PHOTODYNAMIC THERAPY
FOR
BARRETT'S OESOPHAGUS
with use of 5-Aminolevulinic Acid

Jolanda van den Boogert-Kluin
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Front cover: laser scanning microscopy image of a rat oesophagus at 3 hours after administration of 5-aminolevulinic acid (with the help of GJ, Fred and Vivian).

Back cover: Desiderius Erasmus.

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PHOTODYNAMIC THERAPY
FOR BARRETT'S OESOPHAGUS
with use of 5-Aminolevulinic Acid

FOTODYNAMISCHE THERAPIE
VOOR BARRETT OESOFAGUS
met gebruik van 5-Aminolevulinezuur

PROEFSCHRIFT

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aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
prof.dr. P.W.C. Akkermans M.A.
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Since 1970 the incidence of oesophageal adenocarcinoma is rising in a rate greater than that of any other malignancy. It is assumed that the majority of these adenocarcinomas arise in metaplastic transformed epithelial cells lining the oesophagus, known as Barrett's oesophagus. Treatment of patients with Barrett's oesophagus involves antireflux medication or antireflux surgery. Although the reflux complaints disappear, these therapies have no effect on the Barrett's epithelium itself and malignant degeneration can still proceed. Photodynamic therapy with use of 5-aminolevulinic acid is a new treatment option ideally leading to selective endoscopic ablation of the Barrett's epithelium.

Before photodynamic therapy can routinely be used in clinical practice the technique must be optimized to ensure complete ablation of all (pre)malignant cells and minimize the occurrence of unwanted side effects. The preclinical studies described in this thesis were set up to provide insight in the therapy and to investigate the modalities for effective results. This thesis forms the fundamentals for future clinical studies with photodynamic therapy employing 5-aminolevulinic acid to treat oesophageal lesions.

For a long time both clinical and experimental research on Barrett's oesophagus have been points of interest of the Department of Surgery from the University Hospital Rotterdam Dijkzigt. The experiments described in this thesis were conducted at the Laboratory for Experimental Surgery, Erasmus University Rotterdam and were supported by the Netherlands Digestive Diseases Foundation. The studies for this project were carried out in close collaboration with the Department of Gastroenterology and Hepatology, University Hospital Rotterdam Dijkzigt, the Department of Pathology, Erasmus University Rotterdam and the Department of Clinical Physics, Dr. Daniël den Hoed Cancer Centre, Rotterdam, the Netherlands.

Rotterdam, December 1999
Jolanda van den Boogert-Kluin
CONTENTS

Outline of the Thesis 13

PART I: GENERAL INTRODUCTION

Chapter 1 Barrett's Oesophagus: Pathophysiology, Diagnosis and Management  
Scand J Gastroenterol 1998;33:449-453 19

Chapter 2 Endoscopic Ablation Therapy for Barrett’s Oesophagus: Prospects for Photodynamic Therapy  
Am J Gastroenterol 1999;94:1153-1160 31

Chapter 3 Fundamentals of 5-Aminolevulinic Acid-Based Photodynamic Therapy 49

PART II: ANIMAL MODEL

Chapter 4 Animal Model for Barrett's Oesophagus in the Rat and Opossum  
Gastroenterology (submitted) 63

Chapter 5 Oesophagojejunostomy Provides a Unique, Reproducible Model for Barrett's Oesophagus  
J Surg Oncol (submitted) 79

Chapter 6 Gastric Perforation in Rats after Oesophagojejunostomy  
Lab Anim Sci 1999;38:49-52 93

