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Research Article

Delivery of Indocyanine Green, a Photosensitizer, Into Balloon-Injured Atherosclerotic Plaque of Hyperlipidemic New Zealand Rabbits

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- Indocyanine green
- Local delivery

Abstract

Introduction: Indocyanine Green (ICG) has been demonstrated to be a potent photosensitizer for photodynamic therapy. Although most of the work on ICG has been focused on its potential applications as photosensitizer in Photodynamic Therapy (PDT) for cancer treatment, ICG may have potential clinical use in the treatment of atherosclerosis, both de novo and in restenosis.

Methods: The purpose of this study was to determine the uptake of ICG in balloon-injured atherosclerotic plaque in the rabbit. Twenty New-Zealand rabbits were rendered atherosclerotic and after balloon dilatation of the plaque, ICG was injected locally in the aorta. Different contact times of ICG with the plaque were tested.

Results: Twenty minutes after local ICG injection, fluorescence of atheromatous plaques of the aorta and iliacs was detected with a fluorescence microscope (Zeiss Axiovert).

Conclusion: This study proves accumulation of ICG in atherosclerotic plaque after local delivery. These findings provide a basis for the utilization of ICG for plaque identification and for ICG-PDT in atherosclerosis.

INTRODUCTION

Cardiovascular disease is recognized as the leading cause of death and disability in the world. All current forms of therapy for end-stage atherosclerosis, including bypass surgery and the less invasive endovascular arterial procedures, are limited by restenosis or further progression of atherosclerosis [1]. Restenosis severely limits the overall efficacy of these interventions and can occur in up to 80% of patients. The process of and mechanism behind restenosis is complex. After injury of the vessel wall, migration and proliferation of Smooth Muscle Cells (SMCs) are induced. Some clinical evidence has revealed that SMCs are involved in intimal hyperplasia of vessels. Vascular remodeling and the effect of the extracellular matrix also play crucial roles in restenosis. Many researchers have focused on finding effective inhibitory treatments for the proliferation and migration of medial vascular SMCs. Antiplatelet drugs, antithrombotic drugs, drug-eluting stents and balloons have been used to overcome restenosis after Percutaneous Transluminal Angioplasty (PTA) [2-4].

Although further developments and refinements of the endovascular armamentarium including drug coated devices are of obvious importance to solve the problem of restenosis, rrestenosis continues to be a major problem limiting the effectiveness of revascularization procedures [5]. Improvements in PTA techniques, stent technology should be accompanied by equal research efforts concerning stabilization/regression of the atherosclerotic plaque [6].

Among several new therapeutic approaches to tackle the problem of peripheral atherosclerosis, Photodynamic Therapy (PDT) presents a promising alternative [7,8]. PDT involves the use of a Photosensitizer (PS), light and endogenous molecular oxygen to kill cells. The PDT response starts with accumulation of PS in the target tissue. Light activation of the PS generates cytotoxic Reactive Oxygen Species (ROS). The resulting

photobiological response is direct cell apoptosis and delayed necrosis from neovascular damage [9,10]. The rationale for the use of PDT in treatment of atherosclerosis is based upon the selective uptake and retention of the PS by atherosclerotic plaque as compared to the adjacent normal arterial tissue [11,12]. The appropriate choice of the PS is one of the keys to success. For PDT in atherosclerosis, de novo lesions and restenosis, the PS needs to target the plaque without harming the normal vessel wall. Accumulation of the PS in the plaque is another crucial factor associated with the PDT effect. Other key features are 1) low or no toxicity in absence of light, 2) selectivity for the plaque macrophages, 3) deep tissue penetration and 4) targeted activation [13]. Another point of concern is that PDT generally involves systemic administration of the PS which is allowed to circulate for a specific period of time before there is accumulation in the atherosclerotic plaque. Systemic administration of PS often causes inconvenient cutaneous photosensitivity. Together with major improvements in laser and fiber optic technology, which make local intravascular drug and light delivery possible and as such eliminates problems of insufficient drug/light selectivity, there is a promising role for PDT in treatment of atherosclerosis [14,15]. However, the ideal PS for the use of PDT in de novo or restenotic atherosclerotic lesions is still an unresolved question.

