Historical review of irreversible electroporation in medicine

Abstract. The objective of this chapter is to present a historical review of the field of irreversible electroporation (IRE) in the context of its medical applications. Although relevant scientific observations were made since the 18th century, the electroporation phenomenon was not identified as an increase of membrane permeability until mid 20th century. After that, multiple applications of reversible electroporation emerged *in vitro* (DNA electrotransfer) and *in vivo* (electrogenetherapy and electrochemotherapy). Irreversible electroporation was tested commercially in the 60s as a bactericidal method for liquids and foods but its use in the context of medical applications was not studied until the early 2000s as an ablative method. The cell destruction mechanism of IRE is not based on thermal damage and this fact provides to IRE an important advantage over other physical ablation methods: the extracellular scaffolding, including the vessels, is preserved. Several surgical applications are now under study or even under clinical trial: ablation of hepatocarcinomas, ablation of prostate tumors, treatment of atrial fibrillation and treatment of vascular occurrences such as restenosis and atherosclerotic processes.

Introduction

Electroporation is the phenomenon in which cell membrane permeability to ions and macromolecules is increased by exposing the cell to short high electric field pulses. Such increase in permeability is, presumably, related to the formation of nano-scale defects or pores in the cell membrane; from which the term electro-"poration" stems. Under some conditions (e.g. extremely large field magnitude), membrane permeabilization is permanent and the process leads to cell lysis. It is in this sense of permanent permeabilization that most authors define irreversible electroporation (IRE). However, it must be noted that temporary permeabilization can also cause a severe disruption of the cell homeostasis that can finally result in cell death, either necrotic or apoptotic. Therefore, in a broader sense, IRE could be defined as the permanent or temporal membrane electroporation process that causes cells to die.

The primary use of irreversible electroporation is to induce the death of undesirable cells without causing excessive heating. Recently, irreversible electroporation has begun to be employed as tool in the surgeon armamentarium for minimally invasive ablation of undesirable tissue. The goal of this chapter is to present a historical review of the field of irreversible electroporation from the first reports of the phenomenon in the 18th century to modern applications in minimally invasive surgery. We intentionally omit to mention membrane electroporation theories [1] and models; the present book contains the most recent study of models for the electroporation phenomenon.

The 18th and 19th centuries

Probably the first scientific description of a phenomenon suggestive of irreversible electroporation in tissue can be found in the 1754 book of J.A. Nollet "*Recherches sur les causes particulieres des phénoménes électriques*." [2]. In experiments with electrical fields Nollet noticed the formation of red spots on the skin of humans and animals in areas where electrical sparks were applied. This same phenomenon has been studied recently by J.P. Reilly [3] and, according to him, the red spots may be the consequence of stratum corneum degradation due to thermal damage. However, while thermal Joule heating effects cannot be ruled out, it is also quite probable that those red spots were in fact caused by damage to the capillaries by irreversible electroporation; two facts would support this hypothesis: 1) erythemas are common in skin electroporation [4, 5] and 2) it does not seem likely that the electrical generators at the time, (static electricity generators having been invented by Otto von Guericke in 1663), should be able to cause significant heating.

The interest in the science of electricity in biological materials emerged throughout the 18th century. As a matter of fact, discoveries in electricity and physiology were interlinked at those dates. In a series of experiments started in 1780, Luigi Galvani discovered that when a dead frog was placed on an iron grating and a bronze hook touched the spinal cord the frog's muscle twitched. His explanation to the phenomenon was based on what he called "animal electricity". However it was Alessandro Volta who found the correct explanation: the presence of two different metals in the same electrolyte (frog's body fluids) had created a DC current that had stimulated the frog's muscles. That lead to the invention of the voltaic pile (he replaced the frog's fluids by brine-soaked paper), which was the first device able to produce steady electric current and that became a basic element for later discoveries in electromagnetism.

In 1802, J.W. Ritter, who did a significant number of experiments and discoveries in electrophysiology, observed what was later called *Ritter's opening tetanus*: a contraction that occasionally occurs when a strong current passing through a stretch of muscle-nerve preparation is interrupted [6]. This phenomenon was not understood at the time but, as it will be discussed later, it was hypothesized in the mid 20th century that it was due to the "breakdown" of the cell membrane and such hypothesis leaded to the discovery of the electroporation phenomenon as we know it nowadays.

What may be perhaps the first work focusing on an irreversible electroporation phenomenon can be found in the 1898 study by G.W. Fuller entitled "Report on the investigations into the purification of the Ohio river water at Louisville Kentucky." [7]. In his report, an experiment is mentioned in which multiple high voltage discharges have

some bactericidal effect on a water sample. Temperature was found not to increase significantly because of the treatment. Electrolytic effects cannot be completely ruled out (overall the pulses are applied for several minutes). However, on the basis of the way in which irreversible electroporation is currently used for sterilization of fluids, Fuller's reported bactericidal effect is most likely due to irreversible electroporation.