PART III: PHARMACOKINETICS OF 5-AMINOLEVULINIC ACID

Chapter 7 Pharmacokinetics of 5-Aminolevulinic Acid-induced Porphyrin Production in Rats  
<table>
<thead>
<tr>
<th>Chapter 8</th>
<th>Kinetics, Localization and Mechanism of 5-Aminolevulinic Acid-induced Endogenous Photosensitization of Rat Normal and Barrett’s Oesophagus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lasers Surg Med 1999;24:3-13</td>
</tr>
<tr>
<td></td>
<td>Proc SPIE 1997;3191:214-220</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PART IV:</th>
<th>OPTIMIZATION OF PHOTODYNAMIC THERAPY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 9</td>
<td>Timing of Illumination Is Essential for Safe and Efficient Photodynamic Therapy</td>
</tr>
<tr>
<td></td>
<td>Br J Cancer 1999;79:825-830</td>
</tr>
<tr>
<td></td>
<td>Proc SPIE 1998;3563:82-88</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 10</th>
<th>Photodynamic Therapy for Oesophageal lesions: Selectivity depends on Wavelength, Power and Light Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proc SPIE 1998;3563:112-117</td>
</tr>
</tbody>
</table>

| Chapter 11 | Fractionated Illumination: Effect on Ferrochelatase Activity |
|----------------|----------------------------------------------------------------|---|
|                 | J Photochem Photobiol B Biol (submitted)                           | 165|
| Chapter 12 | Fractionated Illumination: Effect on Blood Flow and PpIX Formation |
|             | Lasers Med Sci (submitted)                                         |

<table>
<thead>
<tr>
<th>PART V:</th>
<th>GENERAL DISCUSSION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 13</td>
<td>General Discussion: Considerations for Clinical Application</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Summary and Conclusions</th>
<th>205</th>
</tr>
</thead>
<tbody>
<tr>
<td>Samenvatting en Conclusies</td>
<td>215</td>
</tr>
</tbody>
</table>

**ADDENDUM**

<table>
<thead>
<tr>
<th>Contributing Authors</th>
<th>227</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dankwoord</td>
<td>229</td>
</tr>
<tr>
<td>Curriculum Vitae</td>
<td>233</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>235</td>
</tr>
</tbody>
</table>
To my parents
To Vincent
Respontur tuo unquam est par fama labori
(Horatius)

sed

suavis laborum est praeteritorum memoria
(Cicero)

Je roem zal nooit evenredig zijn aan de moeite die je getroost hebt
maar
zoet is de herinnering aan het voorbijewezen
PHOTODYNAMIC THERAPY
FOR
BARRETT’S OESOPHAGUS
with use of 5-Aminolevulinic Acid
OUTLINE OF THE THESIS

Barrett’s oesophagus is a premalignant lesion of the oesophagus characterized by the presence of columnar epithelium with intestinal metaplasia in the distal oesophagus. Oesophageal adenocarcinoma (the most rapidly increasing cancer in the Western world) is thought to originate from Barrett’s oesophagus following a sequence from metaplasia through dysplasia to adenocarcinoma. To gain insight in the various aspects of Barrett’s oesophagus, the state of the art of its pathophysiology, diagnosis and management is reviewed in Chapter 1, Part I, the general introduction of the thesis.

Both endoscopic surveillance and oesophageal resection are recommended for patients with Barrett’s oesophagus and high-grade dysplasia. This, however, is still controversial as the mortality and morbidity associated with oesophageal resection are considered to be high in view of a preneoplastic disease. Therefore, endoscopic ablation therapies, including photodynamic therapy, have been experimentally used to treat Barrett’s oesophagus. The characteristics of the various endoscopic ablation therapies and their results are summarized in Chapter 2.

The studies described in this thesis aim at making 5-aminolevulinic acid-mediated photodynamic therapy clinically applicable for the endoscopic treatment of Barrett’s oesophagus. For a better understanding of the experimental chapters, Part I is completed with Chapter 3, outlining the fundamentals of 5-aminolevulic acid-based photodynamic therapy.

All experiments in the present thesis are performed in animals. Besides the normal rat oesophagus, rats with Barrett’s oesophagus are used. Part II includes Chapters 4, 5 and 6 and discusses the animal model.

In Chapter 4 it is hypothesized that longstanding reflux of alkaline duodenal content in the oesophagus is involved in the pathogenesis of Barrett’s oesophagus. To test this hypothesis an oesophagojejunostomy, inducing duodeno-oesophageal reflux, is performed in rats and opossums. The opossum is
chosen because the gastrointestinal tract of this species is similar to man.

