperative ECT) (10). In human oncology, ECT has an established use only for the palliation of skin metastases and for some primary skin tumors. The use of ECT for visceral tumors through laparoscopy or endoscopy in human oncology is still in an experimental phase today. In **Figure 2B** some examples of different types of electrodes used for veterinary ECT are depicted.
Below we briefly report the most significant results obtained in the veterinary field and in human oncology with the use of ECT.

FELINE AND CANINE SOFT TISSUE SARCOMA (STS)

STS in pets are aggressive tumors with a bad prognosis (41, 42). In the last years ECT has been successfully defined as an effective therapy of incompletely excised feline and canine STS both in intraoperative and in postoperative modality (43-45). Nevertheless, several protocols of combination therapy have been successfully defined

Figure 2. ***(A)*** *Schematic representation of a syringe for the injection for GET procedure through electric fields;* ***(B)*** *different types of electrodes used for ECT;* ***(1)*** *Reusable and removable electrode plates (steel 3 mm);* ***(2)*** *Reusable and removable two-needle unshielded electrode, steel 40 mm and* ***(3)*** *steel 52 mm;* ***(C)*** *electroporation cuvette inserted in a cuvette holder modified for EP-TVL.*

in pets, thus emphasizing the value of currently adopted protocols (46, 47).

EPITHELIAL TUMORS

Head and neck skin carcinoma is a quite frequent neoplasm in white skinned humans and white-haired pet (48, 49). Moreover, in cats is very common to detect tumors in a very advanced stage. In recent years, some pivotal studies have confirmed the great efficacy of ECT compared to standard therapeutic protocols (surgery and/or radiation therapy), displaying also minor side effects and greater monetary sustainability for the owners (50-55). In **Figure 3** an example of successful treatment with ECT of squamous cell carcinomas of the nasal planus in cats is depicted.

Figure 3. *Successful therapy of an early-stage SCC in a 14-year-old female cat.*

It is mandatory to mention also carcinoma of perianal and anal sac gland in dogs. These tumors, due to the location adjacent to nerves and muscular sphincter, frequently are difficult to treat by surgery and/or radiotherapy. Numerous clinical trials have demonstrated the ability of ECT to control these neoplasms, resulting in extended survival for the dogs, compared to standard therapeutic protocols (56-58).

MELANOMA

Melanoma is frequently described in dogs and in grey horses. Numerous articles have demonstrated the efficacy of ECT both in local control and systemic spread of the disease, probably based also on the stimulation of the immune system (59-61).

ECT CLINICAL TRIALS IN HUMANS

There is a plethora of ongoing studies on electroporation with 306 trials registered on the website clinicaltrial.gov (https://clinicaltrials.gov/search?term=electroporation&city=). Numerous clinical studies have verified the effectiveness and safety of ECT on diverse tumor (melanoma, colorectal, gynecological, pancreatic, liver, head and neck cancer).

Concerning melanoma in advanced stage, the clinical trial NCT00006035 proved the efficacy and safety of ECT combined with bleomycin, when analyzing the healing time and the duration of lesion after these treatments in the designated group of patients (https://clinicaltrials.gov/study/NCT00006035).

In a different study focused on the treatment by ECT and intravenous bleomycin of cutaneous metastases of melanoma in a group of 30 patients and 654 different metastatic nodules, the authors evidenced 72% local tumor control rate after 24 months, claiming an effective role for ECT as a first-line palliative treatment in metastatic melanoma (62).

In a prospective cohort study on 151 patients with metastatic melanoma, Kunte *et al*. were able to demonstrate that the ECT treatment combined with bleomycin was well-tolerated with a thorough response in 58% of nodules (63).

The safety and efficacy of ECT through endoscopic electroporation in colorectal tumor has been assessed in a phase I study with very promising results in term of reduction of intraluminal masses and cessation of bleeding, as verified by instrumental analyses (computed tomography and magnetic resonance imaging) (64). Comparable results have been obtained in a phase I study on esophageal cancer by using A similar endoscopic electron device (65).

