JEAN CARLOS DOS SANTOS DA LUZ

**Eletrogeneterapia com IFN- β em
modelo murino de melanoma**

Dissertação apresentada à Faculdade de
Medicina da Universidade de São Paulo para
obtenção do título de Mestre em Ciências

Programa de Ciências Médicas
Área de concentração: Distúrbios do
Crescimento Celular, Hemodinâmicos
e da Hemostasia

Orientador: Dr. Bryan Eric Strauss

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São Paulo

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RESUMO

Luz JCS. *Eletrogeneterapia com IFN- β em modelo murino de melanoma* [dissertação]. São Paulo: Faculdade de Medicina, Universidade de São Paulo; 2021.

A eficácia de determinadas moléculas terapêuticas com ações intracelulares pode ser comprometida pela incapacidade destas em ultrapassar a membrana celular ou pela curta permanência das mesmas em seu sítio de ação, criando a necessidade do uso de altas doses ou reaplicações, que podem gerar toxicidade. Na terapia oncológica, citocinas administradas de forma sistêmica requerem altas doses para uma eficaz atividade antitumoral, o que frequentemente acarreta severos efeitos colaterais. A eletrotransfecção gênica (GET) é uma técnica que combina o uso de plasmídeos com a eletroporação (ou eletropermeabilização), um método que facilita o transporte de moléculas externas para o interior das células por meio de pulsos elétricos permeabilizantes da membrana celular. A técnica possibilita a expressão de citocinas de forma local e prolongada, evitando efeitos colaterais sistêmicos. Na oncologia, o uso de plasmídeos codificantes de citocinas visa modular o microambiente tumoral, objetivando gerar uma resposta imunológica antitumoral local, sistêmica e de memória. Atualmente, plasmídeos codificantes de interleucina-12 (IL-12) são os mais amplamente utilizados em GET tanto como monoterapia ou em combinação com outras terapias e têm apresentado resultados promissores, entretanto, a IL-12 tem suas ações limitadas à um repertório restrito de células com receptores para tal citocina. Neste trabalho, foi proposto o uso de interferon- β (IFN- β) como molécula imunomoduladora, visto sua ampla variedade de atividades antitumorais e presença de receptores IFNAR em todas as células nucleadas. O presente projeto teve como objetivos estabelecer o protocolo elétrico para eletrotransfecção de plasmídeos em linhagem celular de melanoma murino (B16F10) *in vitro* e avaliar as atividades antitumorais do tratamento proposto, bem como uma avaliação preliminar para o uso do IFN- β murino GET *in vivo*. Como resultados, o protocolo de eletrotransfecção estabelecido para a linhagem B16F10 resultou em uma taxa de eletrotransfecção de plasmídeo codificante de eGFP de até 45,6% com viabilidade próxima a 100%. Além disso, a eletrotransfecção de plasmídeo codificante de IFN- β resultou no acúmulo da proteína no sobrenadante em até 526,1pg/mL, e retardo no crescimento celular de até 66% em comparação com as células controles. Em ensaio preliminar *in vivo*, foi detectado o sinal de eGFP após GET em tumores subcutâneos. Este estudo abre o caminho para investigações mais aprofundadas do tratamento utilizando GET e a possível resposta imunológica antitumoral provocada pela IFN- β .

Descritores: Bleomicina; Neoplasias; Citocinas; Eletrogeneterapia; Antineoplásicos; Terapia gênica; Modelos animais.

ABSTRACT

Luz JCS. *Electrogenotherapy with IFN- β in a murine model of melanoma* [dissertation]. São Paulo: "Faculdade de Medicina, Universidade de São Paulo"; 2021.

The efficacy of certain therapeutic molecules with intracellular actions may be compromised by their inability to cross the cell membrane or by their short retention in their site of action, creating the need for the use of high doses or reapplications, which can generate toxicity. In cancer therapy, cytokines administered systemically require high doses for an effective antitumor activity, which often causes severe side effects. Gene electrotransfer (GET) is a technique that combines the use of plasmids with electroporation (or electropermeabilization), a method that facilitates the transport of external molecules into cells by using electrical pulses to permeabilize the cell membrane. This technique allows the expression of cytokines in a local and prolonged way, avoiding systemic side effects. In oncology, the use of plasmids encoding cytokines aims to modulate the tumor microenvironment, seeking to generate a local, systemic and memory antitumor immune response. Currently, plasmids encoding interleukin-12 (IL-12) are the most widely used in GET either as monotherapy or in combination with other therapies and have shown promising results, however, IL-12 targets a restricted repertoire of cells with receptors for that cytokine. In this work, the use of interferon- β (IFN- β) as an immunomodulatory molecule was proposed, given its wide variety of antitumor activities and the presence of IFNAR receptors in all nucleated cells. This project aimed to establish the electrical protocol for electrotransfection of plasmids in a murine melanoma cell line (B16F10) *in vitro* and to evaluate the antitumor activities of the proposed treatment, as well as a preliminary evaluation for the use of murine IFN- β GET *in vivo*. As a result, the electrotransfection protocol established for the B16F10 cell line using an eGFP-encoding plasmid yielded an electrotransfection rate of up to 45.6% with viability close to 100%. Furthermore, electrotransfection of an IFN- β encoding plasmid resulted in accumulation of the protein in the supernatant of up to 526.1pg/mL, and reduction in cell growth of up to 66% compared to control cells. In a preliminary *in vivo* assay, the eGFP signal was detected after GET in subcutaneous tumors. This study paves the way for further investigations of treatment using GET and the possible anti-tumor immune response elicited by IFN- β .

Descriptors: Bleomycin; Neoplasms; Cytokines; Gene electrotransfer; Antineoplastic Agents; Genetic therapy; Models, animal.

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***“Do interesting things and
interesting things will happen to you.”***

– Sir John Hegarty

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1. INTRODUÇÃO

Câncer é o nome dado a um conjunto de cerca de 200 doenças. Em comum, cânceres se caracterizam pelo crescimento descontrolado de células que apresentam a capacidade de invadir tecidos e formar metástases (CANCER, 2021; WHO, 2021). Apesar do avanço no conhecimento da doença em si, dos avanços tecnológicos para o diagnóstico precoce, e do desenvolvimento de novas estratégias terapêuticas, o câncer continua sendo um desafio à saúde pública mundial. Foram estimados 17 milhões de novos casos somente no ano de 2018 (CANCER RESEARCH UK, 2019), e estima-se para o ano de 2040 que os novos casos anuais aumentem para 27,5 milhões, sendo 16,3 milhões (60%) o número de mortes projetadas (IARC/OMS, 2021). No Brasil, estimou-se aproximadamente 626.030 mil novos casos para o ano de 2020, sendo quase 232.030 mil as mortes causadas pela doença no ano anterior (INCA, 2021). Considerada desde 2013 a grande descoberta da oncologia, a imunoterapia moderna tem revolucionado a medicina com uma mudança no paradigma. É proposta uma forma completamente diferente de tratar câncer: o alvo é o sistema imune e não o tumor em si (OISETH; AZIZ, 2017; COUZIN-FRANKEL, 2013). Os resultados satisfatórios obtidos em pacientes tratados com estratégias já implementadas na rotina clínica (anti-CTLA-4, PD-1 e PD-L1), colocam em evidência o papel importante que o sistema imune tem na resposta antitumoral (PHAN et al, 2003; ROSENBERG; RESTIFO, 2015).

O melanoma, apesar de constituir apenas 1% dos cânceres de pele, é responsável por 60% das mortes pelas malignidades dermatológicas. Devido a sua alta carga mutacional e à expressão de neoantígenos, esta malignidade é um ótimo candidato à imunoterapias como anticorpos anti-CTLA-4 e anti-PD-1 (KHAZAEI et al., 2018; SCHUMACHER; SCHREIBER, 2015). Entretanto, tais estratégias imunoterápicas enfrentam vários desafios, como custo elevado, limites da sua efetividade terapêutica, resistência e toxicidade (GREEN, 2021; SAMBI; BAGHERI L; SZEWCZUK, 2019). Diante desse cenário, existe a necessidade de desenvolvimento de novas estratégias terapêuticas que combinem eficácia antitumoral com a geração imunidade antitumoral protetora e baixa toxicidade.

Para a introdução ao tema, apresentamos uma revisão bibliográfica que aborda princípios teóricos, aspectos imunológicos e respostas antitumorais de terapias baseadas em eletroporação (TBE) e suas aplicações para o tratamento de câncer em humanos e animais, bem como os aspectos antitumorais do interferon- β (IFN- β), citocina utilizada no desenvolvimento deste trabalho.

2. REVISÃO DE LITERATURA

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Clinical Applications and Immunological Aspects of Electroporation-Based Therapies

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Abstract: Reversible electroporation (RE) is an ultrastructural phenomenon that transiently increases the permeability of the cell membrane upon application of electrical pulses. The technique was described in 1972 by Neumann and Rosenheck and is currently used in a variety of applications, from medicine to food processing. In oncology, RE is applied for the intracellular transport of chemotherapeutic drugs as well as the delivery of genetic material in gene therapies and vaccinations. This review summarizes the physical changes of the membrane, the particularities of bleomycin, and the immunological aspects involved in electrochemotherapy and gene electrotransfer, two important EP-based cancer therapies in human and veterinary oncology.

Keywords: bleomycin; electrochemotherapy; electroporation; gene electrotransfer; vaccines.

1. Introduction

The permeabilization of the cell membrane by the application of external electric pulses was first described in 1972 by Neumann and Rosenheck [1,2]. This physical process was coined as “electroporation” in 1982 by the same group [3]. Such permeabilization is characterized by the uptake of external nonpermeant molecules in the cell cytoplasm and leakage of internal substances from the electroporabilized cells [4–6]. Since then, this technique has been used and developed in several areas of biotechnology and medical research, such as bacterial transformation [7,8], cell electrofusion [9,10] transdermal drug delivery [11,12], CRISPR editing [13], CAR-T cell generation [14], DNA vaccines, including for the novel Sars-CoV-2 coronavirus [15–17], gene therapy [18–21], cancer treatment [22–24] and even in food processing [25,26] and environmental sanitation [27,28].

Although electroporation (EP) and electroporabilization are frequently used as synonyms, electroporation is related strictly to the aqueous pores formed in the lipid membranes of the cells, while electroporabilization is related to all the events involved with the membrane permeabilization process, including modulation of membrane channels, and cellular biophysical and biochemical changes [5,29–31]. There are two main classifications of electroporabilization, irreversible electroporabilization (IRE), and reversible electroporabilization (RE), which are related to different effects on cell membranes, depending on the electrical parameters employed [4,6]. IRE is a tissue ablative therapy, causing cell death mainly by apoptosis and necrosis without drug intervention. The irreparable disruption of plasma membrane and, subsequently, the influx and efflux of soluble molecules leads to loss of cell homeostasis [23,32,33]. This technique was introduced as cancer therapy by Dr. Rubinsky’s group in 2005 [34]. Moreover, another IRE technique, named nanosecond pulsed electric field (nsPEF), has also been used in oncology. This approach uses electric pulses to create irreversible pores in plasmatic and also intracellular membranes, leading to cell death [35,36]. In turn, RE is used in oncology for electrochemotherapy (ECT), gene electrotransfer (GET or, the less used term, electrogenetherapy—EGT) and electrochemogenetherapy (ECGT), aiming to permeabilize the cells to

allow the entrance of external therapeutic molecules, but without irreversible damage to the cell [4,35,37].

This review focuses on the physical changes of the membrane, the particularities of bleomycin, and the immunological aspects involved in EP-based therapies and their application for the treatment of cancer both in humans and animals.

2. Plasma Membrane Electroporation

The phospholipidic components of the plasma membrane possess a polar hydrophilic “head” and a nonpolar hydrophobic “tail”. In this amphiphilic structure, the phospholipids are spontaneously organized in the aqueous physiologic environment, arranging their hydrophilic heads together in the outer region of the membrane and aggregating hydrophobic tails in the inner part, forming a stable, bilayered closed vesicle [38,39]. The plasma membrane acts as a regulatory barrier, separating the cell interior from the exterior. This structure is permeable to water, oxygen (O₂) and carbon dioxide (CO₂), relatively permeable to hydrophobic molecules and poorly permeable to hydrophilic molecules, allowing only membrane-controlled ions and molecules to cross in and out of the cell through diffusion channels, and membrane transport proteins [33,39–41]. Thus, cells can naturally maintain an electric potential difference between the internal and external side of the plasma membrane, the so-called resting transmembrane potential [29,41].