Indocyanine Green (ICG) is mainly used as a fluorescent dye. ICG was initially developed in 1950s as a cyan dye for use in the film industry, as the introduction of color into traditional black and white film was occurring. The first description of ICG and its potential use in medicine is attributed to Irwin J, Fox and Earl H. Wood from the Mayo Clinic, who described to the use of indicator-dilution curves to assess cardiac output. Their study led to FDA approval of the agent in 1959 for use in cardiac-output monitoring, leading to the marketing of ICG as "Cardio-green". The success of ICG opened the doors to investigation of ICG in many other applications, including assessment of hepatic function and ophthalmologic angiography. ICG is now proposed as an ideal fluorescent agent for use in intraoperative surgical guidance [16].

Recently ICG has been studied for the imaging of lipid-rich plaques by near infra-red fluorescence. Because rupture of atherosclerotic plaque and the associated thrombotic complications of myocardial infarction, stroke, ischemic limbs remain leading causes of mortality and morbidity, the identification of those plaques is clinically relevant. Vinegoni, et al demonstrated that ICG targets lipid-rich and macrophage-rich human atheroma specimens ex *vivo* [17]. The use of ICG in PDT as a safe PS is well documented. Combining its properties as a particularly good imaging tool in atherosclerotic plaque and its capacities as PS in PDT, ICG can be a good PS for the use of PDT in atherosclerosis or for the inhibition of restenosis [18]. The purpose of this study was to investigate the uptake of locally administered PS ICG in balloon-injured atherosclerotic plaques of New Zealand rabbits.

MATERIALS AND METHODS

Photosensitizer

ICG is a tricarbocyanine, a negatively charged ion that belongs to the large family of cyanine dyes (shown in Figure 1). ICG has both hydrophilic and lipophilic properties [19,20]. It is soluble in water (1 mg/mL) but not readily soluble in saline. Therefore, ICG should first be dissolved in water before diluted with saline if an isotonic solution is needed. It is also well known that ICG molecules tend to aggregate in aqueous solutions. The absorption and fluorescence spectrum of ICG is in the near-infrared region. Depending on the solvent and the dye concentration, ICG absorbs mainly between 600 nm and 900 nm. Fluorescence properties are between 750 nm and 950 nm (Figure 2,3). The peak optical absorption wavelength of ICG is approximately 800 nm in blood plasma, with a fluorescence wavelength of approximately 810 nm in water and 830 nm in the blood [21,22].

The peak excitation and emission wavelengths of ICG are 800 nm and 830 nm in blood, which are both within the optical window. Thus, the ICG fluorescence method can be used to provide

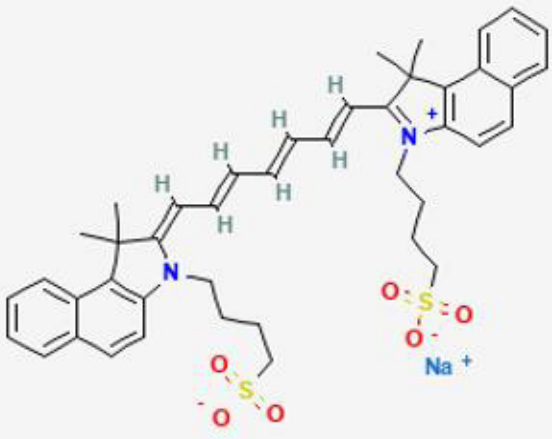

Figure 1 Structure of ICG: National Center for Biotechnology Information (2022). PubChem Compound Summary for CID 5282412, Indocyanine green.

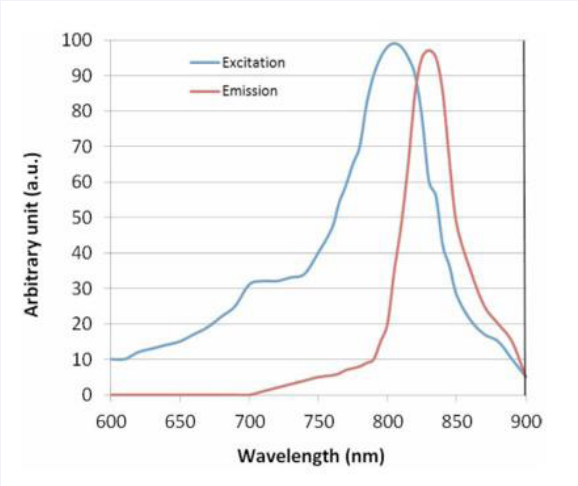

Figure 2 Excitation and emission spectra of ICG. Reprinted from "Indocyanine Green: Photosensitizer or Chromophore? Still a Debate" by Camille Giraudeau, Albert Moussaron, Aurélie Stallivieri, Serge Mordon and Céline Frochot, Current Medicinal Chemistry. 2014; 21(16).

