

Irreversible Electroporation Attenuates Neointimal Formation After Angioplasty

Elad Maor*, Antoni Ivorra, Jonathan Leor, and Boris Rubinsky

Abstract—Restenosis following coronary angioplasty represents a major clinical problem. Irreversible electroporation (IRE) is a nonthermal, nonpharmacological cell ablation method. IRE utilizes a sequence of electrical pulses that produce permanent damage to tissue within a few seconds. **Methods and results:** The left carotid arteries of eight rats underwent *in vivo* intimal damage using two Fogarty angioplasty catheters. The procedure was immediately followed by IRE ablation in four rats, while the remaining four were used as the control group. The IRE ablation was performed using a sequence of ten dc pulses of 3800 V/cm, 100 μ s each, at a frequency of ten pulses per second, applied across the blood vessel between two parallel electrodes. The electrical conductance of the treated tissue was measured during the electroporation to provide real-time feedback of the process. Left carotid arteries were excised and fixated after a 28-day follow-up period. Neointimal formation was evaluated histologically. The use of IRE was successful in three out of four animals in a way that is consistent with the measurements of blood vessel electrical properties. The integrity of the endothelial layer was recovered in the IRE-treated animals, compared with control. Successful IRE reduced neointima to media ratio (0.57 ± 0.4 versus 1.88 ± 1.0 , $P = 0.02$). **Conclusions:** We report for the first time the *in vivo* results of attenuation of neointimal formation using IRE. Our study shows that IRE might be able to attenuate neointimal formation after angioplasty damage in a rodent model of restenosis. This approach may open new venues in the treatment of coronary artery restenosis after balloon angioplasty.

Index Terms—Angioplasty, bioelectric phenomena, biomedical engineering, biophysics, blood vessels, cardiology, electric field effects, electroporation, restenosis.

I. INTRODUCTION

CORONARY artery disease is a leading cause of morbidity and mortality in the Western World [1]. The introduction of coronary angioplasty followed by coronary stent implantation has been shown to reduce both morbidity and mortality associated with ischemic heart disease [2]. Coronary artery restenosis is the major clinical complication following coronary angioplasty with or without stent implantation [3].

Restenosis following angioplasty is a complex process, and its exact mechanism has not yet been determined. Restenosis

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involves both arterial remodeling as well as smooth muscle cell proliferation. Vascular smooth muscle cells (VSMCs) together with their synthetic products elastin, collagen, and other extracellular matrix components play a pivotal role in arterial remodeling, restenosis, and intraluminal loss [4]–[6]. Many of the medical drugs and devices that have been introduced in the last decade have tried to attenuate the restenosis process by inhibiting the hyperplasia of VSMC in the intimal layer. The major breakthrough in recent years has been the introduction of antiproliferative drug-eluting stents, which have been shown to reduce restenosis rates by 70% compared with bare-metal stents [7]. Of late, however, there has been growing concern regarding the safety of this innovative technology [8].

Recently, our group has begun studying the effects of irreversible electroporation (IRE) on different tissues [9]–[13]. IRE is a modality whereby microsecond electrical pulses are applied across the cell to generate a destabilizing electric potential across biological membranes and cause the formation of nanoscale defects in the lipid bilayer. These defects cause the lipid bilayer to lose its semipermeability, thus leading to cell death.

Particular to our approach in using IRE is that the electrical fields are chosen in such a way that damage due to Joule heating is avoided or minimized [9]–[13]. The finding that electrical fields that induce IRE without causing major detrimental thermal effects exists, makes our mode of use of IRE unique among tissue ablation methods. It imparts on our mode of using IRE the ability to affect only the cell membrane without damaging any of the other components of the tissue, such as the extracellular matrix or without causing denaturation of proteins.

Because IRE increases the permeability of the cell membrane, measuring the impedance of the treated tissue and detecting a change provides real-time verification on the successful application of IRE [14]. In a recent paper, we investigated the effect of IRE on intact large blood vessels and demonstrated the ability of IRE to substantially ablate VSMC in a rodent carotid artery [15]. We have also verified that IRE changes the electrical properties of the blood vessels, and therefore, this measurement may serve as real time control for the successful application of IRE.

The purpose of this study was to investigate the effect of IRE on the process of neointimal formation following intimal damage in a rodent model of carotid artery restenosis.