Cell membrane EP requires well-defined electric pulse protocols, regarding the number, duration, voltage amplitude, frequency and electric current applied to the tissue depending on its intended use. These electric pulses are generated by specialized equipment and delivered to the cells or tissue through electrodes (commonly needle or plate electrodes) with positive and negative poles, with the electric field occurring between those poles, positioned around the site of interest [30,42–44]. While *in vivo* RE uses electric parameters varying around eight square pulses of milli- to microseconds of duration and 100–1000 V of amplitude and only causes transient permeability with subsequent membrane resealing, *in vivo* IRE uses more pulses (usually around 80–100 pulses), decreased pulse durations and higher voltages (around 3000 V), leading to cell

death due to incapacity of cells to recover [4,6,35,36]. Hypothetically, it is possible to achieve EP in all cell types, *in vitro* and *in vivo*, as long as its particular characteristics are respected, such as physical (e.g., cell and electrode sizes and shapes), biological (e.g., cytoskeleton structure), tissue localization and adequate electric parameters [45–47].

When an external electric pulse exceeds a threshold value, it creates an electric field that can modify the resting transmembrane voltage (RTV), leading to an induced transmembrane voltage (TMV), causing structural and molecular changes to the plasma membrane components [5,29,48]. Below the threshold of TMV, no detectable EP occurs. With an adequate electric field, the TMV threshold is achieved, enabling an RE during the pulse application. In the presence of stronger electric fields, a nonthermal IRE takes place, which leads to cell death, and may be used as a cancer treatment. Otherwise, with even stronger electric fields, thermal IRE takes place, leading to thermal damages to the tissue, and consequently cell death. This approach is not used in cancer treatment [49,50].

Kotnik and collaborators [29] reviewed the RE process and divided it in five phases — Initiation: changes in plasma membrane electrical (conductivity) characteristics in the first nanoseconds and increase in its permeability in the following microseconds, when TMV exceeds a threshold value; Expansion: with the persistence of TMV above the threshold value, augmented conductivity and permeability of plasma membrane remains and/or intensifies until the pulse ceases; Partial recovery: with the pulse cessation, the TMV returns below the threshold value and membrane conductivity and permeability decrease partially (microsecond and millisecond, respectively), remaining stable with ions and small molecule diffusion; Resealing: the gradual recovery of the membrane homeostatic impermeability in RE of cells (and nonrecovery in case of IRE), taking seconds to minutes at temperatures around ~20–37 °C, and up to hours at temperatures around ~4 °C; and, finally, Memory: normal membrane permeability with cellular physiological alterations and reactions to stressors during the first hours post pulse cessation; after this period cell recovers its homeostatic state [29,48].

From a physical perspective, the pore formation can be divided into: (A) normal non-electroporated membrane; (B) early formation of unstable water

channels; (C) metastable pore formation (reversible electroporation); and (D) pore destabilization (irreversible electroporation) [29,43,51] as demonstrated in Figure 1.

Figure 1. Formation of membrane pores. (a) Normal non-electroporated phospholipid bilayer membrane (yellow) and water (blue dots). (b) Early formation of a small unstable water channels. (c) Reorganization of the hydrophilic heads (arrow) and consequent formation of a metastable pore (reversible electroporation); with pulse cessation the membrane tends to return to the normal state (a); and (d) electric pulses beyond the RE threshold parameters leads to enlargement and destabilization of the pore with consequent loss of cell homeostasis (irreversible electroporation — IRE). Dashed lines representing the pore size.

Beyond pore formation, modulation (opening) of voltage-gated channels and clustering of transmembrane proteins (creating temporary pseudotunnels) also contribute to the crossflow of physiological and inoculated substances [12,29,31]

3. Clinical Aspects of EP-Related Therapies

3.1. Bleomycin

Bleomycin and cisplatin are the most widely used chemotherapeutic drugs in electrochemotherapy. Both present poor cell membrane penetration, and their cytotoxic potential is increased up to 8000 times and up to 80 times, respectively, when combined with EP [24,52]. Furthermore, bleomycin, the drug highlighted in this review, has other important features: it has minor side effects at the recommended doses for EP-therapies, presents selectivity for tumor cells,

reduces tumor oxygen and nutrients supply by its antivasular effects and can be used intratumorally and systemically (intravenously) in humans, dogs, and cats [53–58].

The cytotoxic potential of bleomycin relies on its ability to cause single- and double-stranded DNA breaks, acting as an endonuclease [58,59], and alternatively also cleaves RNA molecules such as mRNA, tRNA and rRNAs [60]. Inside the cell, bleomycin can cause two different types of cell death, depending on the number of molecules internalized by the tumor cell [59,61]. The level of internalization is related to the route used to administer the drug, that is, intratumoral (IT) or intravenous (IV) systemic administration. When administered intravenously, the interstitial concentration of bleomycin at the time of application of the electrical pulses is low, thus allowing the establishment of mitotic cell death; in cases of IT application, the internalized concentration is higher, leading to the so-called pseudoapoptotic cell death [58,59,61,62].

The antitumor selectivity of bleomycin resides in the mitotic cell death (i.e., intravenous route), characterized by the appearance of TUNEL and caspase-3 positive cells and atypical mitotic cells. When low numbers of molecules are internalized, nonlethal damage occurs in the DNA. Such damage is shown to be cytotoxic only at the moment of cell division, since the cells' ability to repair the DNA damage is impaired; the cells in constant replication (such as tumor cells) are led to cell death by apoptosis, while non-dividing healthy cells around the treated tumor are spared, as the chances of such DNA damage affecting the expression of vital genes is almost null. This characteristic is particularly important in cases of narrow safety margin resection, allowing a "cleaning" of possible residual tumor cells in healthy tissues, when this approach is used as adjuvant to surgeries [31,58,59,61,63]. In the case of intratumoral administration, pseudoapoptosis predominantly takes place, characterized by a rapid appearance of apoptotic cells due to the high number of bleomycin-induced breaks in the double-strand DNA. In this case, the characteristic of tumor cell selectivity is diminished due to the numerous breaks of DNA strands, acquiring a cytotoxic profile even in the absence of cell division, causing cell death in the treated area [58,61,62].

Recommended doses of bleomycin for human treatment in intravenous

bolus injection is of 15,000 IU/m² (patient's body area) and intratumoral dose according to tumor nodule size: bleomycin dose of 250 IU/cm³ for nodules >1 cm³, bleomycin dose of 500 IU/cm³ for nodules >0.5 cm³ and smaller than 1 cm³ and 1000 IU/cm³ for nodules smaller than 0.5 cm³ [24]. For dogs and cats, the recommended dose in intravenous bolus is 300 IU/kg of patient weight and intratumoral dose according to nodule size: 1500–3000 IU/cm³ for tumor nodules >1 cm³ and 1500 IU/cm³ for tumor nodules smaller than 1 cm³ [54]. A benefit of the systemic injection of bleomycin lies in the possibility of treating multiple nodules or large nodules after a single drug injection, avoiding repetitive bleomycin application and its toxic cumulative dose of 400,000 IU/m² in human, dogs, and cats [54,64].

3.2. Electrochemotherapy

The term electrochemotherapy (ECT) was coined by Mir et al. in 1991 in one of the first studies to introduce the use of ECT, where they described the potentiation of bleomycin's cytotoxic effects in murine models [65]. The first human clinical trial using ECT was also performed in 1991 [66] and the first veterinary clinical trial in 1997 [67]. Since then, ECT evolved from a rescue to a primary option for the treatment of cancer, both in humans and animals. This approach gained prominence in 2006, mainly due to the European Standard Operating Procedures of Electrochemotherapy (ESOPE), a highly successful multi-institutional effort that aimed to establish standard operating procedures for ECT in humans. Currently, ECT is increasingly being introduced into clinical guidelines and evolving with multi-institutional collaborative research groups (e.g., InspECT, EURECA, Gisel, and ReinBone) and new technologies and procedures [24,42,68,69], with important contributions from veterinary patients in these developments [70,71].

Nowadays, ECT has been included in several guidelines from many countries for the treatment of different cancer types, including melanoma, breast cancers, head and neck cancers, carcinomas, soft tissue sarcomas and bone metastases [42,72]. Among the different indications of ECT for cutaneous and subcutaneous primary cancer or skin metastasis, the following are included: palliative treatment of skin metastases in advanced diseases; neoadjuvant

cytoreductive therapy prior to conventional treatment; organ and function sparing treatment; patients not suitable for or relapsed after treatment by surgery, radiotherapy or systemic therapy, and treatment of highly vascularized nodules due to its antivasculature effect [42,73–76]. Additionally, in veterinary medicine ECT is used as an adjuvant therapy for the treatment of incompletely resected tumors, aiming to remove possible residual infiltrating cancer cells at the surgical site, when a wide safety margin resection is not possible [31,77,78]. The use of ECT in noncutaneous deep-seated and internal organ tumors is under investigation in a number of human clinical trials, with preliminary results indicating its feasibility and safety [42,72,79–83]. Currently, novel approaches using ECT are under investigation in pancreas, bone, liver, bladder, prostate and gastrointestinal tumors; other studies indicate the potential of ECT in lung and brain tumors, suggesting that new future approaches might be translated between human and veterinary care [68,70,79,84,85].

Technically, ECT can treat any type of solid tumor as long as it is accessible to electrodes, whether cutaneous or deep-seated, and accessible through endoscopy, laparoscopy or open surgery [64], but it may present different levels of effectiveness in different tumor types, possibly due to insufficient drug and/or electric field coverage and also due to patient biological features, such as previous treatments, tumor size, intrinsic tumor cell sensitivity to the drugs, drug distribution through tumor vascularization, tumor immunogenicity and patient immune status [72,86–90]. This approach has numerous advantages when compared to other locoregional antitumor therapies, such as ease of administration, low cost, minimal side-effects (generally limited to mild local inflammation and transitory pain in the treated site), possibility of repetition if necessary, and high response rate, consistently obtaining approximately 80% objective response (OR) rates in all tumor histologies and around 60–70% CR (complete response) rates after a single treatment in humans [42,53,70,76,87]. As an example, in a study of 52 patients with cutaneous or subcutaneous metastasis of different tumor types, Campana and colleagues (2009) obtained approximately 96% (50 of 52) OR (RECIST) one month after the treatment with ECT plus bleomycin. Specifically, 50% (26 of 52) of those patients had a CR and 46% (24 of 52 — RECIST) had partial response (PR); there was no change in

tumor size in the other two (4% — RECIST) patients after treatment. Between the partial responders and nonresponders, an improved response was obtained after, at least, one additional ECT session. Among the 26 complete responders, 17 were disease-free at the treated site after a mean follow-up time of 9 months. However, 7 of the 26 complete responders developed new lesions in untreated areas, one presented local recurrence after seven months and another two patients died from systemic disease progression [91], indicating a localized ECT efficacy in those patients, though further studies with larger cohorts are warranted. Similarly, Campana and colleagues (2016), in a prospective analysis of 376 patients with different tumors, obtained CR above 80% (RECIST) regardless of tumor histotypes, treated by single or multiple sessions of ECT plus bleomycin, however, no observation of regression in untreated tumors, reinforcing the strictly local effectiveness characteristic of the technique [92].

Similar to surgical approaches as a local treatment, an adequate application of ECT must uniformly cover the whole tumor tissue and its surrounding safety margins with an appropriate electric field and sufficient drug at the moment of the delivery of electric pulses [42,89]. To accomplish this, the pulses must be applied between 1 to 10 min after intratumoral bleomycin application and between 8 to 28 min if intravenously applied, consistent with the period of bleomycin's pharmacokinetic peak [24,93,94]. After drug administration, the electrodes must be systematically moved and the electrical pulses applied in the new site (Figure 2), treating the entire tumor area [42].

Figure 2. Application of electrochemotherapy in a tumor. After drug administration, the electric pulses are delivered by electrodes in contact with the tumor. The electrodes must be systematically moved to the adjacent area, and pulses applied again until the entire tumor area is covered. In the image, a model of needle electrode is exemplified.

Indeed, the efficiency of EP in RE therapies depends on several factors, such as the electrical parameters, tumor location and composition of the extracellular matrix and the type and dose of plasmid administered [95]. Moreover, different types of electrodes can be used to deliver genes: needle electrodes [96], microneedles [97], plate electrodes [98] and noninvasive multi-electrode array (MEA) [99]. In general, plate electrodes are used for superficial tumors and needle electrodes are used for deeper tumors [42]. Internalization of plasmid vectors represents a crucial point for GET therapies and further studies aiming to reach a major plasmid intake and expression are mandatory [100,101]. One strategy is the employment of hyaluronidase enzyme. This enzyme disrupts the extracellular matrix, allowing the DNA to reach the cell membrane more easily, where it will be internalized by the cells when pulses are administered [102,103].