Towards the end of the 19th century, therapeutic uses of electricity are frequently reported in the medical literature. Most applications are based on thermal effects or on electrochemical phenomena. Use of short strong pulses is not cited. However, a book by A.D. Rockwell [8] reports experiments performed during the late 1800s in which "Under the discharges of the Leyden jar the red corpuscles (of the blood, i.e. red blood cells) change their shape and lose their color". This is probably a description of hemolysis induced by irreversible electroporation [9]. Leyden jars were the original capacitors and they were used to accumulate the charge generated by the electrostatic generators so that it was possible to produce high-voltage and high-current short pulses as it is required for electroporation of cells in suspension.

As a conclusion for this brief review on irreversible electroporation during the 18th and 19th centuries it can be said that some effects that we now know to be the consequence of IRE had been already observed before the beginning of the 20th century. Nevertheless, it seems that no explanation based on an increase of cell membrane permeability or based on membrane rupture was proposed.

First half of the 20th century

Observations on the effects of electrical fields on tissue made in the early 20th century can, in hindsight, be related to electroporation. In a 1913 set of lectures, A.J. Jex-Blake reviews knowledge on the lethal effects of human made electricity and lighting [10]. He notes that burns observed in industrial accidents with electricity are related to thermal effects whereas electrical injuries from lighting do not seem to be always from thermal origin. At the present it is accepted that some of the injurious effects of lighting are caused by irreversible electroporation [11].

An extraordinary non-lethal effect of lighting on humans is the emergence of red Lichtenberg figures on the skin that disappear in a few days. Quite probably these figures have the same origin than Nollet's red spots ("It is thought that they represent red blood cells extravasated into the superficial layers of the skin from capillaries secondary to the dielectric breakdown of the skin and subsequent massive electron shower" [12]).

Jex-Blake also cites works of the XVIII century in which animals were recovered from heart failure induced by electrical current (fibrillation term not coined at the time) by

means of another electrical shock. In the context of electroporation this is relevant because now it is thought that electroporation plays a significant role in defibrillation [13].

In the 1930s the thermal effect of electrical fields on biological materials was well established, (e.g. [14, 15]). The 1936 report of G.M. McKinley [16] is relevant to this review in the sense that, from his own observations and from experiments performed during the 20s and 30s by other researchers, McKinley concludes that damage caused to living tissues by high frequency fields (10 to 100 MHz) cannot be only from thermal origin, particularly in the case of nervous tissue. He even proposes that this special "agent" associated with the electrical field can be used as a minimally invasive ablative method that will be selective to some specific tissues. However, in his study using chick embryos, there is not enough methodological data to conclude that electroporation was being performed. In fact his description of inductive current effects suggests that this may be a thermal effect. Nevertheless, the paper proposes that electrical fields produce other mechanisms of damage to biological cells in addition to thermal. Therefore, it is quite likely that attempts to understand the nature of these mechanisms were made in the ensuing years.

As indicated earlier, in 1951 A.L. Hodgkin [17] proposed that the Ritter's opening tetanus phenomenon is associated with " …(the breakdown)… of the insulating properties of the membrane … under the influence of the abnormally high potential difference". It is obvious that this explanation describes a phenomenon akin to what is now referred to as irreversible electroporation. The wording is suggestive of the concept of breakdown of a cell membrane viewed as a dielectric layer. As a matter of fact, the notion that the cell membrane could be modeled as a thin dielectric layer had appeared quite earlier. For instance, in 1925, H. Fricke [18] was able to hypothesize a reasonable value for the membrane thickness (30 nm instead of the actual 7 nm) by analyzing the passive electrical properties of red blood cells under the assumption that cell membrane acts electrically as a thin dielectric layer. Once the cell membrane was viewed as a thin dielectric it was reasonable to expect that some sort of dielectric rupture phenomenon could exist in the case of living cells as it happens in most dielectrics. A common breakdown mechanism in dielectrics is the *avalanche breakdown*: when the dielectric is subjected to a sufficiently high electric field, some bound electrons are freed, accelerated and then those electrons can liberate additional electrons during collisions in a process that leads to a dramatic conductivity increase and, in some cases, to permanent physical damage of the dielectric material. Now it is accepted that electroporation is not due to dielectric rupture by electron avalanche [19] but the idea that membrane breakdown could be caused by excessive transmembrane voltage surely helped to understand some experimental observations that are related to electroporation.

These highlights from research in the first half of the 20th century suggest that during this period further observations on the effects of electrical fields on biological materials were made that were consistent with the phenomenon of irreversible electroporation. The concept that the cell membrane is a dielectric and that it can irreversible breakdown under the application of an electrical field seems to have become accepted. Perhaps the central characteristics of the findings relevant to irreversible electroporation during this period is the realization that while electricity can induce damage to biological materials through thermal effects there is also another mechanism associated with electricity that induces damage and which is not thermal. In addition evidence seems to be building that electrical fields can produce irreversible damage to the cell membrane.