II. METHODS

A. Experimental Protocol

Eight Sprague–Dawley rats weighting 300–350 g were used in this pilot study. All animals received humane care from a properly trained professional in compliance with both the Principles of Laboratory Animal Care and the Guide for the Care and

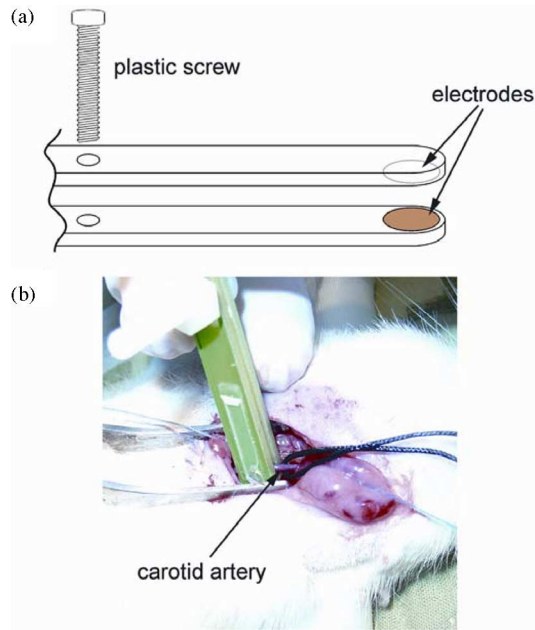


Fig. 1. Custom-made electrode clamp employed to induce irreversible electroporation of the carotid artery. (a) Clamp consists of two printed circuit boards (1.5 mm thickness) with disk electrodes (diameter = 5 mm) made of copper (70 μm thickness) plated with gold (manufacturing process by Sierra Proto Express, Sunnyvale, CA). (b) Clamping of the carotid artery, the distance between electrodes was approximately 0.3 mm.

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Each animal was anaesthetized throughout the procedure. The left common carotid artery was exposed, and intimal denudation was performed as previously described [15], [16]. Briefly, the left external carotid artery was incised, and a 2F Fogarty arterial embolectomy catheter (Edwards Lifesciences) was advanced through the incision to the left common carotid artery. The balloon was inflated and drawn back three consecutive times. At the end of the procedure, the balloon was deflated, extracted, and the left external carotid artery was ligated.

Four rats were used as control, and their skin incision was sutured immediately at the end of the procedure. In the remaining four rats, a custom-made electrode clamp with two parallel disk electrodes (diameter = 5 mm) was applied on the left common carotid artery, very close to its bifurcation to the internal and external carotid arteries, at the exact site of intimal damage (see Fig. 1 for further details). The measured distance between electrodes was approximately 0.3 mm. A sequence of ten dc pulses of 115 V (i.e., electrical field of approximately 3800 V/cm), 100 μs each, at a frequency of ten pulses per second, was applied between the electrodes using a high-voltage pulse generator intended for electroporation procedures (ECM 830, Harvard Apparatus, Holliston, MA). Current and voltage were recorded by means of special oscilloscope probes (current probe was AP015 and high-voltage probe was ADP305, both from LeCroy Corporation). From these two signals, conductance was obtained during the pulses. The procedure was applied in three successive locations along the common carotid artery. At the end of the procedure, the skin incision was sutured

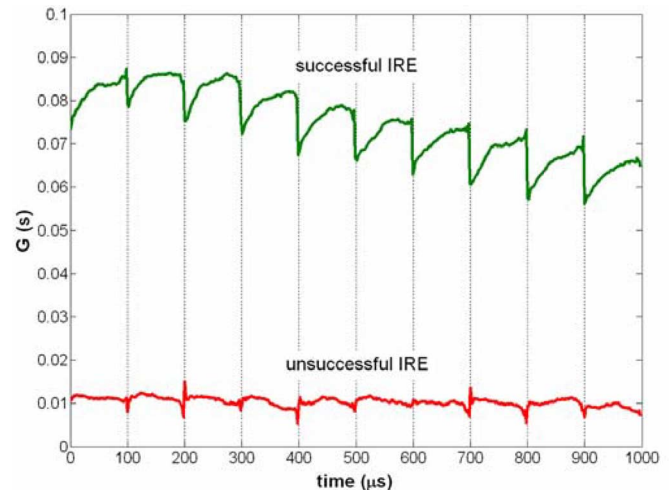


Fig. 2. Examples of conductance of the arterial wall during repetitive dc pulses. Conductance is measured only during the 100 μs pulses, and here, it is displayed without the 100 ms intervals between pulses. Two cases are shown: a trial in which successful irreversible electroporation was achieved, and a case in which the voltage pulses apparently were not able to cause electroporation.

and animals were kept alive for a follow-up period of 28 days until they were euthanized.