The voltage is also important for drug delivery. Typically, small molecules (e.g., bleomycin and cisplatin) require short high-voltage pulses, while large molecules need pulses of longer duration that are either low-voltage or a combination of high- and low voltage. The electric field is distributed according to the electrode geometry and tissue conductivity [104], and the cells that first come into contact with the proper electric field will be the first to be electropermeabilized. [46,105]. Thus, electrical dosage in tumors depends on the tumor's conductivity, volume, position and also electrode configuration [106]. Recent papers have reported the increasing importance of the target tissue in the efficacy of gene electrotransfer, which electrical parameters should be determined for each tissue type, such as liver, skin, muscle or tumor, due intrinsic differences between them [107].

4. Immunological Aspects of Reversible Electroporation and Electrochemotherapy

While ECT is recognized for its cytotoxic clinical benefit, the immunological potential of RE alone and ECT deserve particular toxic clinical benefit, the immunological potential of RE alone and ECT deserve particular attention. As described here, these effects play an important role in promoting antitumor immune responses.

According to the “danger theory”, introduced by Matzinger in 1994, apoptotic and necrotic cells could act as immunostimulants by releasing damage-associated molecular patterns (DAMPs) [108–110] that trigger proinflammatory responses. This type of cell death is known as immunogenic cell death (ICD) [111]. The damage caused to the tissue by EP-related therapies can elicit ICD and promote a proinflammatory milieu and the recruitment of antigen presenting cells (APC) where tumor-specific antigens (TSA) and tumor associated antigens (TAA) might be captured and presented by APCs to T cells, leading to adaptive immune responses [112–117]. In fact, RE alone or in combination with bleomycin can lead to the leakage of cytoplasmatic molecules related to ICD and the activation of proinflammatory markers [114,118–120]. The main immunostimulatory molecules related to RE include: adenosine triphosphate (ATP) release, calreticulin (CRT) translocation to cell membrane, release of the non-histone, protein high mobility group box 1 (HMGB1), induction of heat shock protein family (HSP70) stress response and other proinflammatory genes [114,121,122]. These markers contribute to the induction of the host’s adaptive immune response.

ATP, a major immunostimulatory DAMP related to ICD, promotes the recruitment, maturation and cross-presentation by APCs [114,121] supporting immune responses in EP-related therapies. Calvet and colleagues (2016) demonstrated that RE alone was capable of releasing ATP from CT26 murine colon cancer cells immediately after the electric pulses and lasting up to hours. However, no ATP was detected 30 h after the electric pulses alone. In contrast, a significant amount of ATP was detected 30 h after EP plus bleomycin, even in cells that were washed to removed ATP released by the electric pulses, indicating a continued leakage of ATP even after the cell membranes completely reseal, and pointing to the mandatory presence of bleomycin for the continued release of ATP at the treated site [114].

CRT promotes the secretion of type I interferons and the uptake of dying cells by APCs [121]. Two possible mechanisms for CRT release are: (i) direct ER stress caused by the electric pulses and/or (ii) the generation of reactive oxygen species (ROS), which causes ER stress and indirectly leads to CRT exposure on the cell surface [114,123,124].

Schultheis and collaborators (2018) evaluated the effects of electric pulses alone on tissues using RE in the Hartley guinea pig model. They showed that CRT translocation to cell membrane was upregulated after RE alone, corroborating a previous study using ECT plus bleomycin *in vitro* [114,119], and CRT was detected along the entire area of the electric field, returning to normal levels after 24 h post-RE. Furthermore, infiltration of APCs such as dendritic cells (DCs) and macrophages at the treatment site was also reported, with its peak level coinciding with the peak of CRT translocation to the cell membrane [119]. In fact, melanoma patients exhibited CRT+ cells after treatment with ECT plus bleomycin as detected by fluorescent immunochemistry up to 14 days later, and this longer duration of CRT exposure may be related to the internalization of bleomycin by the cells [114,125].

HMGB1 is a protein that stimulates the synthesis of several proinflammatory cytokines, such as TNF, IL-1 α , IL-1 β , IL-6, IL-8, MIP-1 α , MIP-1 β and is able to drive the maturation and cross-presentation of antigens by APCs [121,126]. Calvet et al. (2014) showed that RE alone did not trigger the release of HMGB1 in CT26 tumors *in vivo*, possibly due to its dependence on the cell death processes. However, in the presence of bleomycin alone or in combination with electric pulses (ECT), cell viability decreased with consequent HMGB1 release, where ECT was associated with a higher level of HMGB1 release than bleomycin alone [114].

Gene expression profiles have been shown to be altered after RE. In SK-MEL28 melanoma cells exposed to RE, differences were revealed in seven genes related to HSP70, which is recognized for promoting the uptake of dying cells by APCs, repression of H4 histone and protein synthesis downregulation [121,122]. Moreover, no changes of major tumor suppressor genes, oncogenes or genes involved with DNA stability were observed, supporting the notion that EP does not induce tumorigenesis [122]. On the other hand, RE caused the activation of proinflammatory chemokine genes, including the expression of MIP-1 α (CCL3), MIP-1 β (CCL4), MIP-1 γ , IP-10, MCP-2, and XCL1, which trigger the influx of inflammatory APCs to the treated site [127].

Moreover, EP can also induce a bystander effect, seen when using conditioned medium, possibly through the release of microvesicles, with a

decrease of viability in the cell lines exposed to medium from RE and IRE treated cells. The number of microvesicles and the induction of cell death upon exposure to the conditioned medium are increased as the electric field intensity and the number of applied pulses rise, and also varies according to cell type [128].

Regarding immune status and ECT responses, Sersa and colleagues (1997) compared the outcomes in C57BL/6 and Swiss nude mice after treatment with ECT plus cisplatin aiming to evaluate the antitumoral response of immunocompetent and immunodeficient mice [129]. Nude mice are characterized by an absence of thymus that consequently leads to a lack of T cells (both CD4+ and CD8+) while keeping intact innate populations such as natural killer (NK) cells [130]. In their experiment, up to 82% (14 of 17) of immunocompetent mice bearing LPB sarcomas were cured by ECT plus cisplatin, whereas no therapeutic response was observed in nude mice. Furthermore, after a rechallenge with LPB tumor cells, 75% of the cured mice previously treated by ECT rejected the tumor, whereas no rejection was observed in the control group [129]. A similar outcome was described by Mir and colleagues (1991) using ECT plus bleomycin, where immunocompetent mice demonstrated better outcomes when compared to immunodeficient nude mice [65]. Both studies confirm the importance of host immune responses, especially T-lymphocytes, in the cure rate of the treatment.

However, the immunological response seems to be limited. For example, melanoma patients exhibited increased tumor infiltration by CD3+ CD8+ and CD3+ CD4+ and decreased CD4+ FOXP3+ Tregs after treatment with ECT plus bleomycin. Among the treated patients, 60% (6 of 10) demonstrated CR of treated lesions and 40% (4 of 10) patients displayed PR. Even so, four patients presented new lesions despite CR or PR after the previous treatment. In addition, only a low level of calreticulin exposition was observed on the first day post-treatment, followed by even weaker exposition at day 14 post-treatment [125].

Together, these findings indicate that despite the induction of ICD and local proinflammatory milieu, ECT by itself is not enough to induce antitumor effects on distant untreated lesions but retains an intrinsic potential for systemic and long-lasting effects when combined with immunostimulants [63,113,131–134]. In this way, the rationale is that the proper activation of APCs and other innate cells within the tumor microenvironment upon EP-related therapies ultimately must

lead to the priming of adaptive immune cells, that not only contribute to the killing of tumor cells, but also lead to the establishment of immunological memory that will protect in case of tumor relapse or metastasis. To this end, two different approaches can be mentioned, gene electrotransfer and electrochemo-genetherapy, both of which aim to boost the local immunological response by taking advantage of the delivery system provided by the electric pulses.

5. Gene Electrotransfer (GET) and Electrogenechemotherapy (EGCT) Alone or in Combination with Other Immunotherapies

Due to recent advances in understanding the biology of the immune system, new strategies such as those based on gene transfer have been proposed to treat infectious and cancer diseases. Genetic immunization transfers into the host a gene sequence encoding antigens or immunomodulatory molecules in order to induce an immune response. To date, the vast majority of gene therapy clinical trials have addressed cancer (67.4%), monogenic diseases (11.6%), cardiovascular and infectious diseases (5.8%) [135,136]. Among the nonviral vectors, plasmid DNA represents a good option for gene therapy purposes due to its safety, flexibility in design, cost-effective production in large scale, and stability at different temperatures. On the other hand, plasmid DNA itself is poorly immunogenic, and its introduction into host cells may require assistance by chemical or physical approaches [137,138].

Electroporation emerged as a suitable strategy for introducing plasmid DNA into target cells, paving the way for gene therapy mediated by electroporation, namely, GET, to be recognized as a useful approach for treating infectious and cancer diseases. Due to the perturbation of the cell membrane induced by the administered pulses, EP has been demonstrated to yield a higher number of transfected cells and larger quantities of plasmid permeating into each cell [139,140]. Furthermore, the local tissue damage induced by EP has been shown to play a role in enhancing both arms of the immune response, thus favoring immunization protocols [141]. GET-based preclinical protocols have been tested in different animal species (mice, rats, rabbits, and pets such as cats, dogs, horses) and tissue-specific protocols have been set up in skin, muscle, tumor, liver, cornea, lung, kidney, brain, bladder, and testis. These studies have

led to the establishment of different delivery conditions making efficient DNA electrotransfer feasible while minimizing possible electrical damage and maintaining effective protein expression [4,142,143].

Currently, human (Table 1) [144] and veterinary [145] clinical trials have been approved for antitumor therapies based on EP delivery of antitumoral drugs (bleomycin) or plasmids (cytokines and neoantigen vaccines) combined or not, are ongoing, mainly in the USA and Europe. The most advanced anticancer immune therapy based on plasmid DNA and electroporation is the GET of interleukin-12 (IL-12), which is currently on fast-track in the USA for orphan drug status [146].

Table 1. Current human clinical trials in the recruitment phase for electrochemotherapy, electrotransfer gene and combinations. Accessed on: 1 June 2021. Link: <https://clinicaltrials.gov/ct2/results?term=electroporation+NOT+irreversible+NOT+calcium+NOT+infection&recrs=a>

Research Aim	Neoplasms	Clinical Trial	Reference Centers
VGX-3100 and electroporation in treating patients with HIV positive high-grade anal lesions	<ul style="list-style-type: none"> Anal intraepithelial neoplasia High grade squamous intraepithelial neoplasia and others 	NCT03603808	<ul style="list-style-type: none"> UCLA CARE Center, United States University of California, United States and three more
Tavo with E electroporation, pembrolizumab and epacadostat in patients with unresectable head and neck cancer	<ul style="list-style-type: none"> Metastatic head and neck squamous cell carcinoma Recurrent head and neck squamous cell carcinoma Unresectable head and neck squamous cell carcinoma 	NCT03823131	<ul style="list-style-type: none"> University of California, United States
Neoadjuvant immunotherapy with Tavo + electroporation in combination with Nivo. in melanoma patients	<ul style="list-style-type: none"> Melanoma 	NCT04526730	<ul style="list-style-type: none"> Moffitt Cancer Center, United States
REVEAL 2 Trial (Evaluation of VGX-3100 and Electroporation for the treatment of cervical HSIL)	<ul style="list-style-type: none"> Cervical dysplasia Cervical high grade squamous intraepithelial lesion HSIL 	NCT03721978	<ul style="list-style-type: none"> Visions Clinical Research, United States Western Connecticut Health Network, United States and 44 more
HPV DNA vaccine via electroporation for HPV16 positive cervical neoplasia	<ul style="list-style-type: none"> Human papillomavirus type 16 Cervical intraepithelial neoplasia grade II cervical intraepithelial neoplasia, grade III 	NCT04131413	<ul style="list-style-type: none"> Johns Hopkins University, United States
Tavo and pembrolizumab in patients with stage III/IV melanoma progressing on pembrolizumab or nivolumab treatment (Keynote 695)	<ul style="list-style-type: none"> Stage III/IV melanoma 	NCT03132675	<ul style="list-style-type: none"> The University of Arizona Cancer Center, United States Yuma Regional Medical Center, United States and 35 more