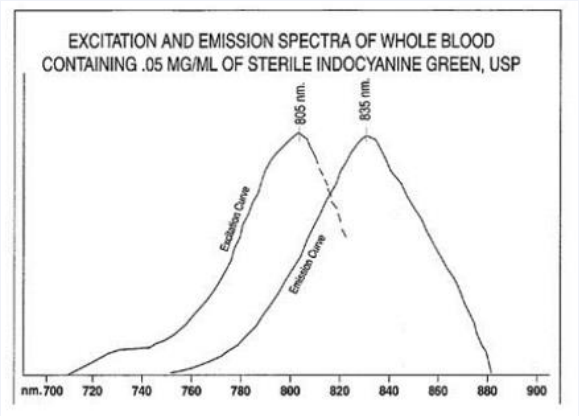

Figure 3 Excitation and emission spectra of ICG in blood plasma. Retrieved from: https://www.drugs.com/pro/indocyanine-green.html

assessment of blood vessels located relatively deep in the tissue. Because ICG has amphiphilic characteristics, it binds to many plasmatic proteins and thus ICG is able to remain intravascular for a long time [20]. Its fast binding to plasmaproteins does not seem to alter protein structures, which is one sign of nontoxicity. Since ICG is also not absorbed by the intestinal mucous membrane, it has extremely low toxicity. The safety of intravenously applied ICG in humans is very well documented with severe adverse reactions only in 0.05% of the cases. The half-life is 2 to 4 minutes, and it is removed from the circulation exclusively by the liver to bile juice [21]. For the experiments, Verdye® 25 mg, provided by Pulsion Medical Systems AG (München, Germany) was used. It is a sterile, lyophilized green powder containing the active ingredient ICG. ICG was dissolved with sterile water for injection to a final concentration of 5 mg per 1 mL.

Experimental Design - Animal Model and Procedure

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Animal Care commission and the Ethical Committee of the KU Leuven approved the experimental protocol (project number 103/2016).

De design of the study is an exploratory research. The goal is to answer the question if after local injection of ICG, we have accumulation of ICG in atherosclerotic plaques. A total of 20 New-Zealand White (NZW) rabbits (all approximately 4-5 kg, 4 months old) were fed a diet containing 1% cholesterol for 12 weeks to create an atherosclerotic rabbit model (Ssniff experimental diet for rabbits, 4.9% coconut fat, 1% cholesterol) [23]. General anesthesia is induced by administrating ketamine (35 mg/kg) and xylazine (5 mg/kg) intramuscularly. During the surgical procedure, the rabbits are ventilated with a mask with 50% air - 50% O2 mixture. A bilateral groin incision with exposure of the femoral arteries and a laparotomy with exposure of the infrarenal abdominal aorta and iliac arteries is performed. After administration of 500 IU/kg heparin intravenously, dilatation of both common iliac arteries with a 2.5*20 mm balloon for 2 minutes is performed. The infrarenal aorta and external/internal iliacs are clamped and the blood in the clamped vessel segment is aspirated. This aspirated volume is replaced with ICG at concentration of 5 mg/mL. ICG is locally injected into the clamped segment. After a fixed contact time the remaining non-absorbed ICG is extracted from the aorta. The aorta/iliacs are unclamped and the circulation is restored for 5 minutes. The rabbits are sacrificed by intravenous injection of sodium pentobarbital (100 mg/kg). The previous clamped segment of aorta and iliacs is removed, rinsed in water, and immersed in Liquid Nitrogen (LN2) for cryopreservation (shown in Figure 3).

Histology

Cryotherapy with Liquid Nitrogen (LN2) was used to preserve the fluorescent properties of ICG. The tissue was cut with a cryostat into slices of 8 µm. The distribution of ICG in the arterial wall was studied using a fluorescence microscope (Zeiss Axiovert) with a specific ICG filter cube mounted (Edmunds optics).

RESULTS

A total of 20 rabbits started with the diet containing 1% cholesterol for 12 weeks to create an atherosclerotic rabbit model (Table 1). Three rabbits died before performing the surgical procedure. One died during the period of diet of unknown cause. Two rabbits died shortly after administration of the anesthetic agents. In 17 rabbits the surgical procedure was performed as planned (Figure 4A-4D). All 17 rabbits had macroscopically signs of plaque in the aorta and iliacs. Introduction of the sheath and balloon through the femoral artery was often difficult due to the small diameter (2.5-3 mm) of the arteries in addition to the calcified aspect of the vessels. In all rabbits microscopy showed plaque in the removed vessel segment (Figure 5A,6A). Different sets of contact time of ICG with the plaque were used, ranging from 5 to 30 minutes. Table I gives an overview of the experiments with different contact times and subsequent results on microscopy. One rabbit served as a control. In this rabbit a

Table 1: Overview of the experiments with different contact times and subsequent result on microscopy.