1950 to 1970

Considering that research on nerve stimulation with electricity was done since Galvani, it should not be surprising that the first systematic work on what is now known as irreversible and reversible electroporation was done on nerves. In 1956, B. Frankenhaeuser and L. Widén publish a study that attempts to explain the phenomenon of *anode break excitation* [20]. The anode break excitation phenomenon is described as the finding that that there is a change in the normal nerve conductivity behavior when electrical pulses are applied on nerve nodes with (in their study) amplitudes that are up to ten times the normal threshold and the pulse duration increased from less than 1 ms to more than 100 ms. Frankenhaeuser and Widén state that the actual phenomenon is known since, at least, 1898 [21]. In an explanation to the phenomenon, which is indicative of reversible and irreversible electroporation, they write "It is, therefore, concluded that the strong (electrical) shock damaged to a large extent the nodes… and that these nodes were more or less inactivated". In addition they write "It may be concluded that the effect caused by the strong shock is to a fair extent reversible." It should be emphasized that several prior studies, including that of Hodgkin [17], are brought in support of their conclusion.

R. Stämpfli produced, between 1950 and 1960, in collaboration with A. F. Huxley and others, a series of studies, describing irreversible and reversible electroporation on a frog nerve membrane. In a paper entitled "*Membrane potential of a Ranvier Node measured after electrical destruction of its membrane*" Stämpfli and Willi [22] write: "We had confirmed the observation of Frankenhaeuser and Widén [20], showing that anode break excitation in myelinated nerve can be elicited by strong positive pulses. We were able to show that such pulses produce a breakdown of membrane resistance and potential, if they increase the membrane potential by 70 to 110 mV, which corresponds to a voltage gradient of approximately half a million V/cm across the membrane. If only one short pulse is given the membrane recovers immediately after the breakdown like an

electrolytic condenser. If very strong positive pulses of the order of 10 V are applied, the membrane is destroyed irreversibly." In a further study with a single insulated Ranvier node of a frog nerve fiber, entitled "*Reversible electrical breakdown of the excitable membrane of a Ranvier node*", Stämpfli reports that 5 second pulses which induce membrane potentials of about 120 mV to 140 mV (corresponding to voltage gradients across the membrane of roughly half a million V/cm) can cause the breakdown of the membrane resistance. Under certain conditions this breakdown is irreversible whereas in others it is reversible [23]. He describes the phenomenon as akin to the breakdown of the dielectric field of a capacitor. The observed reversibility is attributed to the fact that in his particular device the electrical field is stopped at the onset of the breakdown of the membrane and therefore the membrane can recover.

Throughout the history of irreversible electroporation the field has advanced with research carried out in parallel in the area of biomedicine and in the area of food processing technology. In food technology the irreversible electroporation topic is referred to as *Pulsed Electric Field* processing or *Electroplasmolysis* in reference to the lysis of cell membranes in tissue, for extracting their contents, or the bactericidal effect in treatment of fluids. The bactericidal non-thermal effects of electrical fields, reported first in 1898 [7], remained an area of research in the food industry through the first and second half of the 20^{th} century and until today [24]. However, apparently during this period it was not obvious to researchers whether electric fields had a bactericidal effect beyond those expected from thermal or electrochemical causes. For instance, A.J.H. Sale and W.A. Hamilton, who produced seminal papers in electroporation in 1967 (see next paragraph), cite a review published in 1949 that finds accounts both for and against such non-thermal effects of electric fields [25] and the same author of the report, H. Burton, publishes a letter in *Nature* in 1950 [26] in which he opposes the conclusions from a previous paper in *Nature* in which it is claimed that it is possible to destroy a large proportion of bacteria in a liquid suspension at sub-lethal temperatures by the application of high radiofrequency electric fields [27]. Nevertheless, in 1961, H. Doevenspeck [28] describes commercial installations using electrical pulses to break apart cellular components for industrial food related processing of animal meat through non-thermal means, which resemble irreversible electroporation. These involve the electrical discharge of electrical pulses from carbon electrodes through the treated material. It should be emphasized that the paper does not specifically refer to the breakdown of the cell membrane. Neither does it provide specific values for the electrical pulses used. However, the outcome reported is clearly non-thermal ablation of the cell membrane. Furthermore, Doevenspeck also reports results showing that these electrical pulses can inactivate micro-organisms with what he considers a non-thermal effect producing a small increase in temperature of at most 30 °C.

The interest in the so-called "bactericidal action of electrical fields" motivated three outstanding and seminal papers by Sale and Hamilton which set the basis for the field of irreversible electroporation and contain the ingredients of many of the future studies in electroporation in general [29-31] . The goal of the first of the three papers was to demonstrate that high field DC electrical pulses can kill cells without a thermal effect. They evaluated the non-thermal bactericidal effect by using ten DC electrical pulses that were very short, between 2 to 20 μs, and separated by long intervals of seconds, to minimize the temperature rise. A systematic study with several types of bacteria and two species of yeast demonstrated that the effect is not related to the stage of growth of the cells, the pH, electrolysis or heating. The measured temperature raise was at most 10 °C. They concluded that the parameters which affect cell killing are, in order of importance, electrical field magnitude first and then the time extent the field is applied. The electrical fields required to completely ablate the cells were found to be quite substantial, (e.g. 6 kV/cm for Saccharomyces cerevisiae and 16 kV/cm for Escherichia coli).