Tavo and pembrolizumab with or without chemotherapy in patients with inoperable locally advanced or metastatic TNBC	<ul style="list-style-type: none"> • Triple negative breast cancer 	NCT03567720	<ul style="list-style-type: none"> • Stanford University Medical Center, United States • Moffitt Cancer Center, United States • and five more
Electrochemotherapy for noncurable gastric cancer	<ul style="list-style-type: none"> • Gastric cancer 	NCT04139070	<ul style="list-style-type: none"> • Zealand University Hospital, Denmark
A study of JNJ-64300535 in healthy participants	<ul style="list-style-type: none"> • Healthy 	NCT04736147	<ul style="list-style-type: none"> • SGS Life Science Services, Belgium.
INO 5401 vaccination in BRCA1/2 mutation carriers	<ul style="list-style-type: none"> • BRCA1/2 mutation 	NCT04367675	<ul style="list-style-type: none"> • University of Pennsylvania, United States
DNA plasmid-encoding interleukin-12/HPV DNA plasmids therapeutic vaccine INO-3112 and durvalumab in treating patients with recurrent or metastatic human papillomavirus associated cancers	<ul style="list-style-type: none"> • Human papillomavirus-16 positive • Human papillomavirus-18 positive • Metastatic malignant neoplasm and others 	NCT03439085	<ul style="list-style-type: none"> • MD Anderson Cancer Center, United States
Neoantigen DNA vaccine alone vs. neoantigen DNA vaccine plus durvalumab in triple negative breast cancer patients following standard of care therapy	<ul style="list-style-type: none"> • Triple negative breast cancer • Triple negative breast neoplasms • Triple negative breast cancer and others 	NCT03199040	<ul style="list-style-type: none"> • Washington University School of Medicine, United States
A study evaluating UCART019 in patients with relapsed or refractory CD19+ leukemia and lymphoma	<ul style="list-style-type: none"> • B cell leukemia • B cell lymphoma 	NCT03166878	<ul style="list-style-type: none"> • Chinese PLA General Hospital, China
GNOS-PV02 personalized neoantigen vaccine, INO-9012 and pembrolizumab in subjects with advanced HCC	<ul style="list-style-type: none"> • Hepatocellular carcinoma 	NCT04251117	<ul style="list-style-type: none"> • Johns Hopkins Hospital, United States • Icahn School of Medicine at Mount Sinai, United States • Auckland Clinical Studies, New Zealand
Safety and immune response to a mammaglobin-A DNA vaccine in breast cancer patients undergoing neoadjuvant endocrine therapy	<ul style="list-style-type: none"> • Breast cancer • Breast carcinoma • Malignant neoplasm of breast 	NCT02204098	<ul style="list-style-type: none"> • Washington University School of Medicine, United States
Platform study for prostate researching translational endpoints correlated to response to inform use of novel combinations	<ul style="list-style-type: none"> • Metastatic castration-resistant prostate cancer 	NCT03835533	<ul style="list-style-type: none"> • Angeles Clinic, United States • University of California San Francisco, United States • and four more.
Electrochemotherapy of gynecological cancers	<ul style="list-style-type: none"> • Gynecological cancers 	NCT04760327	<ul style="list-style-type: none"> • Institute of oncology Ljubljna, Slovenia
EndoVE endoscopic treatment for esophageal and gastric cancer	<ul style="list-style-type: none"> • Esophageal cancer • Esophageal disease • Gastric cancer 	NCT04649372	<ul style="list-style-type: none"> • Nottingham University Hospital Nottingham, United Kingdom

Abbreviations: VGX-3100: human papillomavirus DNA plasmids therapeutic vaccine; HIV: human immunodeficiency virus; Tavo: tavokinogene telseplasmid; DNA plasmid that encodes human interleukin-12. Nivo: nivolumab. HSIL: high-grade squamous intraepithelial lesions. HPV: human papilloma virus; TNBC: triple negative breast cancer; JNJ-64300535: proposed DNA vaccine to prevent hepatitis B; INO 5401: DNA plasmid that encodes different tumor antigens; BRCA1/2: breast cancer 1 and breast cancer 2 genes, respectively; INO-3112: DNA plasmids that encodes E6 and E7 HPV related cancer antigens and human interleukin-12; UCART019: Universal CD19-specific CAR-T cell; GNOS-PV02: a personalized neoantigen DNA vaccine; INO 9012: DNA plasmid that encodes genes for human interleukin-12; HCC: hepatocellular carcinoma.

IL-12, the most widely used cytokine in GET and EGCT protocols, connects the innate and adaptive immune responses, and presents inherent antitumor activities [142,147,148]. IL-12 is released by APCs after activation by pathogen-associated molecular patterns (PAMPs) and DAMPs and triggers the secretion of interferon- γ (IFN- γ) by T cells and NK cells. This cytokine is a key signal of cross-presenting by DCs to naive CD8⁺ T cells during priming and polarizes T cells towards the Th1 and Tc1 cytotoxic profile, promoting their conversion into IFN- γ producer cells and shifting myeloid-derived suppressor cells to functional APCs [149–154]. In turn, IFN- γ may also act directly on tumor cells, upregulating the expression of HLA class I and II, enhancing their recognition by CD8⁺ T cells through MHC class I processing and presentation of TSA and TAA [147,155,156]. In other words, IL-12 can transform noninflamed tumors into inflamed Th1-polarized tumors [149]. B16F10 tumors treated with IT IL-12 GET demonstrated a rapid induction of IL-12 expression, increase in T cell infiltration (TILs), upregulation of antigen-presenting genes, recruitment and polarization of antitumoral M1 macrophages, decrease of PD-1 expression and induction of memory T cells when compared to control groups [142,157,158]. Interestingly, it was observed that distant untreated lesions also had increased TILs, particularly of KLRG1^{hi} CD8⁺ effector T cells, a population that can differentiate into a memory T cell subset capable of mounting highly effective anti-tumor responses with improved resistance to T cell exhaustion by PD-1 and CTLA-4 immune checkpoints [142,159].

Mechanistically, IT delivery of IL-12 plasmid applied once in B16F10 cells resulted in 70% (14 of 20) of tumor remission in treated animals, with significant therapeutic effects against untreated tumors; the lack of perforin or IFN- γ and the blockage of CD8⁺ T cells abolished IL-12 GET efficacy, whereas the blockage of CD4⁺ T cells or NK cells did not. IL-12 GET induced a specific CD8⁺ T cell population (against epitope Trp2180-188) with approximately 33.3% (2 out of 6) of the cured animals being protected against tumor rechallenge. Concurrent Trp2 peptide vaccination increased protection and in turn of 85.7% (6 of 7) cured mice were able to reject tumors [160]. As a result, preclinical animal models treated by EP delivery of IL-12 demonstrated tumor regression, long term survival and resistance to rechallenge [99,158,161,162]. Furthermore, in veterinary oncology,

administration of IL-12 GET alone or in combination with ECT has been performed in client-owned dogs with spontaneous tumors, observing enhanced antitumor effectiveness and safety, opening new perspectives for human treatments [163–165].

Aiming to synergistically join the antitumoral activities of ECT and GET, some studies proposed ECGT, uniting the local ICD effect of ECT with the enhanced proinflammatory milieu of IL-12 (or other proinflammatory proteins) GET, which may lead to memory and systemic (abscopal) immune response even in untreated lesions. In this approach, the tumor antigens released by the treatment act like an autologous vaccine [63,132]. The treatment of B16F10 tumors with ECGT of IT IL-12 GET plus bleomycin led to complete remission in 37.5% (3 of 8) of treated mice by 150 days after treatment, with 100% (3 of 3) of those establishing tumor resistance upon rechallenge [166]. In canine mast cell tumors treated by ECGT, a pronounced and long-term antitumor effect was observed in patients treated by single or multiple peritumoral (PT) IL-12 GET with intratumoral cisplatin or bleomycin, with 72% (13 of 18) CR (RECIST) and prevented local recurrences and distant metastases (median 40 months of follow-up) [163]. Milevoj et al. obtained a 67% (6 of 9) objective response rate (RECIST) in canine patients with oral melanoma treated by cytoreductive surgery plus ECGT of PT IL-12 plus bleomycin in the tumor site. A decline in peripheral Treg cells was reported after the treatment, with no changes in peripheral CD4+ and CD8+ T cell populations [167]. In another study, a clinical response to ECGT of IT IL-12 plus bleomycin was obtained in dogs with different types of spontaneous tumors. CR was obtained in 50% (3 of 6) and the other three had PR to the treatment [165].

Other proposed approaches include the combination of EP-related therapies with immune checkpoint blockade (ICB) or DNA vaccination. It is known that systemic immunotherapy options such as vaccination and ICB have shown limited antitumoral responses due to the lack of tumor antigen availability, poor antitumoral immune cell infiltration and tumor immunosuppressive microenvironment [168,169], representing possible targets for the combined therapy. In a case report of metastatic melanoma treated by ECT and subsequent administration of ipilimumab (a CTLA-4 inhibitor), Brizio and colleagues (2015)

obtained CR of all lesions, even in the untreated internal metastasis, in an abscopal manner [170]. Mozzillo and colleagues (2015) conducted a retrospective analysis of advanced melanoma patients treated with ECT plus ipilimumab [171], concluding that the combination seemed to be beneficial to the patients, with reasonable responses obtained. They also indicated that circulating Treg levels might be a potential predictive biomarker of tumor response for this combined approach [171]. In another case report, Karaca and colleagues (2018) treated a patient with metastatic melanoma with ECT plus nivolumab (a PD-1 inhibitor). Although the patient had previously been treated with different therapies, including chemotherapy, targeted therapy, and immunotherapy, with no consistent tumor response, yet after the combined therapy they obtained a CR with no evidence of cutaneous or internal lesion during 4 years of follow-up [172]. Furthermore, ECT and CTLA-4 and PD1 inhibitors have been evaluated in a retrospective study of melanoma patients. The authors found that ECT plus PD-1 inhibitors had better outcomes than the combination with CTLA-1 in terms of OR (19.2% and 40%, respectively—RECIST) [173].

In a recent phase II clinical trial, patients with nonimmune-infiltrated “cold” melanoma were treated by a combination of IT IL-12 GET plus pembrolizumab (an anti-PD-1 checkpoint inhibitor). Patients had a 41% (9 of 22—RECIST) overall response rate (ORR), with 36% (8 of 22—RECIST) of CR, and exhibited significant local and systemic antitumor response, including those with previous unsuccessful anti-PD-1 therapy, with no new or unexpected toxicities related to pembrolizumab. Upregulation of innate and adaptive immune-associated genes and proinflammatory cytokines and chemokines expression was observed, and enhanced T cell infiltration in treated and untreated lesions and an increase in CD8+ effector memory T cells among peripheral blood was reported [149]. Recently, using a murine model of melanoma, intradermal IL-12 GET plus DNA vaccinations combined with anti-CTLA4/PD-1 blockade led to tumor reduction through long-lasting antigen-specific IgG antibodies together with antigen-specific CD8+ T cells [174]. In another approach, using EP as a delivery method for the intradermal Her2/neu DNA vaccine in murine models, Lamolinara and colleagues (2015) obtained a cytotoxic response in tolerant mice and a humoral (antibody) response in both tolerant and nontolerant mice [175].

6. Variability of Responses in EP-Related Therapies

The different outcomes in EP therapies demonstrated in the literature stem from biological factors, such as the individual, tumor types, intrinsic variability and the diverse protocols used in studies and clinical trials, which include different application routes of drugs (intravenous or intratumoral) and plasmids (intratumoral, peritumoral and intramuscular), different doses and types of therapeutic molecules, as well the diverse electrode designs. In addition, much of the data has come from early phase clinical trials performed in a limited number of patients which may add to the observed variability. In a systematic review of local effectiveness of single-session ECT comprising 413 patients with different tumor types and 1894 nodules (evaluated by WHO or RECIST criteria), the authors showed a higher antitumor response of IT treatment (72.7% of CR and 85.8% of OR) when compared to the IV administration of bleomycin (54.9% of CR and 80.7% of OR), but with no significant difference when comparing tumor response to IT administration of bleomycin and IT cisplatin. Further, they did not observe different outcomes in human patients with different tumor types (melanoma, carcinoma and sarcoma) treated by ECT [176].

The inability to generate a systemic immune response and the regression of untreated lesions in the form of an abscopal effect, even with the modulation of the local microenvironment in the ECT-treated lesions, may be related to previously established immunosuppression in these untreated lesions and intrinsic characteristics of the tumors [133,177], which may also occur in other EP-related therapies. For example, it was shown that a higher number of CD8⁺ cells infiltrating tumors prior to ECT treatment was associated with better local response (7 responders of 9 treated—WHO criteria) when compared to the low presence of those cells (1 responder of 6 treated). Interestingly, the infiltration of FOXP3⁺ cells was not associated with ECT local response, however, it was significantly associated to the development of visceral metastasis [178], corroborating similar results obtained by Ursic and colleagues (2021) in three different tumor models treated by ECT, where the more immunogenic CT26 tumors had better outcomes when compared with less immunogenic 4T1 and B16F10 tumors [179]. In addition, in a comparative study of a murine model of sarcoma (immunogenic) and carcinoma (less immunogenic) treated by ECGT of

IT cisplatin plus single or multiple IM IL-12 GET, it was demonstrated that the antitumor effect depends in part on the immunogenicity of the treated tumor, indicating that more immunogenic tumors had a better response and higher cure rate [90]. Those findings reflect the role of individual and tumor immunologic profiles in the outcomes of EP-therapies.