Although the animal model introduced in Chapter 4 is suitable for studies of the pathogenesis and etiology of Barrett’s oesophagus, for studies concerning photodynamic treatment it is more convenient to have an animal model that induces Barrett’s oesophagus within a shorter time period. Therefore, in Chapter 5 several pathogenetic and possibly synergistic factors are investigated which could hasten the induction of Barrett’s oesophagus in rats by duodenoesophageal reflux.

The occurrence and differential diagnosis of gastric perforation, as observed in some animals in the rat model for Barrett’s oesophagus, is described in Chapter 6.

Prior to the routine use of the endogenous photosensitizer 5-aminolevulinic acid for photodynamic therapy several practical questions must be answered. These questions include general and oesophagus-specific pharmacokinetic aspects of 5-aminolevulinic acid-induced photosensitivity and are studied in Part III.

Chapter 7 is set up to investigate the pharmacokinetics of 5-aminolevulinic acid and 5-aminolevulinic acid-induced porphyrin production in rats. Special attention is given to general pharmacokinetic questions regarding the toxicity of 5-aminolevulinic acid after oral and intravenous administration, the site of porphyrin synthesis and its relation to 5-aminolevulinic acid concentration, and the time course for the development and declination of photosensitization. Additionally, localization of photosensitive porphyrins is studied using laser scanning microscopy.

Chapter 8 focusses on the pharmacokinetics of 5-aminolevulinic acid-induced porphyrin accumulation in the rat normal and Barrett’s oesophagus. For an optimal treatment questions arise as where and at what time after 5-aminolevulinic acid administration do porphyrins accumulate in the normal and Barrett’s oesophagus. In the study in this Chapter biochemical determinations and laser scanning microscopy are carried out to answer these questions.

Possibilities for improving 5-aminolevulinic acid-photodynamic therapy are investigated in Part IV of this thesis.

In Chapter 9 the optimal time between administration of 5-aminolevulinic acid and subsequent illumination, as deducted on a pharmacological base in Chapter 8, is studied in an animal model. Besides the effect of the length of the time-interval on the effectiveness of the treatment in terms of complete ablation of the epithelium, the occurrence of side effects and its impact on oesophageal function in the healing phase are evaluated.
Although in the literature the selectivity of photosensitization has been given more attention than the physical aspects of the applied laser light, these aspects will also have their effect on the achieved damage. Chapter 10 was set up to investigate the effect of the applied wavelength, power output, and total light dose on the epithelial and muscular damage induced by endo-oesophageal 5-aminolevulinic acid-based photodynamic therapy. To make possible proper evaluation of the results the porphyrin fluorescence in the oesophageal wall and the total applied light dose at the oesophageal surface are both measured during illumination.

Finally, the supposed beneficial effects of fractionated illumination are studied in Chapters 11 and 12. Not only the effect on the extent of epithelial ablation but also the possible mechanism of fractionation is studied. Chapter 11 tests the biochemical hypothesis that a fractionated illumination selectively reduces ferrochelatase activity and herewith raises the concentration of the photosensitive porphyrins. Furthermore, this chapter investigates the effect of a second dose of 5-aminolevulinic acid to ensure that the relative ferrochelatase deficit becomes relevant. Chapter 12 focusses on the effect of fractionating the light on oesophageal blood flow and ongoing protoporphyrin IX production. For this, experiments using various fractionating schedules are carried out.

Finally, in Chapter 13, Part V, the general discussion, the consequences of the results of the thesis for 5-aminolevulinic acid-photodynamic therapy for Barrett's oesophagus are briefly discussed, recommendations for future research are provided and the prospects for clinical application are considered.
PART I

GENERAL INTRODUCTION
INTRODUCTION

Since 1970 the incidence of adenocarcinoma of the oesophagus has increased in many Western countries, at a rate that exceeds that of any other malignancy. Most adenocarcinomas arise in a short or long segment of Barrett’s epithelium. Strict follow-up of patients with Barrett’s oesophagus seems therefore of great importance. Consensus about the necessity, the manner and frequency of follow-up has not been achieved. The same holds for the definition, diagnosis, and treatment of Barrett’s oesophagus. This chapter presents the state of the art of the pathophysiology of Barrett’s oesophagus, its diagnosis and its management.

