Electroporation: New strategy to improve the drug uptake and overcome the tumour resistance

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SUMMARY

Electrochemotherapy is an innovative therapeutic strategy to overcome multidrug resistance (MDR) phenomenon of several neoplasms. New anticancer treatment combines the administration of a chemotherapeutic agent with the application of electric pulses (electroporation, EP) having appropriate waveforms to increase drug uptake. Its efficacy as adjuvant therapy has been already demonstrated in the veterinary patients, in combination with several anticancer agents resulting in enhanced cytotoxicity. The main goal of our project is to increase the effectiveness of doxorubicin (DOX) on MDR human colon adenocarcinoma cell line (LoVo DX) and mitomycin C (MMC) on two human breast adenocarcinoma cell lines (MCF-7 WT and MCF-7 DX), by using trains of biphasic pulses. The *in vitro* experiments of the combined treatment (EP plus DOX) showed the enhancement of DOX accumulation and nuclear distribution on LoVo DX cell suspension, evaluated by flow cytometry and confocal microscopy, respectively. Moreover, evident morphological changes were observed by scanning electron microscopy. MTT assay showed that MCF-7 cells treated with EP alone showed the same cell viability as the control; this proves that electrical impulses alter only the membrane permeability favouring drug uptake without inducing a cytotoxic effect on tumour cells. Cell viability assay showed a 20% reduction after the combined EP plus MMC treatment. Further studies will be carried out to confirm the cytotoxic damage and assess the role of electrochemotherapy in the pharmacological resistance phenomenon.

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Statistics on global cancer incidence

Cancer is one of the main public health problems all over the world. In Worldwide cancer data are estimated 18 million cases, of these 9.5 million cases were in men and 8.5 million in women. In the human disease incidence, the breast cancer ranks the second case diagnosed, with a greater incidence in women. It is followed by the colorectal cancer, which ranks third in the global list for both sexes.

Although chemotherapy and radiotherapy are the main strategies for the treatment of cancer, a major problem limiting its success is drug resistance (Büsselberg and Florea, 2017). The drug resistance may be either intrinsic if the tumour cells are naturally resistant to the drug, or acquired if it arises as a consequence of the pharmacological treatment. Multidrug resistance (MDR) phenotype arises when antineoplastic drugs are extruded from the cells, and their intracellular concentration drops below the cytotoxic threshold.

Cancer cells may show resistance to many chemotherapeutic drugs *via* some mechanisms and pathways such as modification of both metabolism and transport of drugs or specific changes (gene mutations/amplifications) related to the drug targets that may lead to cell resistance (Pan *et al*., 2016). Cancer has a dynamic trend: during the course of disease, it becomes more heterogeneous including some genetically distinct tumour cell subpopulations with differential levels of treatment sensitivity (Dagogo-Jack and Shaw, 2018). Therefore, drug resistance results in a treatment failure. The activation of resistance pathways may lead to cross-resistance, thus generating a serious problem in the choice of therapeutic treatments.

The main goal of our project is to increase the effectiveness of the chemotherapy agents used in sensitive and drug resistant human colon and breast carcinoma cell lines by means of electrochemotherapy (ECT), a novel non-toxic and well-tolerated therapeutic modality of local treatment.

Bases of electrochemotherapy

ECT is an innovative medical strategy to overcome the MDR phenomenon of several neoplasms (Pasquali *et al*., 2018). ECT is a loco-regional therapy that associates the administration of antitumor agents with electric pulses having appropriate characteristics (form, voltage, frequency) to increase the drug uptake (Meschini *et al*., 2012). The cellular membrane is the main barrier that prevents the passive diffusion of lipophobic compounds. The application of electric pulses permeabilizes the tumour cell membrane, leading to the formation of transient pores, enhancing the delivery of lipophobic drugs, such as bleomycin, cisplatin, and other compounds. This kind of therapy reduces the patient's morbidity, because ECT allows using lower doses of drug concentration, thus limiting side effects while improving tumour control (Spugnini *et al*., 2012). When electric pulses are applied to cells, two different phenomena can be observed: reversible electroporation (EP) and irreversible EP, both used in clinical practice. Reversible EP occurs if the membrane returns to its normal state after the end of exposure to the electric field. The irreversible EP leads to a disturbance of the cell homeostasis and thus to apoptosis in the treated tissue. Irreversible EP is used in clinical routine as non-thermal form of soft tissue ablation. This method of tissue ablation has found widespread use to destroy malignant tissue. The EP process consists of five steps: induction, expansion, stabilization, releasing and memory effect (Kotnik *et al*., 2019). They happen within microseconds, milliseconds, seconds and hours, respectively. Several studies have been carried out to establish the appropriate electrical parameters to define the most effective ECT protocol (Okino *et al*., 1992). The physical model is too general for ECT of neoplasms, because it does not consider the cellular heterogeneity within the neoplastic tissue and the different orientation of tumour cells in terms of the polarity of the field (Orlowski and Mir, 1993). For these reasons, Sersa *et al*. suggested to divide a train of eight pulses into two perpendicular trains of four pulses each, obtaining better cancer control in mice with solid tumours (Sersa *et al*., 1996). Several factors influence the efficacy of the combined treatment:

1) electrode size, shape and composition;
2) electrical field strength;
3) pulse shape;
4) pulse duration;
5) pulse frequency;
6) number of applied pulses.

It has been demonstrated that the variation of the length and the number of the pulses does not affect the threshold value of effective EP, but modulates the cellular permeability. The role of the impulse form has not been fully explored. Over the years, many experiments have shown that electric pulses induce two phenomena at the cellular level: an initial pore creation phase, followed by a pore enlargement phase during the pulse delivery period (Hibino *et al*., 1991). These initial pores are called "transient electropores" that, after the disappearance of the electric field, reduce in size and stabilize to form the so-called "long-lasting electropores" (Salford *et al*., 1993). Many molecules (greater than several kilodaltons) can only pass through the cell membrane transient electropores. The transport (velocity) of macromolecules is inversely related to their molecular weight and the final concentration of these particles within the cell is far from equilibrium (an intracellular concentration higher than the theoretical value). It can thus be

deduced that mechanisms other than simple diffusion through the electropores are involved in the translocation process (Glogauer and McCulloch, 1992). "Long-lasting electropores" are effective only for the transport of small or medium-sized substances that move by simple diffusion regulated by their intra- and extracellular concentration. Results showed that the EP does not depend on the length of the pulse but rather on the shape and width of the pulse, on the cell size and on the poration coefficient of the cell membrane (Tomov, 1995).

In vitro and *in vivo* application of electrochemotherapy

Treatment of cancer patients is subject to limitations in radiotherapy and chemotherapy. The ECT efficacy, as adjuvant therapy, has been already demonstrated in veterinary and human patients, in combination with several anticancer agents against cutaneous and subcutaneous malignant lesions, resulting in an enhanced cytotoxicity (Spugnini *et al*., 2017). ECT has been used for treatment of cutaneous and subcutaneous metastases located in head or neck, melanoma, non-melanoma skin cancer, or breast cancer metastases to the skin, and for treatment of non-cutaneous metastases located in bone, liver, or soft tissue sarcoma. Moreover, there are clinical trials for the treatment of primary tumours, such as ovary or colon cancer (Probst *et al*., 2018).

Spugnini *et al.* (2011) investigated another approach to increase the effect of the therapy against a chemo-refractory cancer disease (melanoma), using the EP to promote the uptake and efficacy of antisense molecules. The results confirmed an increased internalization of the antisense probe (Spugnini *et al*., 2011).

The application of ECT offers several advantages:

1) decreased pain experienced by laboratory animals;
2) reduction of the risks of anaesthetics, due to a diminished need to restrict and control pain;
3) treatment available to humans, given the decrease in morbidity that is still the main limiting factor to the spread of this therapy.

In our study, we focused the attention on the delivery of two chemotherapeutics, *i.e.* doxorubicin (DOX) and mitomycin C (MMC), combined with EP on different tumour cells. These are antineoplastic antibiotics, derived from natural products produced by species of the soil fungus *Streptomyces*. The ability of chemotherapy to kill cancer cells depends on its ability to halt cell division. Tumour cells, differently from normal tissues, are characterized by a continuous cell division, because they lose the normal checks of the cell cycle. There are different types of chemotherapy drugs: cell-cycle specific, that affect cells only when they are dividing, and cell-cycle non-specific drugs, that affect cells when they are at rest. DOX and MMC are cell-cycle specific drugs, and act during multiple phases of the cycle.