Regarding the route of plasmid administration, it was shown that IT IL-12 GET led to NK and CD8⁺ and CD4⁺ T cell accumulation and tumor elimination in murine squamous cell carcinoma. However, the antitumor response through the IM route was mediated mainly by NK cells, with minimal activation of specific CD8⁺ T cells and, consequently, inefficacy in tumor elimination [162]. A similar response was observed by Lucas and colleagues (2002) comparing both administration routes. The authors found that IT injection promoted the cure of 47% (8 of 17) of B16F10 melanoma tumors, prolonged survival and resistance against tumor rechallenge in 71.4% (5 of 7) of the cured animals, whereas the IM treatment did not result in tumor regression. Furthermore, they found increased levels of IL-12 and IFN- γ , and influx of CD4⁺ and CD8⁺ T cells into the treated tumors. In addition, both IT or IM treatment did not regress B16F10 tumors in the nude mouse group, reinforcing the role of lymphocytes in tumor elimination [180].

In another cancer model, murine subcutaneous fibrosarcoma treated by IT or PT IL-12 GET led to CR in 90% (18 of 20) of the IT treated mice and 15.6% (3/19) in the PT group; among cured mice, 61.1% (11 of 18) from the intratumoral group and 100% (3 out of 3) from the peritumoral group were protected against tumor rechallenge. The treatment in both regions promoted increase of IL-12 and IFN- γ level in serum and into the tumor and delayed the tumor growth of untreated lesions [181]. In humans, some evidence of complete response was observed in patients with Merkel Cell Carcinoma (MCC) treated with intratumoral IL-12 GET; 33.3% (1 of 3—RECIST) patients with local MCC showed pathological complete remission while 16% (2 of 12—RECIST) patients with metastatic MCC experienced prolonged survival. The responses were associated with the increase of peripheral and intratumoral MCC-specific T cells, also present in non-treated lesions, indicating an abscopal effect [182].

Indeed, even the level and duration of protein expression can be modulated to some extent by different plasmids, electrodes, and electric pulse

protocols, resulting in different therapeutic outcomes. Shirley and colleagues (2015), compared three different electrical protocols of IT IL-12 GET (EP1: six rotating 1300 V/cm, 100 μ s; EP2: ten 600 V/cm, 5 ms pulses; and EP3: one 667 V/cm, 100 ms pulse). They demonstrated that, in general, EP1 generated lower levels and a shorter expression time of IL-12 when compared to EP2, while EP3 generated similar levels to EP2, however, its analysis was discontinued after visible damage to the animals. Interestingly, they showed that the EP1 groups had similar tumor regression response to EP2 groups, however, EP1 groups showed higher immune cell infiltrate (CD11b positive cells), higher tumor rejection upon rechallenge and longer long-term survival compared to EP2 higher level expression groups, signaling the role of IL-12 in generating or abrogating an adequate immune response depends on its level and possibly duration of expression. Furthermore, they also evaluated the responses using different IL-12 plasmids, pUMVC3-mIL12 (commercially available) and pAG250-mIL12 (plasmid engineered for higher IL-12 expression), and the same inverse relation was observed, increased IL-12 correlated with reduced antitumor response in the animals. Indeed, they also demonstrated that different electrodes (plates or needles) impact gene expression levels even when using the same electric protocol [143]. Together, those findings demonstrate the importance of the correct choice of plasmids, electric protocols and electrodes for controlled gene expression. Later, the same group further evaluated the immune response unleashed by the IL-12 GET treatment with the pUCMV3-mIL-12 plasmid. They showed that responder mice had increased infiltration of immune cells in the tumor microenvironment and augmented expansion of CD4⁺ and CD8⁺ memory cells (CD44⁺) in peripheral blood, yet also showed downregulation of regulatory cells (MDSCs and CD4⁺ Foxp3⁺ Tregs) or exhausted T cells (CD4⁺ PD1⁺ and CD8⁺ PD1⁺ T cells) [157].

Tumors have an irregular and precarious vascularization when compared to normal tissues, and may also differ among different tumor types and sizes, impacting drug delivery and distribution into tumors, mainly in systemic (intravenous) administration [72,183], affecting tumor outcomes. In fact, it was shown that tumors with better vascular functions had better outcomes when compared to poorly vascularized tumors [184,185]. Further, it was demonstrated

that tumors up to 3 cm³ had better outcomes, with a negative correlation between the tumor size and tumor outcome. Despite the lack of information, it stands to reason that in smaller lesions an IT drug administration allows a uniform drug and electric field coverage that favors EP-therapies, however, there are no data regarding IT administration in larger lesions, and systemic administration of bleomycin is indicated in these cases [42,72]. However, the aforementioned precarious vascularization is an obstacle to drug distribution, mainly in the case of large tumors which are more likely to have areas with limited blood flow, further, larger tumors also present a diminished antivascular effect of ECT, as larger vessels seem to be resistant to RE vascular disruption [72,183]. A combined approach of both IT and IV drug administration has been proposed to overcome those limitations, minimizing the necessity of further ECT sessions [183]. Even so, in larger tumors proper electric field coverage might be difficult, with a higher probability of escape by nonelectroporated cells, demonstrating the importance of a careful and well-designed therapeutic plan [72].

Furthermore, some other biological factors such as previous tumor viral infection, previous treatments, drug resistance, tumor genomics and mutational load and diverse features of tumor microenvironment and of extracellular matrix seem to impact outcomes of EP-based therapies and deserve further investigation [72].

7. Conclusions

Recent developments in the fields of genetic engineering and immunology research give us high expectations for improvements in the efficacy of drugs and plasmid delivery and DNA immunization using EP. In this perspective, the association of ECT and GET can be envisioned as an interesting, combined modality to generate a local response and also achieve systemic antitumor immunization [63,132]. Additionally, a complete and concise description of all material and methods used in EP-related therapies [30,72,186] and further exploration of immunological analysis aiming for reproducibility and better analytical understanding of the outcomes are suggested. Next, the evaluation of results and follow-up of clinical trials will be important for determining the success of EP-based treatments and may pave the way for personalized therapeutic

protocols, assisting the advent of new therapies that improve the quality of human and animal health in the near future.

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2.1 Uso de citocinas em terapias antitumorais

Fisiologicamente, as citocinas são produzidas em resposta à estímulos externos, normalmente desempenhando atividades autócrinas e parácrinas de meia-vida curta, sendo as principais reguladoras dos sistemas imunológicos inato e adaptativo. Também estão envolvidas em processos relacionados à proliferação e diferenciação celular, além de exercerem funções efetoras e de sobrevivência das células imunes, desempenhando funções importantes às atividades antitumorais imunológicas (BERRAONDO et al, 2018; WALDMANN, 2017; ALGAZI et al, 2017).

O emprego de citocinas em terapias é considerado um marco nas imunoterapias, entretanto, o uso de citocinas como monoterapia e a necessidade de administração sistêmica em altas concentrações para uma eficaz atividade antitumoral têm como consequências diversos efeitos colaterais severos, podendo causar hipotensão, insuficiência renal aguda, insuficiência respiratória, neutropenia e sintomas neurológicos, além de apresentarem respostas antitumorais insatisfatórias (CONLON; MILJKOVIC; WALDMANN, 2019; BERRAONDO et al, 2018). Com isso, novas estratégias têm sido buscadas para potencializar os efeitos benéficos das citocinas em terapias antitumorais.

A eletrotransfecção de plasmídeos codificantes de citocinas, por providenciar um meio para expressão local e prolongada, é uma opção terapêutica cada dia mais em evidência (GOTHELF, 2017; KOS et al, 2016), não apresentando os efeitos colaterais do emprego sistêmico de altas doses de citocinas (REED; LI, 2011; HELLER et al, 2000). Até o momento o uso do IFN- β , citocina utilizada neste trabalho, ainda não foi explorada em GET.

2.1.1 Interferon- β e seus efeitos na resposta antitumoral

Os interferons do tipo 1 (IFN- α e IFN- β) são as principais citocinas da resposta imune contra infecções virais, e foram primeiramente identificados por sua ação antiviral no *Influenzavirus*. Tais citocinas também podem ser induzidos em resposta às infecções bacterianas indicando um papel fisiológico mais amplo na imunidade e homeostase do hospedeiro do que era originalmente assumido (GONZÁLEZ-NAVAJAS et al, 2012; ISAACS; LINDENMANN, 1957).

Interferons de tipo I como IFN- α e IFN- β possuem atividades autócrinas e parácrinas e sua ação antiviral permite a eliminação do patógeno pelas células infectadas e o seu efeito sobre as células vizinhas amplificam o seu sinal no chamado efeito *bystander* (MEDRANO et al., 2017; GONZÁLEZ-NAVAJAS et al, 2012; WELSH et al., 2012; SATO, TANIGUCHI, TANAKA, 2001), estando seu respectivo receptor IFNAR, presente em todas as células nucleadas (SCHREIBER, 2017).

Após a ligação do IFN- β ao seu receptor IFNAR, composto pelas subunidades IFNAR1 e IFNAR2, as quinases Tyk2 e Jak1 fosforilam o mesmo e iniciam o recrutamento dos fatores de transcrição STAT1 e STAT2, os quais são fosforilados, dimerizados e translocados ao núcleo. Os dímeros STAT1-STAT1 levam à síntese de genes envolvidos com a resposta inflamatória como *Irf1*, *Cxcl9* e *Cxcl10*. Dímeros STAT1-STAT2 são complexados com ISGF3 e IRF9 e levam à síntese de genes envolvidos com a resposta antiviral como *Oas* e *Mx1* (IVASHKIV; DONLIN, 2014; TRINCHIERI, 2010), conforme observado na Figura 3.

Diversos estudos têm demonstrado que células tumorais em processo de morte podem liberar adjuvantes internos, como os interferons de tipo I, que promovem sinais de ativação para células dendríticas e demais células apresentadoras de antígenos, o aumento da expressão de moléculas co-estimuladoras como CD40, CD80 e CD86, bem como a produção de IL-12 (MEDRANO et al, 2017; FUERTES et al, 2011). Esta, por sua vez, favorece a ativação e polarização de linfócitos T em linfócitos produtores de IFN- γ , promovendo, desta forma, o estabelecimento de respostas imunes antitumorais e de memória (FUERTES et al, 2011; KONO; ROCK, 2008; STORKUS; FALO Jr, 2007). Além disso, IFNs do tipo 1 também regulam a resposta de linfócitos T

para um perfil Th1, seja pela estimulação direta da produção de IFN- γ por linfócitos T ativados ou indiretamente ao inibir a expressão de IL-4 por monócitos (CAMERON MJ; KELVIN DJ, 2003; HIRANO et al., 2000; HEINRICH et al, 1998). A apresentação cruzada de antígenos antitumorais por células dendríticas a linfócitos T CD8+ também é favorecida *in vitro* e *in vivo* pela ação de IFN- β (MEDRANO et al, 2017; FUERTES et al, 2013; LE BON et al, 2003). O IFN- β também inibe a angiogênese (FIGURA 3) por meio da repressão de genes que codificam fatores pró-angiogênicos e pró-homing de neutrófilos infiltrantes de tumor (JABLONSKA et al, 2010), diminuindo o fornecimento de oxigênio e demais nutrientes para o tumor, o que favorece a eliminação de tumores (MEDRANO et al, 2017).

Figura 3. Funções antitumorais e antiangiogênicas dos interferons do tipo I.

Interferons- α/β (IFN- α/β) tem efeitos diretos nas células tumorais, induzindo a interrupção do crescimento e morte celular (esquerda). Após a ligação ao receptor 1 e 2 de IFN- α/β heterodimérico (IFNAR1/IFNAR2), os IFNs do tipo I induzem uma cascata de eventos intracelulares que culmina na expressão de genes cujos promotores contêm o elemento de resposta estimulada por IFN (ISRE). Dessa forma, são produzidas várias citocinas imunorreguladoras, fatores de morte celular e proteínas relacionadas à resposta antiviral, além de mais IFN- α/β , que por sua vez afeta as células vizinhas. Além disso, os efeitos antitumorais do IFN tipo I também podem ser uma consequência de sua função antiangiogênica, prejudicando a formação de vasos tumorais e levando à morte de tumores por falta de oxigênio e nutrientes (direita). O IFN- α/β pode inibir a produção de fatores angiogênicos pelas células tumorais, além de afetar diretamente as células endoteliais (CE), inibindo sua proliferação e secreção de fatores responsáveis pela quimiotaxia da CE e remodelamento da matriz extracelular. Tirosina quinase 2 (TYK2), Janus quinase 1 (JAK1), transdutor de sinal e ativador de transcrição (STAT), fator regulador de IFN 9 (IRF9), fator de gene estimulado por IFN 3 (ISGF3), fator de crescimento endotelial vascular (VEGF), fator de crescimento de fibroblasto básico (bFGF), interleucina-8 (IL-8), molécula-1 de adesão de células endoteliais de plaquetas (PECAM-1), proteína de leucemia promielocítica (PLM), metaloproteinase de matriz 9 (MMP9). Adaptado de: Medrano et al, 2017.