Rabbit	Period Diet	Contact Time ICG (min)	Result Microscopy
1	12 weeks	5	No signal ICG in plaque
2	12 weeks	10	No signal ICG in plaque
3	12 weeks	10	No signal ICG in plaque
4	12 weeks	15	No signal ICG in plaque
5	12 weeks	**20**	**Signal ICG in plaque**
6	12 weeks	**20**	**Signal ICG in plaque**
7	12 weeks	**20**	**Signal ICG in plaque**
8	12 weeks	**20**	**Signal ICG in plaque**
9	11 weeks	**20**	**Signal ICG in plaque**
10	12 weeks	**20**	**Signal ICG in plaque**
11	12 weeks	**20**	**Signal ICG in plaque**
12	12 weeks	**20**	**Signal ICG in plaque**
13	12 weeks	15	No signal ICG in plaque
14	13 weeks	**30**	**Signal ICG in plaque**
15	13 weeks	**25**	**Signal ICG in plaque**
16	13 weeks	**30**	**Signal ICG in plaque**
17 control rabbit	13 weeks	No injection ICG	No signal ICG in plaque

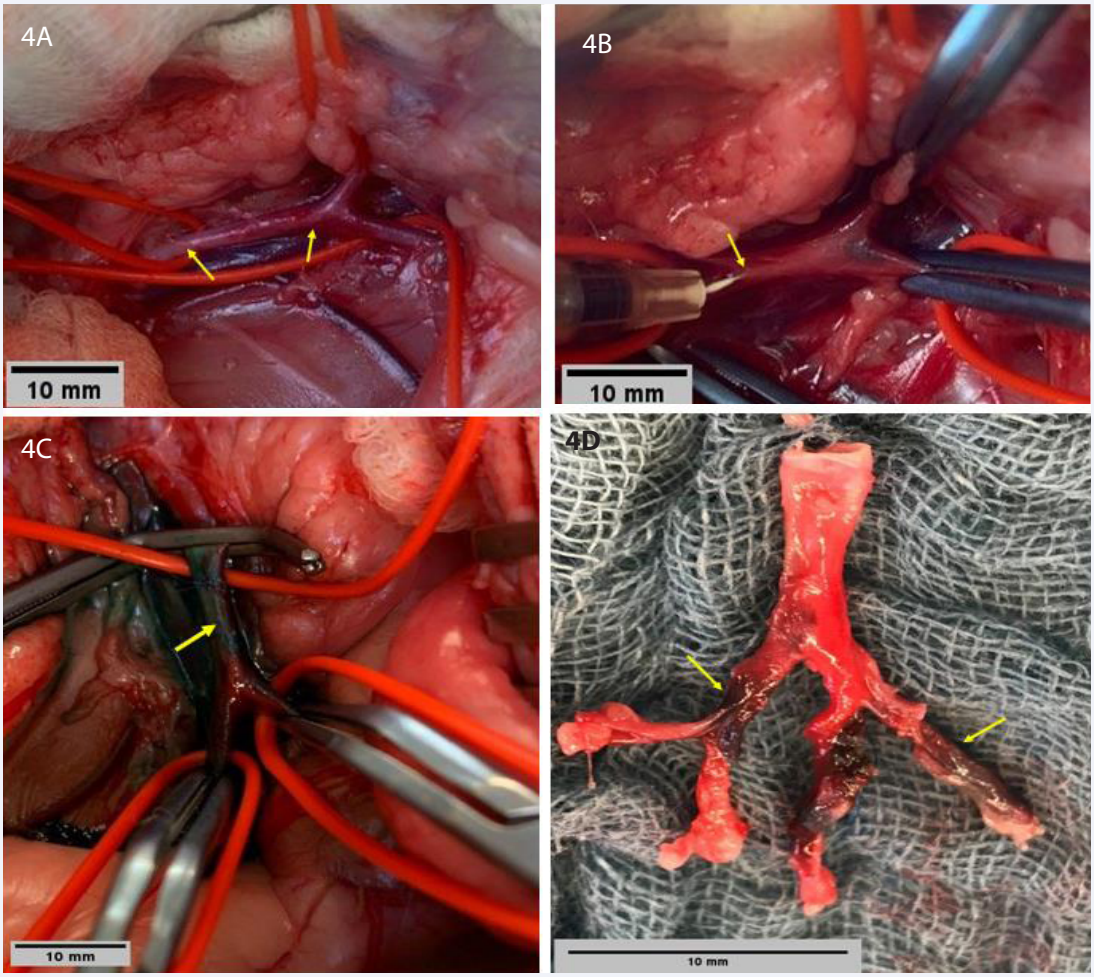