B. Histologic Assessment

Animals were euthanized with an overdose of phenobarbital. The arterial tree was perfused with 10% buffered formalin for 40 min, and the left and right carotid arteries were exposed near the bifurcation of the internal and external carotid arteries. One slice of 1 cm from each artery, at the core of the treated area, was used for histological analysis. Each slice was fixed with 10% buffered formalin, embedded in paraffin, and sectioned with a microtome (5 μm thick). One section was stained with hematoxylin and eosin. The endothelial layer was assessed by lectin immunostaining. Each slide was photographed at X200 magnification, and the following areas were measured: tunica media area, neointimal area, and lumen area. The unequal variance *t*-test method was used to evaluate the statistical difference between the measured areas of the two different groups.

III. RESULTS

All animals survived the procedures. Conductance of the arterial wall decreased during successive direct current pulses [Fig. 2(a)]. During the follow-up period, there were no signs of cerebrovascular events (paraplegia, paraparesis, etc.), and there was no mortality.

Conductance was measured during IRE pulses and was used to monitor the successful use of the electroporation device. Successful IRE was assigned to those cases in which significant conductance increase was observed during applied pulses, as depicted in the case shown in Fig. 2. IRE was successful in three of the four animals. There were no changes in conductance during the pulses applied to the fourth animal and this was considered to indicate an unsuccessful IRE (see also Fig. 2). A constant observation in all successful IRE cases was that,

although conductance increased during each pulse, the overall conductance for the whole sequence decreased.

After 28 days, histological analysis was used to compare the IRE-treated and the control group (Fig. 3). Measurements of neointimal area, tunica media area, and arterial lumen area are summarized in Table I. Compared with control (including the one unsuccessful IRE animal), successful IRE induced a significant reduction in the neointima to media ratio (0.57 ± 0.4 versus 1.88 ± 1.0 , $P = 0.02$). In addition, compared with control (excluding the unsuccessful IRE animal), successful IRE induced a reduction in neointimal to media ratio that was less significant (0.57 ± 0.4 versus 1.67 ± 1.0 , $P = 0.06$).

Examples of the endothelial layer in the different animals are shown in Fig. 3. Endothelial layer seems to have well recovered in the IRE-treated animals compared with control group animals. Endothelial integrity was similar in the IRE-treated group to its appearance in the unharmed right common carotid artery (Fig. 4).

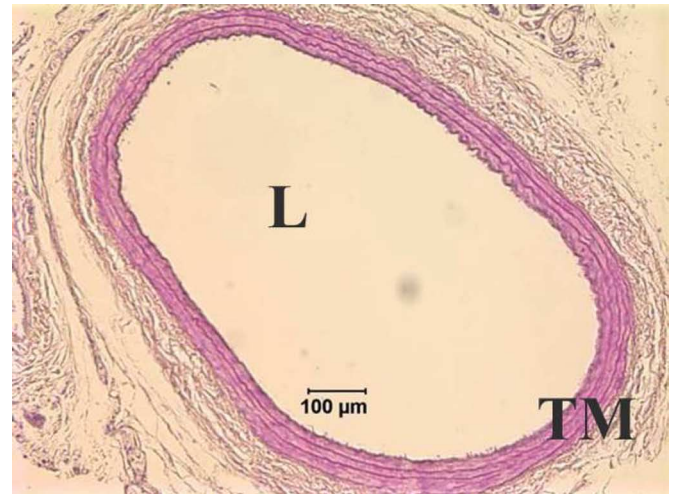
IV. DISCUSSION

To the best of our knowledge, this is the first study showing the ability of IRE to reduce restenosis. There was a tendency toward a lower neointimal formation following successful IRE, compared with control animals. Based on the histological analysis, the extracellular matrix component of the arterial wall was maintained; there was no evidence of necrosis, aneurysm formation, or thrombosis, and there was remarkable recovery of the endothelial layer.