Estudos do perfil molecular de melanomas humanos demonstram que a ativação da via de sinalização de interferons de tipo I parece diferenciar tumores derivados de pacientes que responderam a terapias convencionais e imunoterapia de tumores derivados de pacientes que não responderam (WOO et al, 2015; GAJEWSKI, 2015). Foi demonstrado também que quimioterapias com antraciclinas são capazes de “mimetizar infecção viral” em tumores, ao induzir a produção de IFNs do tipo 1 e, conseqüentemente, possibilitar uma melhor resposta antitumoral ao propiciar uma morte celular imunogênica (SISTIGU et al, 2014).

2.1.2 Eletrogeneterapia com plasmídeo codificante de Interferon- β

Além dos efeitos imunomoduladores, o IFN- β apresenta melhor efeito antiproliferativo em diversos tipos de tumores quando comparado com IFN- α , IL-12 e IFN- γ (MURATA et al, 2006; CHAWLA-SARKAR et al, 2001; JOHNS et al, 1992; ROSENBLUM et al, 1990), apresentando diferentes efeitos antimitóticos, causando parada no ciclo celular (SANGFELT, 2000; COHEN et al, 1995). Entretanto, tal efeito antiproliferativo é dependente da concentração e permanência de IFN- β no tumor, que não pode ser alcançada via parenteral devido sua rápida metabolização e toxicidade sistêmica (QIN et al, 1998; SALMON et al, 1996), sendo a eletrotransfecção de plasmídeos capaz de expressar proteínas de interesse de forma localizada e prolongada (REED; LI, 2011; HELLER et al, 2000).

Outra característica importante é que enquanto todas as células nucleadas produzem receptores para IFN- β , apenas células NK, NK T, linfócitos T e algumas células de origem mielóide e linfócitos B tonsilares expressam receptores para IL-12 (SCHREIBER, 2017; TUGUES et al, 2015), implicando que IFN- β poderá ter um efeito mais abrangente do que encontrado com IL-12.

A transferência gênica de IFN- β pode promover a atividade antitumoral por meio do prolongamento do tempo de fosforilação de moléculas na via de transdução de sinal de IFN- β , como JAK1, Trk2 e STAT1 e a indução de apoptose, inclusive em tumores resistentes a proteínas de IFN (YOSHIDA; MIZUNO; WAKABAYASHI, 2004). O estudo da transferência gênica na imunoterapia de tumores tem demonstrado resultados satisfatórios em diversos

tipos de câncer (KAMRAN et al, 2017; MEDRANO et al, 2016). Desta forma, espera-se que a eletrotransfecção de plasmídeo codificante de IFN- β , combinado ou não com outras terapias, envolvendo seus efeitos antineoplásicos e imunomoduladores de forma sinérgica, possa vir a ser uma futura opção de terapia antineoplásica local e sistêmica.

Com o intuito de desenvolver um tratamento antitumoral por meio da eletrogeneterapia com IFN- β , optou-se pelo modelo murino de melanoma com células B16F10 devido sua aplicabilidade em experimento *in vivo* e *in vivo* e suas similaridades com melanoma humano (OVERWIJK; RESTIFO, 2000).

Tendo como base os resultados obtidos, tal modelo servirá como ponto de partida para futuras investigações de atividades antitumorais local, sistêmica e de memória.

3. OBJETIVOS

3.1 Objetivo geral

Avaliar atividades antitumorais da eletrogeneterapia com plasmídeo de IFN- β em linhagem celular de melanoma murino metastático (B16F10).

3.2 Objetivos Específicos

- Padronizar a técnica de eletroporação *in vitro* com plasmídeo de expressão de GFP sobre células de melanoma murino B16F10.
- Avaliar expressão e as atividades antitumorais com o uso de plasmídeo codificante de IFN- β a partir do protocolo elétrico estabelecido.
- Estabelecer protocolo para eletrotransfecção gênica *in vivo* com plasmídeo de expressão de GFP sobre tumores subcutâneos de melanoma murino B16F10.

4. MATERIAL E MÉTODOS

4.1 Cultivo celular

A linhagem celular de melanoma murino B16F10 (ou simplesmente B16) foi gentilmente fornecida pelo Dr. Roger Chammas (Instituto do Câncer do Estado de São Paulo, São Paulo, Brasil. As células B16 foram cultivadas em RPMI (Thermo Fisher Scientific), com 10% de soro fetal bovino (SFB; Thermo Fisher Scientific), sem adição de antibióticos ou antifúngicos, e mantidas em

estufa a 37°C em atmosfera umidificada com 5% de CO₂.

4.2 Preparação dos plasmídeos

A linhagem de *Escherichia coli* STBL3 foi transformada por eletroporação e mantida para preparação dos plasmídeos utilizando o kit comercial Qiagen EndoFree midi (Qiagen), seguindo instruções do fornecedor. O plasmídeo codificante de IFN- β murino pcmlIFN- β foi gentilmente fornecido pelo Dr. Zhongyun Dong (University of Texas MD Anderson Cancer Center, Houston, Texas, EUA). Utilizou-se como controle o plasmídeo codificante de proteína verde fluorescente (peGFP-N1, Clontech. GFP: do inglês: *Green Fluorescent Protein*). Ambos os vetores utilizam o promotor CMV (*cytomegalovirus*) para direcionar expressão do transgene. A concentração e absorbância dos plasmídeos foram determinadas por espectrofotometria (Nanodrop, Thermo Fisher Scientific), sendo que somente plasmídeos com nível de pureza na razão de A260/280 acima de 1.8 foram utilizados e com qualidade atestada por gel de eletroforese.

4.3 Eletrotransfecção *in vitro*

Células B16F10 em cultivo foram lavadas com PBS, tripsinizadas, bloqueadas com RPMI com 10% de soro fetal bovino (SFB) e, após centrifugação, ressuspensas em PBS para contagem em câmara de Neubauer. Em seguida, as células foram aliquotadas na quantidade desejada, para então, serem centrifugadas e ressuspensas em tampão de eletrotransfecção SBB (*Sucrose based buffer*; Sacarose 250 mM e MgCl₂ 1 mM em PBS; BREES; FRANSEN et al, 2014) de forma que a concentração final de células fosse de 1x10⁵ células a cada 100 μ l de SBB. Então, adicionou-se o plasmídeo de interesse à suspensão de células, de forma que a concentração final desejada fosse obtida em cada 100 μ l de suspensão. As células foram eletrotransfectadas com o eletroporador Neon Transfection System (Thermo Fisher Scientific) com cubeta de 100 μ l, de acordo com o protocolo operacional indicado pelo fabricante. As células foram eletroporadas com o protocolo elétrico de 1 pulso de 1400V por 20ms, e após a eletrotransfecção, foram plaqueadas em placas de 6 poços, em duplicata, como seus respectivos controles, isto é,

células sem tratamento (CC: “controle-controle”) e células receptoras somente de pulsos elétricos (CP: “controle-pulso”). As células foram cultivadas em meio RPMI 10% SFB sem adição de antibióticos ou antifúngicos, e mantidas em estufa a 37°C em atmosfera umidificada com 5% de CO₂.

4.4 Análise da taxa de eletrotransfecção, intensidade de fluorescência e morte celular.

Após 24h, 48h ou 72h de eletrotransfecção, as células foram observadas e fotografadas através de microscopia de fluorescência (EVOS FL- Thermo Fisher Scientific). Em seguida, células eletrotransfectadas com o plasmídeo peGFP-N1 foram fixadas em paraformaldeído 2% para posteriores análises de fluorescência por citometria de fluxo (Attune, Thermo Fisher Scientific). Alternativamente, células eletrotransfectadas com plasmídeo peGFP-N1 foram coletadas nos diferentes tempos e tratadas com LIVE/DEAD™ Fixable Red Dead Cell Stain Kit, for 488 nm excitation (Thermo Fisher Scientific, # L23102) de acordo com as instruções do fabricante.

4.5 Obtenção de sobrenadante para ELISA do IFN-β

As células B16F10 foram eletrotransfectadas com plasmídeo de IFN-β e plaqueadas em placas de 6 poços, com seus respectivos controles. Tais células foram incubadas a 37° C por 24h, 48h ou 72h. Após incubação, os sobrenadantes foram recuperados e centrifugados a 4°C por 5 min a 2000 rpm (rotor A-4-62; eppendorf), de forma a obter um sobrenadante livre de células com a formação do pellet no fundo do microtubo, sendo o sobrenadante armazenado a -20°C em microtubos de 500µl para posterior análise. Os níveis de IFN-β foram obtidos por meio do Kit Mouse IFN-beta DuoSet ELISA (DY8234-05; R&D System Europe) e leitura em 450 nm em leitor de microplacas (Bio-Rad Laboratories).

4.6 Eletrotransfecção *in vivo* e imageamento

O processo de eletrotransfecção foi realizado tendo como base o protocolo estabelecido por Mukhopadyay e colaboradores (2019). Camundongos C57BL6 com seis semanas de idade (CEUA-FMUSP n°1307/2019 - ANEXO),

foram obtidos através do Centro de Bioterismo da Faculdade de Medicina da Universidade de São Paulo (FMUSP) e mantidos no biotério do Centro de Medicina Nuclear (CMN), sendo os experimentos realizados com o apoio da pesquisadora Mara de Souza Junqueira. Foram inoculadas por via subcutânea 5×10^5 células B16F10 para o desenvolvimento do tumor. Quando os tumores atingiram aproximadamente 100 mm^3 , os animais foram tricotomizados na região do tumor e anestesiados com isoflurano 3%. Em seguida, injetou-se por via intratumoral $50 \mu\text{g}$ do plasmídeo peGFP suspensos em $50 \mu\text{l}$ de água estéril. Logo após, aplicou-se o protocolo elétrico (8 pulsos, 400V, 10ms; com intervalo entre os pulsos de 1 Hz) utilizando-se do eletroporador BTX Gemini (Harvard Apparatus) por meio dos eletrodos de placas Tweezertrodes (Harvard Apparatus), sedados gentilmente pelo Dr. Roger Chammas.

Após 48h da eletrotransfecção com plasmídeo peGFP-N1, os animais foram novamente anestesiados com isoflurano 3%, e em seguida, analisados por imageamento *in vivo* (IVIS - PerkinElmer) para visualização qualitativa da atividade do GFP.

5. RESULTADOS

5.1 Estabelecimento do protocolo elétrico

Devido a indisponibilidade na literatura da época de um protocolo elétrico para a linhagem B16F10 com o eletroporador Neon Transfection System (Thermo Fisher Scientific), utilizou-se como ponto de partida os protocolos pré-estabelecidos (Thermo Fisher Scientific, 2020) para duas linhagens de melanoma humano: linhagem G-361 (1 pulso de 40ms de 1050V, como valores mínimos) e linhagem MeWo (3 pulsos de 10ms de 1600V, como valores máximos).

Observou-se que o protocolo indicado para a linhagem G-361 se mostrou ineficiente para a eletrotransfecção de $2,5 \mu\text{g}$ do plasmídeo peGFP-N1 em 5×10^5 células B16F10, pois 24h após a eletrotransfecção, poucas células apresentaram fluorescência à microscopia quando comparado com os 68% de eletrotransfecção descritos para a linhagem G-361 (Thermo Fisher, 2020). Além disso, a morfologia das células receptoras de plasmídeo se mostrou alterada, um indicativo de sofrimento celular, sendo o mesmo observado nas células do

grupo “controle-pulso” (CP: células que receberam apenas os pulsos elétricos). Diferentemente, células do grupo “controle-controle” (CC), que não receberam pulsos elétricos ou plasmídeo, apresentaram morfologia normal, isto é, células fusiformes, com dendritos e aderidas a placa (FIGURA 4).