In the second study [30] Hamilton and Sale try to elucidate what is the mechanism by which the electric field pulses kill the cells and they conclude that the irreversible loss of the membrane's function as a semipermeable barrier is what causes cell death. The paper reports leakage of Escherichia Coli cell content in the medium, detected with spectroscopy, as a measure of the loss of cell membrane integrity. It further demonstrates membrane damage leading to the lysis of erythrocytes and protoplasts and suggests that "the electrical field causes an irreversible loss of the membrane's function as the semipermable membrane between the bacterial cell and its environment and that this is the cause of cell death." Electron microscopy of Escherichia Coli and erythrocytes shows that the complete breakdown of the membrane did not occur and suggests that the damage resulting from the DC pulse is confined to particular areas that were not identified.

In the third paper [31] Sale and Hamilton show that the electric field magnitude for inducing the lysis of various organism ranges from 3.1 kV/cm to 17 kV/cm (fields for 50 % population lysis with a protocol of 10 pulses of 20 μs) whereas the equivalent induced transmembrane voltages only range from 0.7 V to 1.15V. And after this result, they suggest that the transmembrane potential induced by the external field may cause "conformational changes in the membrane structure resulting in the observed loss of its semipermeable properties".

In order to compute the induced transmembrane voltages, Sale and Hamilton employed a model in which the cell was considered to be a conductive sphere isolated from the external conductive medium by a thin dielectric layer. Then they obtained equations that were derived from the equations already described by J.C. Maxwell for calculating the conduction through a suspension of spheres [32, 33]. The transmembrane voltage (V_m) has a maximum at the poles facing the electrodes (i.e. direction of the electric field, **E**)

and its value at those two points is $V_m = (3/2)\times a\times|\mathbf{E}|$ where a is the radius of the cell. At any point of the cell the transmembrane voltage is $V_m=(3/2)\times a\times|\mathbf{E}|\times\cos(\theta)$ where θ is the polar angle measured from the center of the cell with respect to the direction of the field, this expression is usually referred to as the *Schwan's equation* [34].

In summary, at the end of the 1960s it was known that electrical pulses have a permeabilizing effect on the cell membrane, which can lead to cell lysis through an effect that is non-thermal. It was also known that certain electrical pulses can cause reversible breakdown of the cell membrane. Most of the studies in the field were carried out with neurons or in relation to food processing.

1970 to 1990

The 1970s and 1980s produced some of the key advances in the field of reversible electroporation which brought this field into the mainstream of biotechnology and medicine. The research during these two decades was primarily in the field of reversible electroporation and focused on developing new uses and fundamental understanding of the mechanisms. Since reversible electroporation is not the focus of this review we will discuss only some of the highlights.

In their 1972 paper, E. Neumann and K. Rosenheck, apparently unaware of previous studies on electroporation, show that electrical impulses of about 18 to 24 kV/cm and about 150 μsec long produce reversible permeabilization of the cell membrane of chromaffin granules of bovine-medullary cells used as vesicles for epinedrine, norepinephrine, ATP and proteins [35]. Experiments done at 0 °C show that the largest increase in temperature is 6 °C and that the observed effect of reversible permeabilization is therefore not thermal. However, although all their experiments seem to involve reversible electroporation, Neumann and Rosenbeck fail to recognize that the increase of permeabilization is due to an extreme transmembrane potential induced by the external field and they try to relate such phenomenon to the physiological release of hormones and neurotransmitters in neurons.

From observation of discrepancies in the readings of an electrical Coulter counter for cells, U. Zimmerman and his group, also apparently unaware of previous studies by Sale and Hamilton, determined that the electrical field in the counter induces cell membrane breakdown. The methodology they developed, which combines experimentation in Coulter type counters and between parallel plates with the solution of the Laplace equation has produced some of the first systematic data on the electrical parameters required for electroporation in cells. Working with human and bovine red blood cells they explored the dependence of cell membrane breakdown, as expressed by the presence of intracellular contents in the extracellular solution, on increasing pulse length and

amplitude. They found find that the maximal content is reached asymptotically when the pulse length reaches about 50 to 100 μsec and the electrical field strength of about 2.6-2.8 kV/cm. The critical membrane potential difference leading to membrane breakdown was found to be about 1 V. Results from this group of studies were published in a series of papers, since 1974, e.g. [36, 37]. It should be emphasized that the data in this series of papers is extremely relevant to irreversible electroporation parameters. These parameters are the asymptotic values listed in these papers. It is interesting to note that they found different asymptotic values for human and bovine red blood cells, which may suggest the possibility for differential irreversible electroporation in applications in tissue. They also showed that these effects are not thermal. One practical outcome of their work is the suggestion of employing erythrocytes and lymphocytes as drug and enzyme carrier systems.