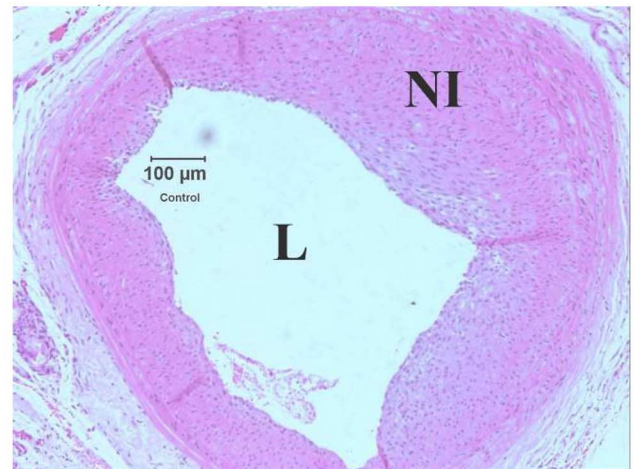
Atherosclerosis, arterial remodeling, and restenosis following angioplasty are complex processes, in which the arterial wall in general, and the vascular smooth muscle cells in particular, play a role [4]–[6]. This study shows that compared with non-IRE-treated controls, there is significant decrease in neointimal formation 28 days after intimal damage in IRE-treated arteries. In a previous study, we showed that in the same model, IRE induced significant reduction in the VSMC population without apparent damage to elastic fibers [15]. Clarke *et al.* [17] investigated the role of VSMC per se in vascular disease. Using transgenic mice expressing human diphtheria toxin receptor on all VSMCs, they showed that apoptosis of 50–70% of the VSMC population in normal arteries induced no endothelial loss, inflammation, reactive proliferation, thrombosis, remodeling, or plaque formation. We believe that by selectively destroying the VSMC population without affecting the extracellular matrix, the specific non-thermal IRE ablation method developed by our group severely reduces the potential ability of neointimal formation, without significant damage to arterial function and overall structure.

To date, different methods to ablate or stop the proliferation of cells in the different layers of the arterial wall have been suggested. These methods include cryoplasty, brachytherapy, photodynamic therapy, drug-eluting stents, and genetic manipulations using gene therapy [8], [18]–[33].

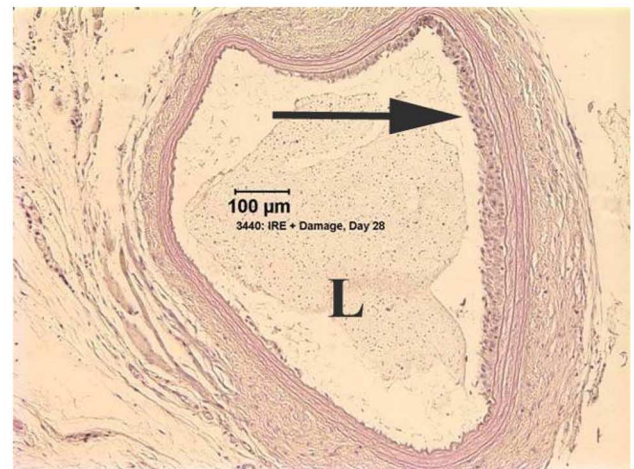
The IRE methodology developed by our group has unique attributes, which may give it advantages over the other methods for reducing restenosis. The nature of the IRE mechanism alone is to produce only nanoscale defects in the cell membrane [34].



(a)



(b)



(c)

Fig. 3. (a) Right common carotid artery. This slide is an example of the appearance of a normal right carotid artery. (L—Intra-arterial lumen; TM—Tunica media). (b) Left common carotid artery, 28 days after intimal damage, showing high neointima to media ratio. (L—Intra-arterial lumen; NI—Neointimal formation). (c) Left common carotid artery, 28 days after intimal damage in an IRE treated rat, showing the scarcity of neointimal formation compared with Fig. 3(b) (L—Intra-arterial lumen; Arrow—Minimal neointimal formation).

TABLE I
MORPHOMETRIC ANALYSIS OF EXPERIMENT SLIDES

Animal #	Intervention	Tunica media area (mm ²)	Neointima area (mm ²)	Lumen area (mm ²)	Neointima/Media ratio	Neointima/Media average ratio*
1	Intimal damage & successful IRE	0.17	0.18	0.27	1.07	0.57±0.4
2	Intimal damage & successful IRE	0.08	0.03	0.32	0.38	
3	Intimal damage & successful IRE	0.16	0.04	0.31	0.28	
4	Intimal damage & failed IRE **	0.08	0.21	0.05	2.69	1.67±1.0
5	Intimal damage alone	0.17	0.17	0.30	0.99	
6	Intimal damage alone	0.11	0.11	0.30	1.01	
7	Intimal damage alone	0.11	0.17	0.35	1.49	
8	Intimal damage alone	0.12	0.38	0.25	3.19	

* P = 0.06.