Figure 4 Different steps of the experiment. **A.** Dissection of the aorta and iliac bifurcation - mark the signs of plaque in the aorta (yellow arrow). **B.** Clamped arteries and local injection of ICG (yellow arrow). **C.** Different sets of contact time of ICG with the plaque were used, ranging from 5 to 30 minutes. At a contact time of 20 min visible signs of ICG in the vessel wall (yellow arrow). **D.** Prelevation of the clamped segment with visible signs of ICG in the vessel wall (yellow arrow).

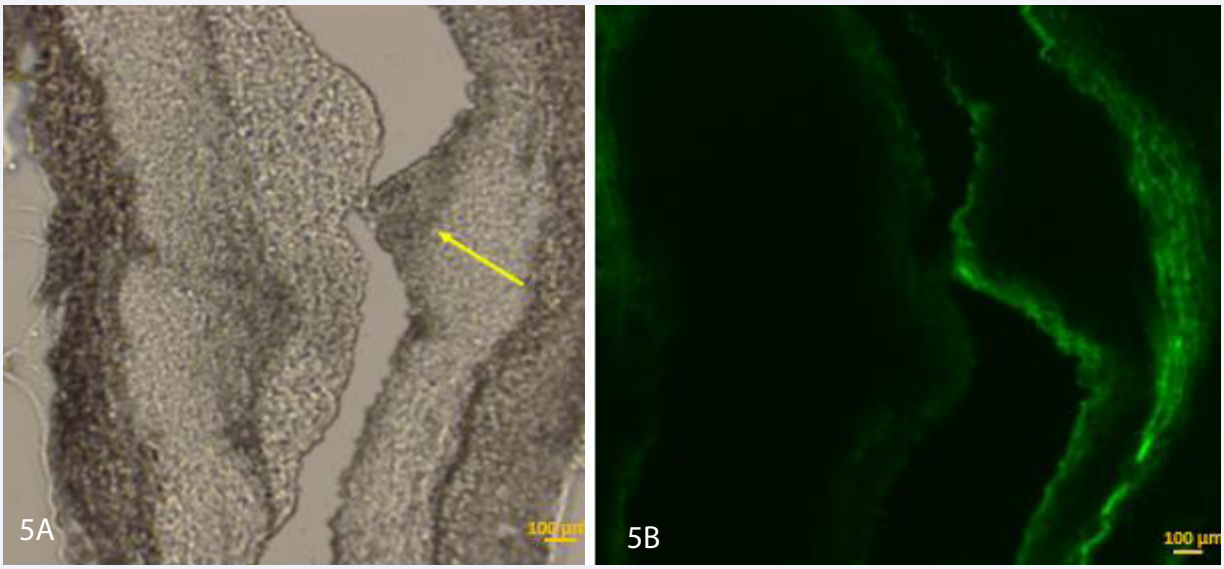

Figure 5 Micrographs of sections of the aortoiliac segments from balloon-injured areas in NZ rabbits after 12 weeks of diet, local injection of ICG and contact time of 15 minutes. **A:** Yellow arrow shows the plaque. **B:** Fluorescence image: no sign of ICG in the plaque - autofluorescence of the elastic lamina. Magnification 100 x.