In particular, DOX inhibits the topoisomerase II enzyme, causing DNA damage and induction of apoptosis. It is also able to intercalate itself within DNA base pairs, causing breakage of DNA strands and inhibition of both DNA and RNA synthesis. It can also limit the DNA synthesis in presence of iron, inducing free radical-mediated oxidative damage. DOX is commonly used in the treatment of solid tumours in adult and paediatric patients, such as soft tissue and bone sarcomas, cancers of the breast, ovary, bladder, and thyroid, and also used in the treatment of acute lymphoblastic leukaemia, acute myeloblastic leukaemia, Hodgkin lymphoma, and small-cell lung cancer. Adverse reactions are common; in particular, a significant cardiac toxicity develops within days of drugs administration.

MMC is an alkylating agent, and its cytotoxicity is due to its ability to generate DNA monoadducts as well as intrastrand and interstrand cross-links. This drug is used for treatment of adenocarcinoma of the stomach or pancreas, and also of anal, bladder, breast, cervical, colorectal, head and neck, and non-small cell lung cancer. Like any other chemotherapy drug, MMC has several side effects that can be more or less serious. The major drawback of MMC is its dose-limiting toxicity, and its non-specific antineoplastic effect (Fang *et al*., 2018).

Other studies have been carried out to test the efficacy of the combinatorial treatment of DOX e MMC with EP on human neoplasms. Shil *et al*. (2006) demonstrated that EP enhances radiation and DOX-induced toxicity in solid tumour. These results suggested that the antitumor effects of a moderate dose of γ radiation and low concentration of DOX can be significantly enhanced by combination with EP (Shil *et al*., 2006). Also, further studies both *in vitro* and *in vivo* carried out by Vásquez *et al.* (2015) on bladder cancer showed increased cytotoxicity and a greater survival rate in mice after the combined treatment.

In our studies, we used two cell lines of different histotypes: human colon adenocarcinoma cell line (LoVo) and human breast adenocarcinoma cell line (MCF-7), either pharmacosensitive (WT) or pharmacoresistant (DX). MDR human cell lines were selected from their drug sensitive parental cell line (WT) by continuous exposure to increased DOX concentrations. Both MDR cell lines express high level of a specialized protein, P-glycoprotein (P-gp), associated with the resistant phenotype. P-gp is an ATP-dependent pump capable of extruding cytotoxic agents, such as DOX out of the tumour cell, resulting in their reduced intracellular accumulation. In our experiments, the cells were treated either individually with a DOX or MMC, or in combination

with EP to evaluate if the combined treatment may promote the enhancing cytotoxic effect.

Our experiments have been made using an instrument (Onkodisruptor®, Biopulse Srl, Naples, Italy) with an EP protocol based on stabilized biphasic waveform delivered in a condensed train (Spugnini *et al*., 2014). The EP treatment consists of a series of eight biphasic pulses, with the voltage of 300 V/cm and duration of 50 + 50 µs each. The frequency is 1 Hz and pause between two following pulses is 10 µs, resulting in the delivery of the whole train of waveforms within 870 µs. This EP is different from the most commonly used instruments, which adopt square waveforms delivered in sequence of individual pulses, thus lengthening the duration of treatment.

Initial studies focused on the effects of DOX on MDR human colon adenocarcinoma cell line LoVo DX by using trains of biphasic pulses, confirming the chemosensitizing effect of the EP. Both qualitative and quantitative analyses of the adjuvant effect were carried out to analyse the cellular morphology, proliferation and survival observing the cellular internalization and distribution of the chemotherapeutics.

Results

The morphological analysis of adenocarcinoma untreated cells was performed by scanning electron microscopy (SEM). LoVo DX cells have an epithelial morphology, are polygonal in shape with microvilli of regular size and grow well attached to the substrate (Figure 1A).

The *in vitro* experiments of the combinatorial treatment (EP + DOX) gave the following results:

1) enhancement of DOX accumulation in cell suspension after the EP evaluated by flow cytometry (*data not shown*);
2) increase of the drug intranuclear localisation through observations made under confocal microscopy (Figure 1C) compared to cells treated only with the drug (Figure 1B).

To validate the synergistic effect of the combined therapy, we tested two human breast adenocarcinoma cell lines (MCF-7 WT and MCF-7 DX), using MMC. We performed MTT test measuring mitochondrial enzymatic activity, to analyse the cell viability of both cell lines after single EP, treatment with MMC alone and combined treatment (EP + MMC). Our results showed a 20% reduction in cell viability after the combined EP + MMC treatment (*data not shown*). Samples treated with EP alone had the same cell viability as the control. This proves that electrical impulses only alter membrane permeability without inducing a cytotoxic effect on tumour cells.