In 1977, K. Kinosita and T. Tsong, [38] proposed that the permeabilization of the cell membrane due to the application of electrical pulses is related to the formation of several pores with radii in the range of a few angstroms. In classical osmotic mass transfer experiments with red blood cells they showed that the size of these pores can be varied and that these pores eventually reseal. They also propose the use of permeabilized cells as reservoirs for transport of chemical species through the body circulatory system. In evaluating the time it takes for the cells to reseal as a function of temperature, they find that "At 37 °C the treated membrane rapidly regains its impermeability to cations, whereas, at 3 °C the cells remain highly permeable even after 20 h." To us this suggests that lowering the temperature may produce an effect akin to irreversible electroporation even when using reversible electroporation parameters.

In 1978, S.V. Belov wrote a paper that it is never cited but that may be relevant in the context of irreversible electroporation as it is probably the first case in which IRE of living tissues is intentionally pursued [39]. In an investigation of coagulation type electrosurgical devices he suggests that surgical coagulation is actually related to cell membrane breakdown due to pulses that have a "high ratio of peak to mean voltages". As a reference to the observation that high ratio of peak to mean voltage produce cell membrane breakdown he mentions a 1938 paper by Tatrinov[1]. Belov's research was performed on frog leg muscle. Histological analysis and measurements of changes in electrical resistance were used to show that 500 pulses of about 8.5 kV/cm and pulse length of 1 μsec and 1000 pulses of about 7.5 kV and pulse length of 2 μsec cause the destruction of the cell membrane in tissue, without producing a thermal effect.

The year 1982 brought about two major discoveries which have led reversible electroporation to the forefront of modern biotechnology and medicine: cell fusion with

[1] V. V. Tatarinov, Arkh. Biol. Nauk, 52, No. 2, 173-177 (1938). Unfortunately we were not able to locate this paper.

reversible electroporation and introduction of genes into cells using reversible electroporation. The use of reversible electroporation to produce fusion between cells is described in the paper by Zimmermann [40]. A more recent review on cell fusion is by C. Ramos and J. Teissie [41]. In a now classical paper Neumann and his collaborators coin the term *electroporation* to describe the membrane breakdown discussed in this review and introduce the use of reversible electroporation for the insertion of genes into cells [42]. They also present a classical thermodynamic analysis of the formation of pores during electroporation. In relation to the field of irreversible electroporation, it is interesting to note that this paper which focuses on reversible electroporation, naturally, caution against the use of irreversible parameters during the procedure. The reason we bring this up here is because during the two decades following this work, irreversible electroporation was studied primarily as an upper limit to reversible electroporation in the context of applications of reversible electroporation. The researches in the application of reversible electroporation have led to numerous other publications in that field, whose review is not within the scope of this paper.

While until the mid 1980s the research on electroporation was primarily related to cells (with few exceptions, [28, 39]) between 1987 and 1989 the research on electroporation began to deal with tissue. During the late 1980s it pursued three important directions, one of which is directly related to irreversible electroporation.

M. Okino and H. Mohri [43] and S. Orlowski et al., [44] proposed , independently, the use of reversible electroporation to reversible permeabilize cells and thereby introduce more effectively cytotoxic agents into malignant cells, for treatment of cancer. This field has subsequently developed to become an important application of reversible electroporation to treatment of cancer.

In 1989, by performing electrical conductance measurements K.T. Powell et al. [45] demonstrated that frog skin can be reversible electroporated. They did not report how that could influence the passage of drugs through the skin but quite probably this paper was influential on the posterior discovery by M.R. Prausnitz et al. [46] in which transdermal drug delivery was enhanced by electroporation.

An important series of studies in the field of irreversible electroporation of tissue began with the 1987 [47] and 1988 [11] papers of R.C. Lee and his co-investigators on electrical discharge induced trauma in tissue. Lee et al write “While it seems likely that Joule heating causes part of the tissue destruction (due to electrical discharge), particularly near the skin contact points, it does not appear to explain the patter of tissue injury frequently observed at sites distant from contact.” . The experiments in [11] were performed on rat muscle cells exposed to electrical fields of 20 to 300 V/cm for periods of 1 millisecond in 30 seconds intervals and 25 V/cm increments. The temperature of the system was monitored during the study. Dissected whole rat muscles were given thirty 1 ms 150

V/cm pulses separated by 5 s. The electrical resistance of the muscle was measured before and after the sequence of pulses. The results show that the cells were irreversible damaged with pulses varying from 50 to 300 V/cm and from 1 to 30 pulses (there are no more precise details), with a temperature rise of less than 0.1 °C. The results with the tissue showed a decrease in electrical resistance following the application of the pulses, which is suggested to be a measure of tissue damage. The non-thermal nature of this damage is proven by measuring an increase in temperature of only 0.7 °C. The study also states that in experiments in which a 100 V/cm electrical field was applied for 1 s a 10 to 20 °C temperature increase is expected. They write, "In this case, cells would be vulnerable to both thermal and nonthermal injury mechanisms. Under these conditions the primary cause of cell rupture would have to be determined since both electroporation and heating may lead to disruption and both may be cytotoxic."

In summary, these two decades end with reversible electroporation and electrofusion becoming important biotechnological and medical tools in cell research and use. A review book that summarizes the state of the art at the end of this period is available [48].