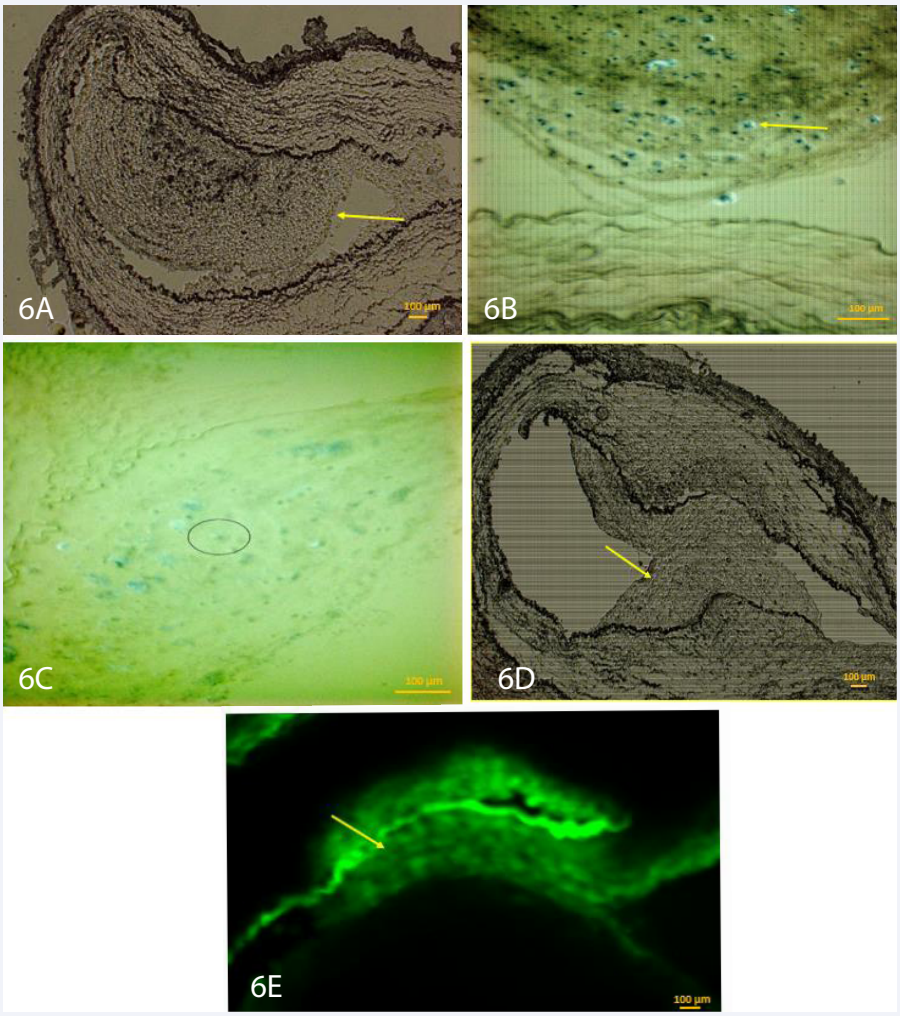

Figure 6 Microscopic images of sections of the aortoiliac segments from balloon-injured areas in NZ rabbits after 12 weeks of diet, local injection of ICG and contact time of 20 minutes. **A.** Photomicropgraphs show clear plaque in the aorta - yellow arrow. **B.** Fluorescent localization of Indocyanine Green (ICG) photosensitizer in the plaque - yellow arrow. **C.** Fluorescent localization of Indocyanine Green (ICG) photosensitizer in the plaque. Black circle indicates spot of ICG. **D.** Clear plaque in the aorta - yellow arrow. **E.** Fluorescent image of the same plaque with accumulation of ICG in the plaque - yellow arrow. Magnification x 100 for image A,D,E - Magnification x 200 for image B,C.

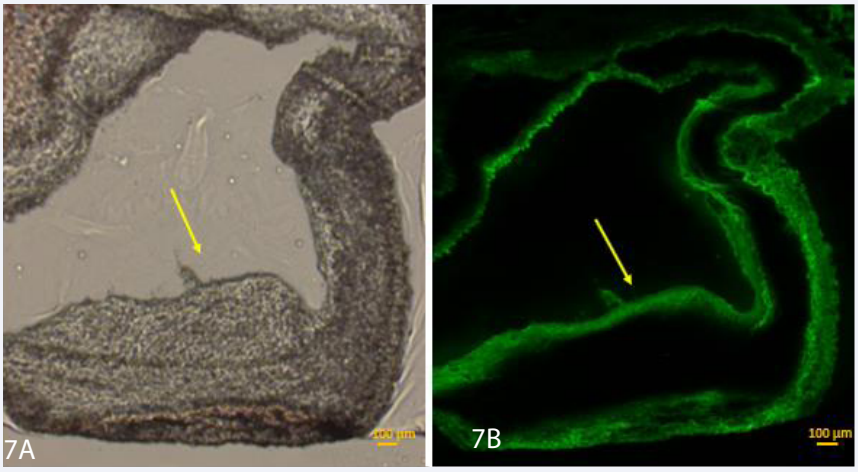

Figure 7 Microscopic images of sections of the aortoiliac segments from balloon-injured areas in NZ rabbits after 12 weeks of diet, but no injection of ICG-control group. **A.** Photomicropgraphs show clear plaque in the aorta - yellow arrow. **B.** Fluorescence image: autofluorescence of the elastic lamina - yellow arrow. Magnification x 100 for image A,B.