** Unsuccessful IRE (See text).

In the absence of thermal damage, IRE does not affect connective tissue, the extracellular matrix, nor does it denaturizes proteins [15], [35]. Therefore, the integrity of the extracellular matrix is retained during the process. The extracellular matrix plays an important role in arterial remodeling and in the elastic properties of the arterial wall [36]. One explanation for the absence of aneurysm formation in our study might be that IRE does not damage elastin or collagen within the arterial wall. One of the problems with an intra-arterial stent is the intense extracellular formation in the later stages of restenosis, probably due to the mechanical damage caused by the stent [37]. Because electrical fields can either produce IRE or not, without any gradual modalities of damage, the margins of the treated region are well delineated and do not extent beyond the area of application of the IRE field. Therefore, with IRE, the effect can be achieved only in the area of interest, without collateral damage. IRE is a nonpharmacological method, and therefore, there is less concern regarding allergic reaction or drug safety.

IRE, and electroporation in general, produces nanoscale defects in the cell membrane and thereby facilitates unimpeded ion transport across the membrane [34]. Therefore, successful IRE results in immediate changes in the passive electrical properties of the tissue that can be measured and employed as a feedback mechanism for real-time control of the technique. In fact, within the context of reversible electroporation, such strategy has been described previously for individual cells [38], cell cultures [39], and tissues [40], [41].

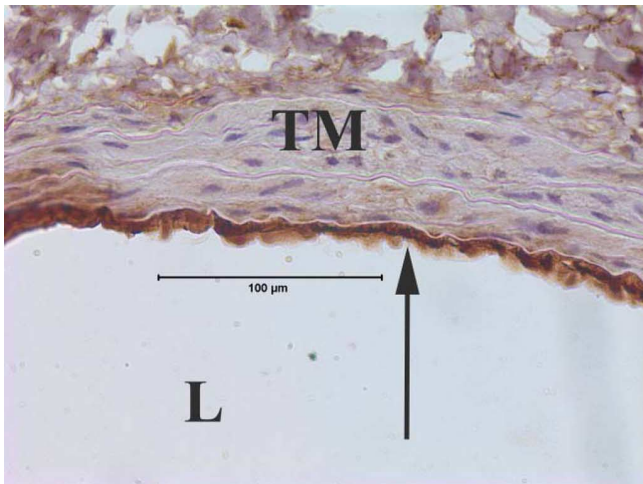
A common and expected observation in previous studies in which *in vivo* conductance has been measured during the application of a sequence of high-voltage pulses, either for reversible or for irreversible electroporation [14], [42], is that electrical conductance increases during the sequence and not only within the pulses. The only exception seems to be the skeletal muscle under IRE. In that particular case, conductance measured at the pulses is quite constant during the whole sequence. However, the cases reported here are probably the first ones in which conductance decreases during the sequence of pulses. We do not have a definitive explanation for such phenomenon. We believe that a plausible hypothesis is that IRE pulses cause contraction of the arteries [43] and that such contraction results in an increase of the impedance of the arteries, particularly of the smooth muscle tissue [44], [45].

We must admit that we do not know for certain why we failed to induce IRE in one of the animals. Nevertheless, we believe that somehow we did not properly apply the electrodes to the artery, and the resulting electrical contact was not good enough over the artery. Direct short-circuiting of the electrodes or through plasma or saline solution does not seem plausible because it would have caused larger conductivity than the measured conductivity during the pulses (Fig. 2).