1990 to 2000

In the nineties, many of the ideas related to reversible electroporation that were conceived in the 70s and 80s reach enough maturity for commercial and clinical applications. We will briefly summarize some of those achievements by referencing books and articles, however, herein after we will focus mostly on aspects related exclusively to irreversible electroporation.

After the design by H. Potter et al. [49] of an electroporation cuvette suitable for cells suspensions, microbiology researchers started to employ electrophoresis power supplies in order to perform gene transfection by electroporation. Soon after, multiple commercial generators specifically intended for electroporation were developed and now this transfection technique is very common in microbiology laboratories. Summaries on the technique and its applications can be found in several edited books, e.g. [48, 50].

The first report on the use of reversible electroporation to introduce plasmid DNA into a living tissue was published in 1991 by A.V. Titomirov et al. [51]. Gene delivery to cells in tissue has also become an area of major importance to biotechnology and medicine in which reversible electroporation plays a central role. It has also found applications in treatment of cancer, e.g. [52, 53]. Some of the reviews and edited books written on this topic include [54-56].

In 1991 the group of L.M. Mir published two breakthrough papers on the use of reversible electroporation to treat cancer by facilitating the penetration of anticancer

drugs, such as bleomycin, in the malignant cells. They coined the term *electrochemotherapy* to describe this procedure [57] and reported the first clinical trial in the field of electroporation [58]. Electrochemotherapy is now one of the most solid applications of reversible electroporation and it is being used clinically to treat cancer patients. Probably the most updated review information on the topic can be found in [59-61].

During this decade, skin electroporation and its use for drug delivery emerged as an important aspect of reversible electroporation of tissue. The study that established skin electroporation for transdermal drug delivery was published in 1993 [46], [4]. Multiple reviews have been written on this application of tissue electroporation, e.g. [62].

The study of the contribution of irreversible electroporation to tissue damage during electrical shock trauma continued since it was first proposed in the 80s and was led primarily by R.C. Lee [63-65]. An interesting new aspect of this research is the suggestion that irreversible electroporated cell membranes could be therapeutically sealed with surfactants [66].

Related to the above paragraph it is convenient to note that most researchers cite necrosis due to excessive permeabilization and consequent disruption of the osmotic balance as the killing mechanism of electroporation. However, in the late 90s, two independent papers were published in which it was shown *in vitro* that electroporation not only caused necrosis but it also induced cell death with features compatible with apoptosis [67, 68]. In both papers it is reported that electroporation leads to chromosomal DNA fragmentation, which is considered to be an unambiguous indication of late apoptosis. Another interesting outcome of the paper by J. Piñero et al. [67] is the following statement written in the abstract: “The possibility of killing tumour cells by electroporation, as a variant of electrotherapy, constitutes, in our opinion, a promising procedure in cancer therapy, avoiding the undesirable side effects normally derived from treatment with cytotoxic drugs.” Quite surprisingly this particular topic is not further developed in the manuscript. In the introduction the authors refer to the use of electrochemotherapy for treating solid tumors and, therefore, we must suppose that the comment in the abstract was actually proposing the use of irreversible electroporation for treating tumors. As far as we know this would be the first time irreversible electroporation is proposed as an ablative method.

In 1998 [69] it was reported a remarkable phenomenon observed during tissue reversible electroporation: blood flow is blocked in the area where the electric field is applied. This phenomenon, referred to as *the vascular lock* [70], has been noticed in muscle [70], liver [69] and tumors [71]. Blood perfusion disruption affects kinetics of drug delivery [70] and is followed by ischemia, which could be beneficial in the treatment of tumors [72]. In fact, it has been proposed the use of electroporation to intentionally interrupt blood flow [73, 74]. J. Gehl et al. [70] suggested two mechanisms that would explain why such

vascular lock is produced: 1) the electrical stimulus, or the permeabilization, induces an immediate reflex vasoconstriction of afferent arterioles mediated by the sympathetic system and 2) the permeabilization of endothelial cells causes an increase of the interstitial pressure and a decrease of the intravascular pressure that leads to vascular collapse.

To sum up, the last decade of the 20th century witnessed the consolidation of reversible electroporation as a standard microbiology laboratory technique and the first animal and clinical tries for using reversible *in vivo* were successfully performed. Irreversible electroporation was well characterized but, in biomedical applications, it was perceived as a drawback, or complication, of reversible electroporation.