Finally, very promising results have been obtained for the use of ECT for gynecological tumor treatment, as well as for pancreatic and liver tumors (clinical trials NCT04760327, NCT04281290, NTC02291133).

Overall, the main clinical studies described are encouraging and prove that ECT can be considered a safe alternative approach to chemotherapy, especially in the case of tumors drug-resistant. The treatment protocol was regulated in the framework of the European Standard Operating Procedure on ECT (ESOPE) released in 2006 (66) and recently updated (67).

CALCIUM-EP

Calcium-EP is an electroporation method in which electrical activity is exploited to allow the entry into the target cells of large quantities of calcium ions, which cause cell death through ATP depletion and mitochondrial dysfunction (68).

Preliminary studies in human oncology on head and neck tumors and in metastatic skin cancers have shown that intracellular calcium has the same efficacy as bleomycin in inducing cell death of neoplastic cells (69, 70).

As far as veterinary oncology is concerned, the most significant studies have been carried out on equine models of sarcoids with promising results, using this method as a first-line therapy (71, 72).

EP-TVL

Extracellular vesicles (EVs) are membrane bound vesicles generated and successively released into the extracellular milieu by vegetal and animal cells. EVs work as shuttles to transport nutrients and transduction signals to nearby or distant cells. Basically, EVs are classified into three categories: exosomes, liposomes and synthetic nano-vesicles. From a therapeutic point of view, EVs have some undeniable properties, such as the ability to precisely load molecules reducing degradation problems and the ability to improve delivery thanks to the ability to move through cellular barriers with extreme ease due to their very nature as an intercellular shuttle. In EP-TVL, electroporation is instrumental to enhance the loading of said vesicles with anti-cancer molecules of different nature (*i.e.*, chemotherapy, photodynamic agents, antibodies, mi-RNA, ricin, *etc*.) (73, 74).

Exosomes are nano-sized vesicle secreted from cells that carry many biomolecules, such as proteins or nucleic acids. Exosomes are wrapped in a lipid bilayer membrane and range from 30-150 nm in diameter. Exosomes are secreted through exocytosis by cells and then are taken up by target cells, functioning as nutrients or as intercellular signaling, and this last activity has a critical role in the regulation of cellular and physiological processes (75, 76). Interestingly, EVs derived from different cell types show different biological properties. For example, tumor cell derived exosomes display tumor growth suppressor properties (77), or hepatic and macrophages derived EVs exhibit anti-viral activity (78), and EV of mesenchymal origin can modulate tissue healing and regeneration (79). Several methods for loading EVs with biologically active molecules have been described and EP has proved to be one of the most promising methods both for loading effectiveness and for maintaining the integrity of the loaded molecules. Nevertheless, there is currently no clear consensus on which experimental conditions are the most effective also because there is a lot of variability based on the origin of EVs themselves (80). **Figure 2C** shows an electroporation cuvette inserted in a cuvette holder modified to be able to convey the electrical impulse through the walls of the cuvette. The exosomes to be electroporated are loaded into these tubes and subjected to the electric field in this modified cuvette holder.

CONCLUSIONS

Electroporation has shown over the last thirty years an abundance of extremely effective applications in medicine, extending from laboratory engineering of EP charged particles to clinical applications in human and veterinary oncology (84). Focusing on cancer therapy, IRE is able to kill cancer cells, while RE can foster effective intracellular drug delivery through the combination of EP and drug treatment (ECT) has proven to be effective, safe and well tolerated by the patient. Several investigations are still needed to better establish the real impact of these methodologies in clinical oncology, but the future of this technology appears optimistic and ripe with promises.

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Conflict of interests

EPS, AB. are founders and stockholders of Biopulse S.r.l.

Availability of data and materials

The data underlying this article are available within it.

Authors' contributions

EPS, AB: conceptualization; writing-original draft preparation: EPS, AB; MC, SC: writing-review and editing; AB: funding acquisition. All Authors have read and agreed to the published version of the manuscript.

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Authors declare no potentially overlapping publications with the content of this manuscript and all original studies are cited as appropriate.

Data falsification and fabrication

All the data corresponds to the real.

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