DEFINITION

In 1950 the British surgeon Norman Barrett published a report that described ulcers in the distal part of the oesophagus lined by columnar epithelium. He believed that the ulcerated columnar-lined tissue was a tubular segment of the stomach in patients with congenitally short squamous-lined oesophagus. Today it is generally thought that the tubular structure is the lower oesophagus, lined with columnar epithelium. This entity is now called Barrett’s oesophagus. Theoretically, in a Barrett’s oesophagus the junction between the squamous epithelium of the oesophagus and the columnar epithelium of the stomach (the so-called Z-line) is located more proximal than the anatomic junction between oesophagus and stomach. As the exact location of the end of the oesophagus and the beginning of the stomach cannot be identified precisely, this definition is not always useful in clinical practice. The only reliable endoscopic landmarks for defining the anatomic gastro-oesophageal junction are provided by the proximal margins of the gastric mucosal folds. Until recently a Barrett’s oesophagus was diagnosed when columnar epithelium was obtained by biopsy at a site minimally 2 to 3 centimetres above the proximal margins of the gastric folds. In 80% of cases the columnar Barrett’s mucosa consists of specialized epithelium with incomplete intestinal metaplasia and characteristic goblet cells. In 20% only cardia-type or gastric fundic-type mucosa is found. Only specialized columnar epithelium with intestinal metaplasia predisposes to adenocarcinoma.

Today, a generally accepted definition of Barrett’s oesophagus is therefore the presence of metaplastic columnar mucosa with goblet cells in the oesophagus, regardless of the length of the segment (Figure 1.1). This replaces the former term “short-segment Barrett’s”, used to indicate columnar glandular mucosa within 2 to 3 centimetres of the proximal edge of the gastric folds. Recently, much attention has been paid to the fact that in biopsy specimens from, or just distal to, a normally
located Z-line intestinal metaplasia can be found.\textsuperscript{9} This intestinal metaplasia of the cardia should not be labelled Barrett’s oesophagus.\textsuperscript{10}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{image1.png}
\caption{Columnar-lined oesophagus with intestinal metaplasia and characteristic goblet cells (arrow) (haematoxylin and eosin staining).}
\end{figure}

\section*{PATHOPHYSIOLOGY}

A Barrett’s oesophagus probably originates from a multipotent undifferentiated stem cell of oesophageal origin.\textsuperscript{11} The pathogenetic stimulus that causes metaplasia in case of Barrett’s oesophagus is still the subject of research. Usually, Barrett’s oesophagus is found in patients who are examined endoscopically because of gastro-oesophageal reflux disease, on the basis of a defective lower oesophageal sphincter or hiatus hernia.\textsuperscript{12} Not all patients with gastro-oesophageal reflux disease develop Barrett’s oesophagus. In animal models reflux of gastric content alone is not enough to induce comparable abnormalities.\textsuperscript{13} One hypothesis is that alkaline (bile) duodenal content reflux is involved in the pathogenesis of Barrett’s oesophagus. As such, patients with complications of gastro-oesophageal reflux disease, like strictures, Barrett’s oesophagus and adenocarcinoma would have mainly duodenogastro-oesophageal reflux.\textsuperscript{14} Analysis of frequency and duration of reflux and reflux material in gastro-oesophageal reflux disease patients with and without Barrett’s oesophagus show conflicting results, partly due to inaccurate detection methods.\textsuperscript{15} The same difficulties are encountered when assessing differences in life-style as potential risk factors for the development of Barrett’s oesophagus. Environmental risk factors for the development of adenocarcinoma in apparent Barrett’s oesophagus have been identified: a higher frequency and long-standing periods of (acid) reflux, smoking and, possibly, excessive alcohol consumption.\textsuperscript{15,16}
EPIDEMIOLOGY