2000 to 2008

In 1997 K.H. Schoenbach et al. [75, 76] reported the first *in vitro* study on the use of very high voltage pulses of "submicrosecond" duration. Numerous papers have been published since then on the use of pulses with a duration of some nanoseconds or tens of nanosecond [75-80]. The main motivation for this research line came from the believe that those ultra-short pulses, known as *nanosecond Pulsed Electric Field* (nsPEF), could be able to induce electroporation of intracellular membranous structures (e.g. mitochondria) without disturbing the cell membrane. In *conventional* electroporation, that is, with pulses larger than 10 μs, the cell membrane is charged up to a stable transmembrane potential in very few microseconds and the intracellular structures become isolated from the external field and hence cannot experience electroporation. On the other hand, since the charging time constant is proportional to the structure size, it seems reasonable that if very short pulses of high magnitude are applied then the small intracellular membranous structures will be charged to a sufficiently high voltage for electroporation before the cell membrane is barely charged. That is the reason why it was expected that it would be possible to electroporate internal structures without causing electroporation of the plasma membrane. However, recent computer models [81] and experimental results [82] indicate that cell membrane electroporation also occurs when nanosecond pulses are applied. That is, with nsPEF all the membranous structures of the cell are electroporated (some authors refer to this phenomenon as *supraelectroporation*). Still, it is important to point out that researchers in this field found out that nsPEF can induce apoptosis [83] and that they even partially succeeded in inhibiting tumor growth after nanosecond pulses were applied *in vivo* [84, 85].

In 2004, perhaps motivated by the results that researchers in the nsPEF field had been obtaining, C. Yao et al. [86] investigated the use of special pulses, to which they referred to as *Steep Pulsed Electric Field* (SPEF), for killing cells and for *in vivo* tumor growth

inhibition. Those SPEF pulses consisted of a fast rising edge (rise time ~ 200 ns) followed by a slow exponential decay (τ ~ 200 μs) which was originated from a capacitance discharge. The authors wanted to combine the intracellular effects from the "high-frequency components" of the rising slope together with the plasma membrane effects from the "low-frequency components" of the falling slope so that it was possible to "destroy both nucleus and membrane". And indeed they succeeded in killing cells and delaying tumor growth. However, taking into account the low amplitude of the applied pulses and the fact that a rise time of 200 ns is quite standard in electroporation, we believe that what they induced and observed does not differ from the effects of *conventional* IRE. Therefore, despite their unawareness, it is very likely that these researchers were the first ones to obtain empirical evidences of the capabilities of IRE as a tissue ablation method for tumors.

Also in 2004, R. Davalos and B. Rubinsky filled a US patent application [87] which followed a provisional application filled in 2003 and that proposed the use of conventional IRE (pulses longer than 5 μs) as a tissue ablation method. In the patent publication, the inventors detail methods for treatment planning by adjusting the electrode configurations and the applied voltages. They also point out the fact that IRE can be easily applied in areas where perfusion is high (e.g. in the vicinity of blood vessels), as opposed to the case of thermal methods for ablation. Nevertheless, probably the most essential aspect of this application, and of the research carried out immediately afterwards by the same authors [88], is the recognition that IRE is an ablation method that is not necessarily accompanied by thermal effects and that such feature has important implications in post-treatment healing.

The subsequent research efforts carried out by Rubinsky's group were aimed at treating hepatocarcinomas by means of irreversible electroporation. First it was shown *in vitro* that IRE is capable of killing human hepatocarcinoma cells [89]. Afterwards it was demonstrated that IRE can ablate selectively areas of non-pathological rodent livers [90]. In this later study the histological assessment three hours after pulses were applied showed some interesting features: the treated areas exhibited microvascular occlusion, endothelial cell necrosis and diapedeses, resulting in ischemic damage to parenchyma and massive pooling of erythrocytes in the hepatic sinusoids. Hepatocytes displayed blurred cell borders, pale eosinophilic cytoplasm, variable pyknosis and vacuolar degeneration. On the other hand, large blood vessel architecture was preserved.

The last published report of this series of studies on liver ablation by IRE was performed on pigs under experimental conditions relatively close to those of a clinical scenario [91]. After exposing the liver, IRE was applied by using 18 gauge stainless needles that were positioned with the assistance of ultrasound sonography. Previously, the size and shape of the lesions had been designed by means of a treatment planning procedure that will be briefly explained later in the context of the paper by J. Edd and R. Davalos [92].

Following the surgical procedure, animals were sacrificed at 24 hours, 3 days, 7 days and 14 days and liver samples were excised for histopathological analysis. Multiple interesting observations and conclusions emerged from this study: 1) all the 14 animals survived the procedure; 2) upon application of the IRE pulses a variable degree of generalized muscle contraction occurred in each animal and such degree appeared to be related to the administered amount of muscle relaxant (Pancuronium); 3) immediately following pulse application, sonography showed a markedly hypoechoic area in the expected location of the IRE lesion and, at 24 hours, the ultrasound image showed the area had permuted its quality and was now uniformly hyperechoic; 4) histological analysis showed that the IRE ablated area was continuously necrotic and that the transition between this area and the adjacent untreated normal parenchyma was abrupt; 5) macroscopic histological analysis also showed that large vascular structures were mainly unaffected; and 6) all animals manifested peripheral lymphadenopathy in the drainage area of the ablated tissue.