dilatation of the aorta/iliacs was performed without injection of ICG. Microscopy showed autofluorescence of the elastic lamina in all cases (Figure 5B). Consistently a clear fluorescence signal in the atheromatous plaque was seen in rabbits with at least 20 minutes contact time (Figure 6A-6E). In all rabbits with injection of ICG but a contact time below 20 minutes, no fluorescence signal in the plaque could be detected (Figure 5A,5B). The control rabbit showed atheromatous changes but no fluorescent signal in the plaque (Figure 7A,7B).

DISCUSSION

PDT is a two-stage process and requires a PS, light and oxygen. In the first stage the PS is taken up by the tissues. Selective accumulation of the PS in the tissue of interest is critically important for the success of PDT. In atherosclerosis, drug lipophilicity and the high lipid content of vascular plaque both appear to contribute to the selective uptake of the PS in the plaque. While specific mechanisms have not been established clearly, passive and active processes appear to be involved in uptake of hydrophobic photosensitizers into atheromatous plaque [24]. Selective accumulation within the atherosclerotic plaques was demonstrated with porphyrin based PS. In a rabbit atherosclerosis model, hematoporphyrin uptake was observed through the thickness of the plaque with a concentration gradient from luminal surface towards aortic wall [25]. In this study hematoporphyrin PDT inhibited Smooth Muscle Cell (SMC) growth and decreases intima/media ratio of atheroma after 7- or 14-day post PDT as compared with the control group. These findings support the hypothesis that PDT can inhibit Intimal Hyperplasia (IH) because SMC migration and proliferation is an important cause of IH.

Usually, PDT involves systemic administration of the PS that is allowed to circulate for a specific period of time to accumulate in atherosclerotic lesions. The PS needs to circulate for a long period of time until enough accumulation in the plaque has been obtained (from 3 to 24h) [26,27]. Non-specific accumulation of the PS in the skin, leading to cutaneous photosensitivity, represents a severe problem associated with the systemic administration of the PS. Important erythema and oedema can, in some cases, last up to 3 months [28,29]. These important side effects are a major hurdle for the clinical use of PDT in atherosclerosis. To reduce the systemic side effects as a result from intravenous injection with PS, local delivery devices were developed. The intra-arterial, local drug delivery approach has been chosen to minimize systemic exposure to the drug and to attain high therapeutic concentrations of the PS directly on the plaque within a short treatment time compatible with the surgical interventions. In a study from Usui et al., an over-the-wire device designed for localized delivery of solutions through openings located in the balloon segment was used (Dispatch® catheter) [30]. Exposure of plaque to a high local concentration of PS can reduce dose and time of exposure and diminish systemic side effects [31]. Together with local delivery of PS, light delivery is another crucial aspect. The group of Mizeret et al. developed an endovascular light delivery system using a cylindrical diffuser. For intravascular PDT, these cylindrical light distributors are introduced in the vessel through a catheter. The light distributor can be centered in the vessel's lumen with an inflatable balloon [32].

ICG has attracted a lot of attention in PDT over past years, since it is already approved by the Food and Drug administration and many data regarding its pharmacokinetics and in vivo use are available [33-37]. The group of Lin et al evaluated the effect of ICG-PDT using extracorporeal near-infrared light on the inhibition of intimal hyperplasia in balloon-injured carotid arteries of rats. This preliminary study confirms the photodynamic effect of systemic administered ICG in reducing intimal hyperplasia in carotid arteries. A limitation is the use of an extracorporeal light source. NIR light has limited depth for tissue penetration and thus clinical use of PDT with an extracorporeal light source is not broad [38].