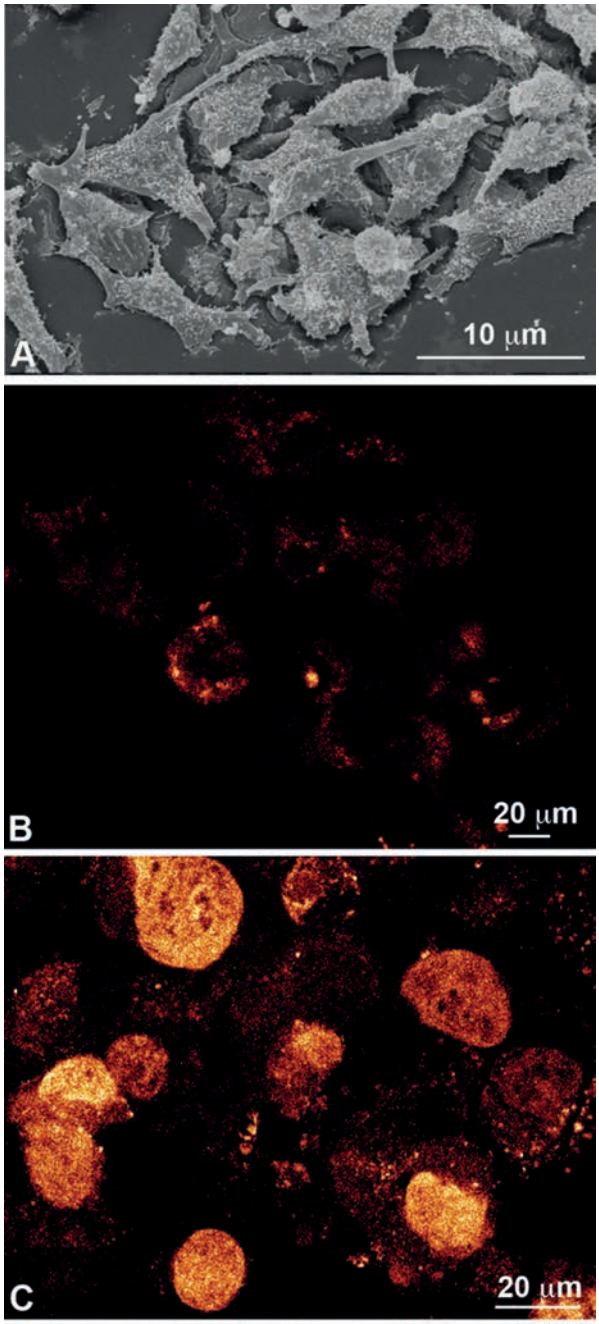

Figure 1. Scanning electron microscopy (SEM) (A) and laser scanning confocal microscopy (LSCM) (B,C) observation of LoVo DX cells. The morphological analysis shows that control cells are polygonal shape and the surface is covered by randomly distributed microvilli (A). Laser scanning confocal microscopy (LSCM) images show the intracellular localization of doxorubicin (DOX) in living LoVo DX cells after 3 h of incubation. The nuclei of the cells treated with DOX (1 µg/ml) alone were negative while the cytoplasm showed a weak fluorescent signal (B). The cells exposed to the combined treatment (electroporation with DOX) displayed an increase of the intracellular drug uptake, preferentially in the nuclei (C).

Conclusions

This preliminary study demonstrates the importance of the combined use of biophysical and chemical techniques with the aim of increasing the effectiveness of chemotherapy with the need of reduced drug concentrations to minimize the side effects of the treatment. ECT induces the formation of transient modifications on the tumour cell membrane and promotes the internalization of the drug that can reach the therapeutic target with higher efficiency.

Future perspectives concern optimization of the EP instrument in order to increase its effectiveness to promote the drug uptake into cancer cells. Our goal will be the validation of the increased effect of chemotherapy due to combinatorial therapy with EP *in vivo*. ECT can be successfully applied on several tumours with a favourable cost-benefit and can be a repeatable treatment integrated with traditional antineoplastic therapies. In conclusion, the results herein reported suggest that ECT is an innovative and promising therapeutic strategy.

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