This last paper by Rubinsky et al. [91] on the ablation of pig liver is included in a special issue of *Technology in Cancer Research and Treatment* (TCRT, August 2007) devoted to irreversible electroporation. It is worth to briefly summarize here the results and conclusions communicated in some of the other papers included in that issue:

1) E. Maor et al., from Rubinsky's group, presented the results from a pilot study in which IRE (ten 100 μs pulses of 3800 V/cm) was applied to the carotid artery of rats that were kept alive for 28 days after the procedure [93]. Histology showed that the connective matrix of the blood vessels remained intact whereas the number of vascular smooth muscle cells (VSMC) was decreased very significantly without pathological observable consequences such as aneurysm, thrombus formation or necrosis. These findings seem to indicate that IRE can be applied safely to the vicinity of large blood vessels. Moreover, the fact that VSMC population was significantly reduced suggests that IRE could be the basis for treating pathologies such as restenosis and atherosclerotic processes. As a matter of fact, Maor and co-investigators are now performing research on the use of IRE for treating cardiac restenosis [94].

2) G. Onik et al, also from Rubinsky's group, applied *in vivo* IRE to canine prostates by means of percutaneous needle electrodes placed under ultrasound guidance [95]. Macroscopic observation of the induced lesions revealed a very distinct narrow zone of transition from normal to complete necrosis. Nearby structures such as urethra, vessels, nerves and rectum were apparently not affected by the IRE procedure despite the fact that the areas covered by the high electric field purposely included those structures. This study was preceded by an *in vitro* study in which prostate adenocarcinoma cells were destroyed by IRE [96].

3) E.W. Lee et al. reported an experimental study on pig livers which is quite similar to the one presented by Rubinky et al. [91] but in this case the electrodes were inserted percutaneously, without exposing the liver, and the histological samples were analyzed with apoptotic markers. Equivalent observations were obtained (lesion manifestation by ultrasonography and sharp transition zone between ablated and normal tissue). Regarding the histological analysis the authors conclude: "… confirmed complete apoptotic cell death by PIE (i.e. IRE) on Von Kossa, BAX, and H&E staining. In summary, PIE can provide a novel and unique ablative method with real-time monitoring capability, ultra-short procedure time, non-thermal ablation, and well-controlled and focused apoptotic cell death."

4) B. Al-Sakere et al. [97] performed *in vivo* IRE of tumors subcutaneously inoculated in mice and studied the immune reactions. The objective of the study was to elucidate what was the role of the immune system in the ablation of tumors by means of IRE. Their main conclusion is that the immune system response is not required in order to successfully ablate tumors by IRE and, therefore, IRE is a feasible option to consider for the treatment of immunodepressed cancer patients. This study was preceded by an investigation of multiple electroporation protocols that lead to complete tumor regression of tumors inoculated in mice [98]. Best results were obtained by using a protocol that consisted of 80 pulses of 100 μs at 0.3 Hz (an interval of 3.3 seconds between pulses) with a field magnitude of 2500 V/cm. With this protocol complete regression was achieved in 12 out of 13 treated tumors and no thermal effects were induced.

5) J. Edd and R. Davalos described how mathematical modeling aided by computer methods can be employed to predict the shape and extent of the lesion created by IRE [92]. The basic principle for such modeling is that any specific tissue region is electroporated if the electric field magnitude is higher than a certain value. Such threshold is specific to the sort of tissue and the features of the IRE pulses (e.g. number of pulses and duration of the pulses). Once this threshold is experimentally obtained, by using numerical methods on computers it is possible to predict the distribution of the field magnitude in a tissue according to the electrode configuration and the applied voltages to those electrodes. This methodology for treatment planning was pioneered before for the case of *in vivo* reversible electroporation by D. Miklavcic's group [99]

Also in 2007, J. Lavee et al. published a study with five pigs in which IRE of the atrium was performed in order to analyze its applicability for the treatment of atrial fibrillation as an alternative to methods based on thermal ablation [100]. Again the demarcation between ablated and normal tissue was clear and sharp. Histological analysis demonstrated complete destruction of atrial cells down to a mean depth of 0.9 cm. The lesions manifested electrical isolation. The authors conclude "we propose and demonstrate here a new and exciting modality to perform atrial ablation, which holds the

potential of providing very swift, precise, and complete transmurality with no local heating effects."

The first commercial system approved for clinical irreversible electroporation of soft tissues started to be produced in 2008 by Angiodynamics, Inc. (Queensbury, NY) under the brand name NanoKnife™. It consists of a high voltage pulse generator [101] and single-use disposable electrodes. Angiodynamics obtained the technology and the intellectual property rights through the acquisition of Oncobionic, Inc. (Irvine, CA) which was founded in part by Prof. Rubinsky.

Finally we want to point out a recent paper by E. Tekle et al. [102] that demonstrates that electroporation induces the exposure of phosphatidylserine (PS) to the outer surface of the cell membrane and that such externalization results in phagocytic clearance of the cells by macrophages. The authors refer to the cells exposed to the electric fields as *apoptosis-mimetic* cells because they consider that those cells are non-apoptotic but exhibit some features typical of cells undergoing apoptosis. This phenomenon is of interest because it could be employed for removal of pathogenic cells through non-inflammatory phagocytosis. Furthermore, this observation could concur with the non-thermal nature of IRE, and the associated preservation of tissue scaffolding, in order to explain the rapid recovery of tissues after an IRE treatment.

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