To the best of our knowledge, there is still no information about uptake of locally injected ICG in atherosclerotic plaque. In this study, we investigated the accumulation of locally injected ICG in atherosclerotic rabbit artery. Additional balloon dilatation of the artery was used to optimize this selective accumulation. The findings of the present study showed that local delivery results in high accumulation of ICG in the plaque relatively shortly after drug administration. Twenty minutes after local injection of ICG, ICG was found in atherosclerotic plaque. This is similar with the findings of other research groups who evaluated local but pressurized application of a benzoporphyrin-derivative, a PS which was provided as a liposomal preparation, in arterial PDT [39]. This time period, often referred to as Drug-Light Interval (DLI), is short in comparison with systemic administration of PS for use of PDT in atherosclerosis that has a much longer DLI (from 3 to 24h) [40]. A long DLI puts PDT out of the scope for cases where it could be used as an adjunctive treatment immediate after angioplasty. A short DLI is critically important for its applicability. Future research combining local administration of the PS and conjugation of the PS with for example an antibody directed towards specific receptors could diminish the DLI.

The reasons for preferential accumulation of ICG in the plaque are not well known. Potential mechanism can be increased endothelium permeability of the plaque after dilatation and affinity of the dye to fibrinogen and platelets that are components of the atherosclerotic plaque.A limitation of the study is that the experiments were conducted in a rabbit model of atherosclerosis. After dilatation in the aorta/iliacs no damage to the arterial wall was seen. We would expect to see a tear in the plaque or a dissection. Although the atherosclerotic lesions of NZ rabbit resemble the characteristics of human atheroma, yet the plaque stiffness, muscularity, and thickness vary to a great extend between NZ rabbit and human aorta. The visualization of intact intima, even after dilatation, has a retarding effect on the penetration of ICG into the vessel wall.

The absence of quantitative analysis in the experiments is also a limitation. We did not analyze the concentration of ICG

across the plaque. However, the first goal was to proof uptake of ICG in the plaque after local injection. For further experiments quantitative analysis regarding concentration ICG in the plaque and its relation to effectiveness of local ICG-PDT can be important.

Another concern could be the sample size of this experiment. As mentioned, the use of PDT for atherosclerosis has faced some important drawbacks. One of them is the search for the ideal PS. The ideal PS allows local application on the plaque and consequently high therapeutic concentration of the PS in the plaque. This is a complex mechanism to evaluate. Most of the studies on uptake of PS, in the broad use of PDT, are assessed in cellular lines. But for PDT in atherosclerosis research on cell lines is not good enough because we need to evaluate the deep penetration. External application of PDT has the same problem, due to superficial penetration, the atheromatous plaque is not reached. We need animal models to study PDT in atherosclerosis in vivo. In animal studies, we can take into account the scattering properties of soft tissue, blood and skin. These are all complex mechanisms to evaluate in vivo so therefore we believe trials of PDT on atherosclerosis have mostly stayed in cell experiments. If in vivo studies on the effect of PDT are performed, animal groups are small (n = 4 for example) [41]. The goal of our manuscript was however not to prove the effect of PDT but the selective uptake of Indocyanine Green (ICG) in the plaque after local administration of ICG in an animal model. In 11 rabbits we were able to prove that ICG was found in the atherosclerotic plaque. The minimal contact time needed was 20 minutes. Every contact time below 20 minutes did not show any sign of ICG in the plaque. This experiment is an exploratory study, it answers a hypothesis (the uptake of ICG after local contact with the plaque). For this kind of pilot experiments, it is recommended to use at least three animals. Of course, for confirmatory trials (e.g., the proof PDT works in atherosclerosis) it is needed to collect more robust evidence [42]. Furthermore, we aim to respect the 3R's principle of animal experiments: Replacement, Reduction, Refinement [43]. In this perspective one rabbit to serve as control is justified: When persistent signal of ICG in the plaque in 11 rabbits after contact time of at least 20 minutes, this result is strong enough to support the goal of this experiment. In conclusion, this study proves accumulation of ICG in the atherosclerotic plaque after local delivery. We assume that endovascular light activation of the accumulated PS could lead to highly selective approach of atherosclerotic plaque or the use of PDT in inhibition of intimal hyperplasia [44]. Further experiments combining local administration of ICG and utilizing endovascular light activation of ICG in atherosclerotic plaque are under way to verify these findings. These experiments will hopefully answer the question if local ICG-PDT could be effective for management of atherosclerosis in de novo or restenosis lesions.

Author Contributions

Conceptualization, M Vuylsteke, S Mordon; methodology, S Houthoofd; formal analysis, S Houthoofd, Th Schueremans; writing-original draft preparation, S Houthoofd; writing-review and editing, S Houthoofd, S Mordon, M Vuylsteke, I Fourneau; supervision I Fourneau All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Animal Care commission and the Ethical Committee of the KU Leuven approved the experimental protocol (project number 103/2016).

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could have a potential conflict of interest.

Data Availability Statement: All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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