The true prevalence of Barrett’s oesophagus is difficult to determine. From an autopsy study at the Mayo Clinic it appears that only one of 20 patients with Barrett’s oesophagus is clinically diagnosed. On the basis of these findings the estimated prevalence of Barrett’s oesophagus in the general population is 1%. In patients with gastro-oesophageal reflux disease (10% to 30% of the general population) prevalence is 12%. The prevalence of Barrett’s oesophagus increases with age and occurs predominantly in the Caucasian race, with a male to female ratio of 2:1. The high number of unrecognized Barrett’s oesophagus can be explained by the fact that Barrett’s oesophagus itself does not cause any symptoms. Moreover, not all patients with symptoms of gastro-oesophageal reflux disease will be examined endoscopically. Finally, not all abnormalities will be recognized at endoscopy. The risk of oesophageal adenocarcinoma in patients with Barrett’s oesophagus appears to be about 30- to 50-fold higher than that in general population, with an estimated incidence of 1 in 150 patient-years. 

Since 1970 a marked epidemiological change in oesophageal carcinoma has appeared in many Western countries: incidence rates for oesophageal squamous cell carcinoma are fairly stable, whereas the incidence of adenocarcinoma increases at a rate surpassing that of any other cancer. The incidence of oesophageal adenocarcinoma in the USA in 1990 is estimated to be about three times the incidence of 1978. In many Western countries the mortality due to oesophageal cancer has increased during the past 25 years (for example, a threefold increase in The Netherlands between 1970 and 1994) (Table 1.1).

<table>
<thead>
<tr>
<th>Year</th>
<th>Men</th>
<th>Women</th>
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<td>1970</td>
<td>238</td>
<td>141</td>
<td>379</td>
</tr>
<tr>
<td>1980</td>
<td>288</td>
<td>143</td>
<td>431</td>
</tr>
<tr>
<td>1990</td>
<td>547</td>
<td>286</td>
<td>833</td>
</tr>
<tr>
<td>1994</td>
<td>657</td>
<td>317</td>
<td>974</td>
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Unfortunately, in most countries data concerning the percentage of adenocarcinoma are not available. It is estimated that approximately 75% of all adenocarcinomas of the oesophagus originate from Barrett’s oesophagus according to the former definition of >2-3 cm Barrett’s epithelium. Unrecognized short-segment columnar metaplasia may account for a large proportion of the remaining 25%.
CLINICAL DIAGNOSIS

The diagnosis of Barrett’s oesophagus depends on endoscopic examination with biopsy of the abnormal epithelium. At endoscopy squamous epithelium appears glossy, pearly white or pinkish-tan, whereas columnar mucosa has a salmon-pink velvety-like aspect (Figure 1.2).

The junction between squamous and Barrett’s epithelium can therefore readily be seen in most cases, so that an experienced endoscopist can diagnose Barrett’s oesophagus mostly on sight. However, in 18% of the endoscopically negative patients glandular mucosa with intestinal metaplasia can be found in the distal oesophagus. These, formerly called short-segment Barrett’s with intestinal metaplasia, may underlie the increasing frequency of cancer of the gastro-oesophageal junction in the USA and Europe (Figure 1.3). Thus, acquisition of biopsy specimens is also required when glandular mucosa is just seen within 2 to 3 cm of the gastric folds (that is, a normal appearing Z-line). Experimental diagnostic techniques, like cytopathology, are only used in situations in which biopsy is difficult (for example, stricture).

Malignant degeneration. At present, dysplasia is the best known marker for malignant change in Barrett’s epithelium. The grade of dysplasia is indistinguishable at endoscopy. Neoplastic transformation has to be clearly differentiated from reactive and regenerative changes. Dysplasia is defined as "indefinite" when this distinction cannot be made unequivocally. Dysplasia is classified histologically in accordance with a modification of the guidelines for
inflammatory bowel disease: high-grade or low-grade, on the basis of the grade of
distortion of crypt architecture and the extent of nuclear abnormalities. In high-
grade dysplasia nuclei often vary markedly in size, shape and polarity. It is
unnecessary and probably impossible to distinguish reliably between high-grade
dysplasia and carcinoma \textit{in situ}. Use of the latter term has now been abandoned to
prevent confusion with the classification intramucosal carcinoma.