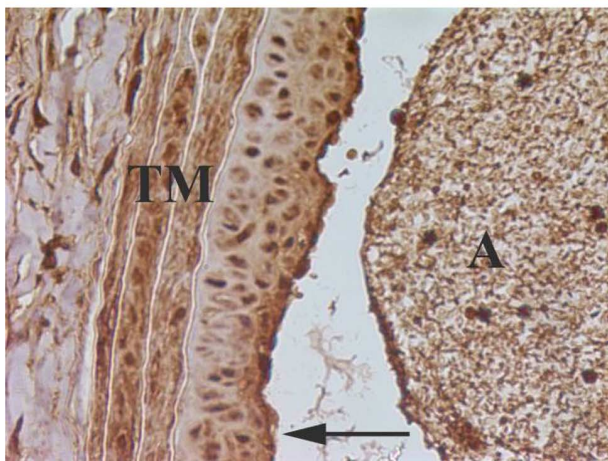
Successful IRE depends on parameters such as electric field magnitude, pulses length and frequency. The reason for choosing the particular electrical parameters used in this study are consistent with the mode of application of IRE that we have developed. These are electrical parameters that were assessed to be high enough to ensure irreversible electroporation [9]–[16], [48], but that do not cause damaging levels of Joule heating. We used a sequence of ten dc pulses of 115 Volts (i.e., electrical field of approximately 3800 V/cm), 100 μ s each, at a frequency of ten pulses per second. These parameters were partially based on previous reports that showed successful tumor cell ablation with IRE [11], [13], [46]. Since the arterial wall has different morphology, and since we did not have data regarding the specific susceptibility of vascular smooth muscle cells to IRE, we used an electrical field that was higher than any previous report, but low enough not to produce thermal damage within the constraints of the treated tissue dimensions. We believe that further research will enable us to better understand the electrical field needed for successful IRE of the arterial wall, as well as the relation between conductance measurements during the procedure and IRE efficiency.

We used a rodent carotid artery model. This model is an acceptable animal model of restenosis [16], [47], but it is important to clarify that our experiments were performed on arteries that were not atherosclerotically changed. Further research will need to address the efficacy of IRE in atherosclerotically changed arteries.

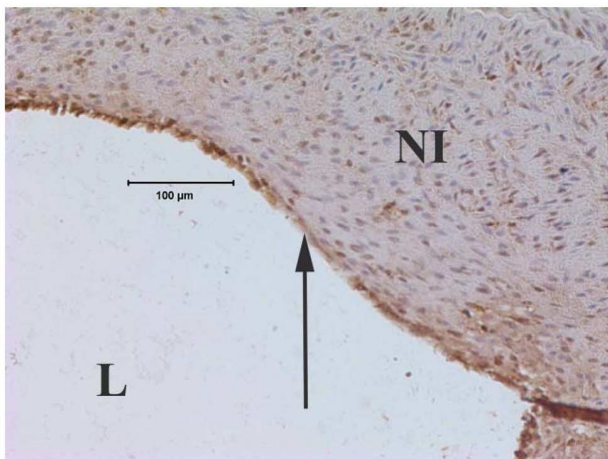
Our electrodes were clamping the artery on its outer surface, but this does not imply that this method will be used as an invasive procedure. Previous reports have already demonstrated the ability to design and use intravascular devices in order to induce reversible electroporation of the arterial wall [48]. We believe that similar designs can be used in the future in order to achieve IRE using intravascular devices.



(a)



(b)



(c)

Fig. 4. (a) Right common carotid artery. This slide is an example of the appearance of a normal control endothelial layer (L—Intra-arterial lumen; TM—Tunica media; Arrow—Endothelial layer). (b) Left common carotid artery, 28 days after intimal damage and IRE. This slide shows the overall preserved appearance of the endothelial layer. (A—Intra-arterial lumen artifact; TM—Tunica media; Arrow—Endothelial layer). (c) Left common carotid artery, 28 days after intimal damage. This slide shows the damaged and irregular endothelial layer in the control group. (L—Intra-arterial lumen; NI—Neointimal formation; Arrow—Irregular endothelial layer).

We are aware of the preliminary nature of this pilot study. Nevertheless, we propose and demonstrate here for the first time the *in vivo*, long-term results of new nonthermal, nonpharmacological strategy to attenuate neointimal formation following intimal damage. It holds the potential to assist in the treatment of restenosis following coronary angioplasty and deliver the treatment with real-time control over the application.

We believe that substantial further investigation is needed to help in understanding the precise molecular mechanism underlying IRE, as well as the exact role for this method in the different vascular pathological states. IRE could theoretically help in preventing and/or ablating coronary and peripheral restenosis process, while also playing a role in attenuating atherosclerotic processes in clinically important locations, such as coronary, carotid, and renal arteries.

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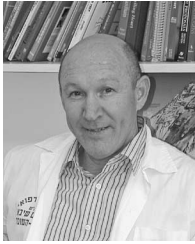
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