Figura 4. Células B16 após 24h de eletrotransfecção de 2,5µg de peGFP-N1 com o protocolo G-361. A. Microscopia de luz; células “controle-controle”, com sua morfologia normal. **B.** Microscopia de luz; células “controle-pulso”. **C.** Microscopia de luz; células receptoras de plasmídeo (GFP) peGFP-N1. **D.** Microscopia de fluorescência; células receptoras de plasmídeo. (Três experimentos independentes, sendo que cada condição foi realizada com duplicata técnica).

Com a utilização do protocolo elétrico indicado para a linhagem MeWo (3 pulsos, 1600V, 10ms) observou-se que este também se mostrou ineficaz para a eletrotransfecção na linhagem celular B16F10, apresentando resultados diferentes da taxa de 71% na eletrotransfecção de plasmídeo codificante de GFP descrito para a linhagem MeWo (Thermo Fisher, 2020). Após 24h da eletrotransfecção do plasmídeo peGFP-N1, as células B16F10 estavam desaderidas em sua totalidade e não apresentavam fluorescência à microscopia, sendo o mesmo observado no grupo CP, enquanto que células do grupo CC apresentaram morfologia normal (FIGURA 5).

Figura 5. Células B16 após 24h de eletrotransfecção de 2,5ug de peGFP-N1 com o protocolo MeWo. A. Microscopia de luz; células “controle-controle” (sem pulso). **B.** Microscopia de luz; células “controle-pulso”. **C.** Microscopia de luz; células receptoras de plasmídeo peGFP-N1. **D.** Microscopia de fluorescência; células receptoras de plasmídeo. (Três experimentos independentes, sendo que cada condição foi realizada com duplicata técnica).

Desta forma realizou-se diversas modificações entre os três parâmetros (número de pulsos, voltagem e duração dos pulsos), tendo os protocolos supracitados como referências máximas e mínimas, até a obtenção do protocolo que resulta em transfecção da maioria das células sem causar danos aparentes, seguindo indicações do guia para otimização de eletrotransfecção (BTX, 2020).

Após vários testes, estabeleceu-se o protocolo adotado (protocolo B16F10) com os seguintes parâmetros: 1 pulso de 20ms de 1400V, que até então era o primeiro protocolo elétrico para a linhagem celular B16F10 de melanoma murino com o *Neon electroporation system*.

Utilizando este protocolo, observou-se uma alta taxa de eletrotransfecção das células na microscopia de fluorescência, morfologia habitual e aderência na placa, indicativo de que o protocolo foi bem tolerado, sendo o mesmo observado no controle CP (FIGURA 6). Observa-se também que existem diferentes intensidades de fluorescência entre as células GFP+ positivas (FIGURA 6D).

Figura 6. Células B16 após 24h de eletrotransfecção de 2,5µg de pEGFP-N1 com o protocolo B16. A. Microscopia de luz; células “controle-controle” (sem pulso). **B.** Microscopia de luz; células “controle-pulso”. **C.** Microscopia de luz; células receptoras de plasmídeo pEGFP-N1. **D.** Microscopia de fluorescência; células receptoras de plasmídeo. **E.** Sobreposição da microscopia óptica e de fluorescência das células B16 após 24h de eletrotransfecção. (Dois experimentos independentes, sendo que cada condição foi realizada com duplicata técnica).

5.1.1 Análise qualitativa da fluorescência de GFP em linhagem B16F10 com o protocolo elétrico adotado.

Com o estabelecimento do protocolo elétrico para linhagem B16F10, foram realizados eletrotransfecções com diferentes quantidades do plasmídeo peGFP-N1 (1µg, 2,5µg e 5µg /100µl da suspensão de células em SBB) a fim de estabelecer qual a melhor quantidade para os experimentos subsequentes. Neste momento, optou-se pela redução da quantidade de células utilizadas, de 5×10^5 para 1×10^5 células B16F10, para possibilitar o acompanhamento das células por até 72h, evitando que os poços ficassem superpopulados.

Após 24h, observou-se que a taxa de eletrotransfecção é dose-dependente em relação ao plasmídeo peGFP-N1 e que a quantidade de até 5µg do plasmídeo peGFP-N1 foi bem tolerada (FIGURA 7), sem aparente toxicidade.

Figura 7. Células B16 após 24h de eletrotransfecção de diferentes concentrações do plasmídeo peGFP-N1 com o protocolo B16. A. Microscopia de luz; células receptoras de 1µg de plasmídeo e sua respectiva microscopia de fluorescência **(B)**. **C.** Microscopia de luz; células receptoras de 2,5µg de plasmídeo e sua respectiva microscopia de fluorescência **(D)**. **E.** Microscopia de luz; células receptoras de 5µg de plasmídeo e sua respectiva microscopia de fluorescência **(F)**. (Três experimentos independentes, sendo que cada condição foi realizada com duplicata técnica).

Após 48h observou-se mesmo padrão de atividade de GFP de acordo com a dose do plasmídeo eletrotranfectado, e também no que se refere a diferentes intensidades dentre as células (FIGURA 8).

Figura 8. Células B16 após 48h de eletrotransfecção de diferentes concentrações do plasmídeo peGFP-N1 com o protocolo B16. A. Microscopia de luz; células receptoras de 1µg de plasmídeo e sua respectiva microscopia de fluorescência **(B)**. **C.** Microscopia de luz; células receptoras de 2,5µg de plasmídeo e sua respectiva microscopia de fluorescência **(D)**. **E.** Microscopia de luz; células receptoras de 5µg de plasmídeo e sua respectiva microscopia de fluorescência **(F)**. (Três experimentos independentes, sendo que cada condição foi realizada com duplicata técnica).

De forma semelhante, após 72h observou-se mesmo padrão de atividade de GFP de acordo com a dose do plasmídeo eletrotransfectado, e também diferentes perfis de intensidades (FIGURA 9).

Figura 9. Células B16 após 72h de eletrotransfecção de diferentes concentrações do plasmídeo peGFP-N1 com o protocolo B16. A. Microscopia de luz; células receptoras de 1µg de plasmídeo e sua respectiva microscopia de fluorescência (**B**). **C.** Microscopia de luz; células receptoras de 2,5µg de plasmídeo e sua respectiva microscopia de fluorescência (**D**). **E.** Microscopia de luz; células receptoras de 5µg de plasmídeo e sua respectiva microscopia de fluorescência (**F**). (Três experimentos independentes, sendo que cada condição foi realizada com duplicata técnica).

5.2 Análise por citometria de fluxo.

5.2.1 Análise de positividade de fluorescência.

Realizou-se análise por citometria de fluxo das células B16 após eletrotransfecção de plasmídeo peGFP-N1. Assim como observado na microscopia de fluorescência (FIGURAS 7, 8 e 9), a análise por citometria apresentou a correlação entre o número de células fluorescentes (%GFP+) com a concentração do plasmídeo empregada, sendo que as células receptoras de 5µg do plasmídeo foram as que mais apresentaram atividade de GFP.

Entretanto, pode-se observar também que apesar das células que receberam maiores concentrações do plasmídeo apresentarem maior porcentagem de células GFP+, essa diferença não ocorreu de forma proporcional (FIGURA 10).

Figura 10. Análise por citometria das células B16 eletrotransfectadas com diferentes concentrações do plasmídeo peGFP-N1 após 24h, 48h e 72h. Legenda – CC: “Controle-Controle”, células não eletroporadas e não receptoras de plasmídeo; CP: “Controle-Pulso”, células somente eletroporadas. (Três experimentos independentes, sendo que cada condição foi realizada com duplicata técnica).

Desta forma, realizou-se análise de intensidade de fluorescência das células após 24h, 48h e 72h de eletrotransfecção.

5.2.2 Análise da intensidade de fluorescência.

De forma semelhante ao observado na microscopia de fluorescência (FIGURAS 7, 8 e 9) e por citometria (FIGURA 10), observou-se que a intensidade de fluorescência também apresentam um padrão de correlação com a dose de plasmídeo empregada, sendo que as células receptoras de 5µg do plasmídeo peGFP-N1 apresentarem atividade de GFP de forma mais intensa (FIGURA 11).

Figura 11. Análise por citometria da intensidade de fluorescência das células B16 eletrotransfectadas com diferentes concentrações de plasmídeo peGFP-N1 após 24h, 48h e 72h. Legenda – CC: “Controle-Controle”, células não eletroporadas e não receptoras de plasmídeo; CP: “Controle-Pulso”, células somente eletroporadas. (Três experimentos independentes, sendo que cada condição foi realizada com duplicata técnica).

5.2.3 Análise de morte celular.

Para analisar os efeitos deletérios do protocolo estabelecido, bem como possíveis citotoxicidades do uso do plasmídeo codificante de GFP, realizou-se análise de citotoxicidade (Live&Dead) por citometria em células eletrotransfectadas com diferentes quantidades do plasmídeo peGFP-N1 em três diferentes tempos, bem como seus respectivos controles (FIGURA 12). Pode-se observar que em todos os grupos o percentual de células mortas foi de aproximadamente 1% nos momentos analisados (24h, 48h e 72h), ou seja, uma viabilidade próxima da 100%, demonstrando ser um protocolo pouco deletério para as células. Como comparação, foi relatado que os protocolos G-361 e

MeWo, apresentaram 80% e 64% de viabilidade, respectivamente (Thermo Fisher, 2020).

Figura 12. Análise por citometria de fluxo a citotoxicidade provocada pelo protocolo elétrico estabelecido em células B16 eletrotransfectadas com diferentes concentrações do plasmídeo peGFP-N1 após 24h, 48h e 72h. Legenda – CC: “Controle-Controle”, células não eletroporadas e não receptoras de plasmídeo; CP: “Controle-Pulso”, células somente eletroporadas (Três experimentos independentes, sendo que cada condição foi realizada com duplicata técnica).

5.3 Eletrogeneterapia de plasmídeo codificante de IFN- β

Após o estabelecido do protocolo elétrico e a validação do mesmo utilizando plasmídeo peGFP-N1, iniciamos a padronização da dosagem do plasmídeo codificante de IFN- β , sendo optado por 1 μ g como dose inicial.

Após acompanhamento por 24h, 48h e 72h, observou-se através da microscopia uma menor densidade das células B16 receptoras do plasmídeo codificante de IFN- β quando comparada com seus respectivos controles, indicando uma possível redução de proliferação (FIGURA 13).

Figura 13. Microscopia de luz de células B16 após 24h, 48h e 72h após eletrotransfecção com plasmídeo codificante de IFN- β . A, B e C. Células “controle-controle”, após 24h, 48h e 72h de cultivo, respectivamente. D, E e F. Células “controle-pulso”, após 24h, 48h e 72h de eletroporação, respectivamente. G, H e I. Células receptoras de 1 μ g de plasmídeo após 24h, 48h e 72h de cultivo codificante de IFN- β , respectivamente. (Dois experimentos independentes, sendo que cada condição foi realizada com duplicata técnica).

Após análise inicial, realizou-se novos experimentos, utilizando-se de três diferentes concentrações do plasmídeo pcmlFN- β (1 μ g, 2,5 μ g e 5 μ g). Na Figura 14 é possível observar imagens de microscopia em três diferentes tempos (24h, 48h e 72h) pós tratamento, melhor visualizadas após tratamento de imagem por ImageJ (Figura 15).

Figura 14. Células B16 após 24h, 48h e 72h de eletrotransfecção de diferentes concentrações do plasmídeo pcmIFN- β com o protocolo B16. A. Microscopia de luz; células receptoras de 1µg de plasmídeo e sua respectiva microscopia de fluorescência (B). **C.** Microscopia de luz; células receptoras de 2,5µg de plasmídeo e sua respectiva microscopia de fluorescência (D). **E.** Microscopia de luz; células receptoras de 5µg de plasmídeo e sua respectiva microscopia de fluorescência (F). (Um experimento onde cada condição foi realizada com duplicata técnica).

Figura 15. Células B16 após 24h, 48h e 72h de eletrotransfecção de diferentes concentrações do plasmídeo pcmlFN- β com o protocolo B16 analisadas através de ImageJ. A. Processamento de imagens de microscopia de luz por ImageJ. **A.** Células receptoras de 1 μg de plasmídeo. **C.** Microscopia de luz; células receptoras de 2,5 μg de plasmídeo e sua respectiva microscopia de fluorescência (**D**). **E.** Microscopia de luz; células receptoras de 5 μg de plasmídeo e sua respectiva microscopia de fluorescência (**F**). (Um experimento onde cada condição foi realizada com duplicata técnica).

Com a quantificação das células pelo ImageJ, pode-se comparar a densidade celular entre os diferentes tratamentos analisados (Imagem 16).

Figura 16. Quantificação por ImageJ das células B16 após 24h, 48h 72h de eletrotransfecção de diferentes concentrações do plasmídeo pcmlFN- β com o protocolo B16. Legenda – CC: “Controle-Control”, células não eletroporadas e não receptoras de plasmídeo; CP: “Controle-Pulso”, células somente eletroporadas. (Um experimento, sendo que cada condição foi realizada com duplicata técnica).