Since there is a considerable inter- and intra-observer variation in the
histological diagnosis of dysplasia in Barrett's oesophagus, biopsy specimens should
be examined by a second pathologist. More objective markers to determine the
potential risk of cancer development are extensively studied. Flow cytometric
analysis of DNA content in Barrett's epithelium showed that aneuploidy or
increased G2/tetraploidy fraction is an independent risk factor for malignant
degeneration in younger patients. More data on DNA content in Barrett's
oesophagus without dysplasia is required before flow cytometry can be used in
clinical practice. Specific genetic changes, like mutations and loss of the p53
tumour suppressor gene (resulting in p53 protein overexpression) and
Y-chromosome loss, are found to go along with increasing dysplasia. Increased
expression of epidermal growth factors and their receptors, reduced or absent cell
adhesion molecule expression (such as E-cadherine), and endoscopic laser-induced
fluorescence spectroscopy may also identify Barrett's oesophagus at risk of
neoplastic progression. These markers can not be applied for clinical diagnosis
yet, since long-term follow-up studies of these features are lacking.

\section*{Endoscopic Surveillance}

There is no consensus on the management of patients with Barrett's
oesophagus. Prospective studies on the effect of surveillance on survival are not
available. Early detection of adenocarcinoma during endoscopic surveillance for
known Barrett's oesophagus leads to a better survival. Regular endoscopic
surveillance is therefore advised by many authors. A recommended protocol
for surveillance requires four biopsies, one from each quadrant, every 2 cm along
the visible length of Barrett's mucosa, with additional specimens from any
abnormal-appearing area. Cost-effectiveness studies advise endoscopic surveillance
every 2 to 3 years. Other retrospective studies, however, could not find a
significant difference in life expectancy between patients who are and who are not
under endoscopic surveillance. This is mainly due to the relative low absolute
incidence of oesophageal adenocarcinoma (ranging from one case per 46 to 441
patient-years). Other causes of death play a more important role in patients with
Barrett's oesophagus. These data, strengthened by the substantial costs of
surveillance programs, form an argument to examine patients with Barrett’s oesophagus only when they are symptomatic. Arguments for both views considered, ideally every patient should undergo an individual surveillance program, based on co-morbidity and the relative risk of malignant degeneration. Estimating this relative risk for each individual is the main problem; at present only male sex, smoking, (possibly excessive alcohol consumption), considerable length of the Barrett’s epithelium (> 8 cm), time of existence, and dysplasia count for risk factors. As yet, surveillance programs are being carried out in a few clinics only.

**THERAPY**

The choice of therapy in patients with Barrett’s oesophagus depends mainly on the existence and grade of dysplasia.

**Barrett’s oesophagus without dysplasia.** Because reflux is the presumed inciting factor in the pathogenesis of Barrett’s oesophagus, the therapy has been aimed at reducing the acid exposure of the oesophagus. By far most patients are treated medically with a H-2 receptor blocker or a proton pump inhibitor. Although reflux symptoms can mostly be controlled successfully, medical therapy usually does not result in consistent reduction in the extent of existing Barrett’s epithelium and consequently does not lead to a reduction of the risk of developing adenocarcinoma. Especially in young patients whose reflux complaints are not controlled after a 12-week course of medical therapy, a Nissen fundoplication is optional. Besides the disadvantages of morbidity and a (small) chance of mortality, surgical treatment does not lead to regression of the length and grade of dysplasia of the Barrett’s epithelium either. The chance of progression is probably smaller than after conservative treatment. Once surveillance is considered, endoscopy every 2 years seems to be the best option.

**Barrett’s oesophagus with indefinite or low-grade dysplasia.** Patients who are diagnosed as being indefinite for dysplasia should undergo a repeat endoscopic examination after an 8- to 12-week course of intensive acid suppression (for example, 40 mg omeprazole daily). In case of low-grade dysplasia, antireflux therapy (either conventional or surgical) is recommended, followed by endoscopic surveillance every 6 to 12 months.