5.4 Análise de expressão de IFN- β por ELISA

Realizou-se análise por ensaio de imunoabsorção enzimática (ELISA/ R&D System Europe) da concentração de IFN- β no sobrenadante das células após tratamento (FIGURA 17). Foi possível observar a presença de IFN- β somente no sobrenadante das células tratadas com pcmlFN- β , com maior nível de expressão em 48h pós tratamento, e ausência do mesmo nas células controle (CC e GFP). Observou-se também correlação positiva entre o nível de expressão do IFN- β com a concentração utilizada do plasmídeo.

Figura 17. Quantificacao de IFN- β por ELISA a partir de meio condicionado.
 Legenda - CP: células “Controle-Pulso”, células não recebedoras de plasmídeo.
 GFP: células eletrotransfectadas com plasmídeo peGFP-N1. IFN: células eletrotransfectadas com plasmídeo pcmlFN- β . (Um experimento, sendo que cada condição foi realizada com duplicata técnica).

5.5 Eletrotransfecção *in vivo* e imageamento

Após a determinação do protocolo elétrico *in vitro* e a fim de se estabelecer o protocolo elétrico *in vivo*, realizou-se também a eletrotransfecção de 50 μ g plasmídeo peGFP-N1 intratumoral em modelos murinos C57BL6 em tumores subcutâneos de células B16F10, por meio do eletroporador BTX Gemini (Harvard Apparatus), utilizando o protocolo elétrico para eletropermeabilização *in vivo* descrito por MUKHOPADHYAY e colaboradores (2019), sendo este: 400V, 8 pulsos, 10ms; com intervalo entre os pulsos de 1 Hz.

Após 48h da eletrotransfecção, os animais foram analisados por imageamento *in vivo* e posteriormente *ex vivo* (FIGURA 18. IVIS - PerkinElmer), sendo possível observar a atividade da proteína fluorescente.

Figura 18. Análise *in vivo* e *ex vivo* por IVIS de tumores B16F10 após 48h de eletrotransfecção do plasmídeo peGFP-N1. A. Imageamento *in vivo* animal controle-pulso (recebedor somente de pulsos elétricos). B, C, D, E. Imageamento *in vivo* após 48h da eletrotransfecção com 50 μ g do plasmídeo peGFP-N1 por via intratumoral. F, G, H. Imageamento *ex-vivo* dos tumores do animal controle-controle (F) e dos recebedores do plasmídeo (G,H, animais B e D, respectivamente). I. Imagem ilustrativa do eletrodo de placas utilizado para a entrega dos pulsos elétricos (Tweezertrodes, BTX, Harvard Apparatus).

6. DISCUSSÃO

Diferentes linhagens celulares requerem diferentes protocolos elétricos para uma eficaz eletrotransfecção. Conforme foi observado, mesmo linhagens celulares de um mesmo tipo tumoral (neste caso, linhagens melanoma murino) apresentam uma grande variação na taxa de eletrotransfecção, bem como efeitos deletérios com o uso de protocolos impróprios (Figuras 3 e 4). De forma semelhante, tal variação na eletroporação pode ser um dos fatores que contribuem para diferentes níveis de respostas antitumorais em pacientes tratados por terapias baseadas em eletroporação (TBE), reforçando a necessidade do estabelecimento de protocolos elétricos tumor-específico para tratamentos clínicos, o que vem sendo alvo de pesquisas (SERSA et al, 2021).

O estabelecimento do protocolo elétrico considerado como eficaz e não deletério para a linhagem celular B16F10, até o momento não havia sido estabelecido para o aparelho de eletroporação *Neon transfection system* (Thermo Fisher). Recentemente, Ishibashi e colaboradores (2020), utilizaram-se de um protocolo elétrico composto de 1 pulso de 30ms de 1100 V e obtiveram resultados positivos no *knock-out* de células B16F10 através de CRISPR-Cas9 com o mesmo aparelho de eletrotransfecção, entretanto, não foram compartilhadas informações sobre viabilidade celular ou taxa de

eletrotransfecção por terem realizado seleção das células eletrotransfectadas com antibióticos (ISHIBASHI et al, 2020), impossibilitando análises de fins comparativos.

Conforme observado, o protocolo elétrico estabelecido apresentou baixo nível deletério (morte celular) quando utilizado sozinho, bem como quando utilizado em combinação com o plasmídeo controle peGFP-N1 em diferentes quantidades (Figura 12). Tal característica é de suma importância para uma eficaz síntese da proteína pelas células eletrotransfectadas. Contudo, vale ressaltar, que mesmo com baixa citotoxicidade, é sabido que o protocolo elétrico sozinho também colabora com uma resposta antitumoral *in vivo* ao induzir a expressão de DAMPs como calreticulina, HMGB-1 e ATP (SCHULTHEIS et al, 2018; CALVET et al, 2014).

Outro fator importante a ser considerado é que devido a natureza transiente da eletrotransfecção de plasmídeos circulares (POTTER, 2003), com o passar do tempo, a relação entre células eletrotransfectadas *versus* células não receptoras de plasmídeo tende a cair (Figura 10), visto que células “filhas” não terão plasmídeos incorporados para expressarem a proteína de interesse. Essa é uma característica importante a ser levada em consideração, principalmente em experimentos *in vivo* onde os modelos experimentais são acompanhados por períodos maiores. Entretanto, devido ao efeito *bystander*, isto é, a geração de um *feedback* positivo gerado pela presença do IFN- β sintetizado pelas células eletrotransfectadas, células vizinhas passarão a sintetizar tal citocina, aumentando a ação antitumoral no microambiente (ROSSI et al, 2015).

Conforme observado nas Figuras 15 e 16, apesar de não terem sido realizado experimentos para avaliação qualitativa como Anexina-PI, por exemplo, pode-se observar que na presença do IFN- β houve uma menor proliferação quando comparadas com seus respectivos controles, possivelmente por parada no ciclo celular, uma característica esperada em terapias que se utilizam de tal citocina (SANGFELT, 2000). Observou-se também o mesmo perfil de correlação positiva da quantidade empregada de plasmídeo, tanto na microscopia (Figura 16) como na presença de IFN- β presente no sobrenadante (Figura 17). O IFN- β também apresenta diversas atividades anti-angiogênicas

(VIEIRA et al, 2019; MEDRANO et al, 2017), assim como a eletroquimioterapia (JARM et al, 2010), podendo gerar uma ação sinérgica prejudicando ainda mais o fornecimento de oxigênio e demais nutrientes ao tumor tratado, o que futuramente pode ser explorado.

Além disso, a eletrotransfecção de plasmídeo codificante de IFN- β pode apresentar resultados melhores quando comparados com a IL-12, citocina mais utilizada atualmente em TBE, visto que esta tem receptores em um número limitado de células, enquanto que todas as células nucleadas apresentam receptores para IFN- β (SCHREIBER, 2017; TUGUES et al, 2015).

Estudos prévios do grupo utilizando-se de vetores virais codificantes de IFN- β em combinação com outros genes, bem como terapias farmacológicas e imunoterapias, tem demonstrado efeitos promissores. Por exemplo, estudos demonstraram os efeitos terapêuticos da transferência gênica de IFN- β juntamente p19Arf em modelo de melanoma B16 *in vitro* e *in vivo*, bem como a resposta imune antitumoral mediada por células *natural killer* e linfócitos T CD4+ e CD8+ em modelos de terapia *in situ* ou vacinação (STRAUSS et al, 2018;; HUNGER; MEDRANO; STRAUSS, 2017; MEDRANO et al., 2016; MERKEL et al, 2013).

Desta forma, acreditamos que o uso de pulsos elétricos permeabilizantes como método de *delivery* gênico de IFN- β sozinho ou em combinação de outros genes ou quimioterápicos podem ser uma alternativa para transfecção de células *in vitro* e *in vivo*, em especial com o uso da bleomicina, fármaco com baixa penetração na membrana celular com diversas características antitumorais previamente mencionadas.

Ademais, é sabido que doses maiores de plasmídeo podem conferir maior síntese da proteína de interesse, fato também observado nas experimentações *in vitro* através da visualização por microscopia (Figura 7, 8 e 9), análises por citometria da proteína fluorescente GFP (Figura 10) e da mensuração de IFN- β através de ELISA (Figura 17). Entretanto, vale salientar que nem sempre tal característica tem potencial benéfico em terapias antitumorais, principalmente em modelos *in vivo* e pacientes, devido todas as interações do microambiente tumoral. Em modelos murinos de melanoma, Shirley e colaboradores (2015), observou que animais recebedores de protocolos terapêuticos com maior

expressão de IL-12 apresentaram atividades antitumorais inferiores aos grupos com menores expressões de tal citocina. Tal observação é digna de ser experimentada também em futuros experimentos *in vivo* utilizando-se GET com IFN- β .

Durante a administração dos pulsos elétricos ocorreram interrupções motivadas por movimentos de contrações musculares involuntárias abruptas, comuns durante este tipo de terapia (MIKLAVCIC et al, 2005). Tais interrupções resultaram em uma menor taxa de expressão de GFP *in vivo* (Figura 18 C e 18 E), salientando a importância de uma completa aplicação do protocolo elétrico nos modelos experimentais, evitando assim possíveis vieses em resultados. A incorreta aplicação dos protocolos elétricos também é considerada como fator contribuinte nas diferentes respostas encontradas em pacientes (CADOSSI; RONCHETTI; CADOSSI, 2014). Outra observação nos modelos *in vivo* foi o surgimento de queimaduras superficiais leves em alguns dos animais, efeito colateral conhecido que pode se apresentar em tecidos mais sensíveis (como no caso dos tumores em questão) e que pode estar relacionado também com protocolo terapêutico utilizado (De VIRGILIO et al, 2018; KOS et al, 2016).

7. CONCLUSÕES

Este estudo possibilitou o estabelecimento de um protocolo elétrico para eletrotransfecção de células B16F10 que até o momento não havia sido descrito para o eletroporador *Neon transfection system* (Thermo Fisher Scientific), resultando em uma taxa de eletrotransfecção do plasmídeo peGFP-N1 até 45,6% com viabilidade próxima a 100%. Além disso, o uso do protocolo estabelecido possibilitou a eletrotransfecção do pcmIFN- β nesta mesma linhagem celular, o que resultou no acúmulo de IFN- β no sobrenadante em até 526,1pg/mL, e retardo no crescimento celular de até aproximadamente 60% em comparação com aos grupos controles devido as atividades proliferativas desta citocina. No ensaio preliminar *in vivo*, detectou-se o sinal de GFP após GET em tumores B16F10 subcutâneos.

Até o momento, o uso de IFN- β em terapias baseadas em eletroporação não foi proposta na literatura, entretanto, visto suas inúmeras características imunomoduladoras, antitumorais e anti-angiogênicas, acreditamos que sua aplicação como monoterapia ou em combinações tem grande potencial terapêutico, conforme já demonstrado em resultados do grupo com vetores virais. Este estudo abre caminho para futuras abordagens experimentais e terapêuticas utilizando-se da eletrotransfecção gênica e da resposta imunológica antitumoral provocada pelo IFN- β .

8. ANEXO

Termo de autorização da comissão de ética no Uso de Animais da Faculdade de Medicina da Universidade de São Paulo (CEUA-FMUSP nº1307/2019) para o presente projeto.

Faculdade de Medicina da Universidade de São Paulo
Avenida Dr. Arnaldo, 455
Pacaembu – São Paulo – SP

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Certificamos que a proposta intitulada “**AVALIAÇÃO DA EFICÁCIA E INDUÇÃO DE IMUNIDADE ANTITUMORAL DE ELETROQUIMIOTERAPIA EM MODELO MURINO DE MELANOMA METASTÁTICO**”, registrada com o nº **1307/2019**, sob a responsabilidade de **Bryan Eric Strauss** e **Jean Carlos dos Santos da Luz**, apresentada pelo ICESP - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) da Faculdade de Medicina da USP em 05.12.19

Finalidade	() Ensino (x) Pesquisa Científica
Vigência da autorização	Início: 01-06-2019 Término: 29-06-2021
Espécie/linhagem/raça	Camundongo C57Bl/6
Nº de animais	182
Peso/Idade	5 semanas
Sexo	machos
Origem	Biotério FMUSP

A CEUA FMUSP solicita que ao final da pesquisa seja enviado Relatório com todas as atividades.

CEUA-FMUSP, 05 de Dezembro de 2019

Dr. Eduardo Pompeu
Coordenador
Comissão de Ética no Uso de Animais

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