**Barrett’s oesophagus with high-grade dysplasia.** There are two main schools of thought with regard to the stage of the disease that requires surgery. According to the first school, an intensive (every 3 to 6 months) endoscopic biopsy protocol
with multiple biopsies can differentiate high-grade dysplasia from early adenocarcinoma in Barrett's oesophagus.\textsuperscript{41} In the second school an oesophagectomy is performed already in the stage of high-grade dysplasia. The latter is based on studies showing that multiple biopsies can fail to detect malignant lesions owing to sampling error and that the distinction between high-grade dysplasia and early adenocarcinoma is not always possible by biopsy.\textsuperscript{42} In our opinion no general treatment protocol can be advised for patients with high-grade dysplasia. Instead, therapy must be prescribed individually on basis of histological findings and co-morbidity, after discussing the risks and alternatives (experimental therapies).

**EXPERIMENTAL THERAPIES**

Neither medical nor surgical treatment in fact eliminates Barrett's epithelium itself: progression of the length, the grade of dysplasia, and development of adenocarcinoma can still occur. Experimental therapies focus on removal of the abnormal epithelium. Endoscopic mucosectomy is one of these new modalities, mainly investigated in China and Japan.\textsuperscript{43} A second approach is thermal therapy using laser light that causes destruction of the superficial epithelium (for example, ablation or coagulation). In combination with acid suppression, restoration of squamous epithelium has been shown to occur.\textsuperscript{44,45} Another form of laser therapy is photodynamic therapy. As will be outlined in Chapter 3, this type of therapy is based on the accumulation of exogenously administered or endogenously produced photosensitive porphyrins.\textsuperscript{46} Porphyrins ideally accumulate semi-selectively in Barrett's epithelium, and subsequent intraluminal illumination with light of an appropriate wavelength creates a photochemical reaction that results in destruction of the Barrett's epithelium. As will be reviewed in the next chapter, in some clinics (mainly in the USA) photodynamic therapy has already been applied clinically, with various degrees of success.\textsuperscript{47-50} For both forms of laser therapy it is not clear whether the risk of adenocarcinoma development is indeed decreased. Recently, it has been reported that beneath the area that is re-epithelialized with squamous epithelium, areas with Barrett's epithelium are still apparent.\textsuperscript{44,48} Probably this can be avoided by optimizing the treatment factors. The described laser therapies require additional long-term acid suppression to prevent new development of Barrett's oesophagus. Another question concerns the long-term consequence of laser therapy. It is known that a corrosive lesion is a risk factor for the development of squamous cell carcinoma. Long-term follow-up studies are therefore required.

26
CONCLUSIONS

Barrett's oesophagus is a complication of long-standing gastro(duodeno)-oesophageal reflux. Since the exact pathophysiology is not known, prevention is hardly possible. The diagnosis of Barrett's oesophagus requires the presence of intestinal metaplasia in at least one biopsy from the lower oesophagus. Barrett's oesophagus is a premalignant condition with an increased risk for the development of adenocarcinoma. The incidence of oesophageal adenocarcinoma is increasing sharply. Cost and time-consuming surveillance programs -with still doubtful efficacy- are carried out in few clinics only. Research on objective markers to determine the risk of malignant degeneration must lead to efficient surveillance of a selected group of patients in the future. Antireflux therapy seems to have little effect on the Barrett's epithelium itself. Experimental therapies focus on the removal of the columnar epithelium with restoration of the squamous epithelium. Laser therapy and especially photodynamic therapy seem to be promising modalities.
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Review of Barrett's oesophagus


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CHAPTER 2

ENDOSCOPIC ABLATION THERAPY FOR BARRETT'S OESOPHAGUS: PROSPECTS FOR PHOTODYNAMIC THERAPY

This chapter has been adapted from Van den Boogert et al. American Journal of Gastroenterology 1999;94:1153-1160.
INTRODUCTION

A generally accepted definition of Barrett’s oesophagus is the presence of columnar epithelium with intestinal metaplasia in the distal oesophagus (Chapter 1). This intestinal type with characteristic goblet cells is especially at risk for neoplastic progression. Carcinogenesis of Barrett’s oesophagus is thought to follow a sequence from intestinal metaplasia, to low-grade dysplasia (LGD) through high-grade dysplasia (HGD), to adenocarcinoma. The choice of therapy of Barrett’s oesophagus mainly depends on the existence and grade of dysplasia. In Barrett’s oesophagus without dysplasia, therapy is focussed on the reflux complaints of the patients. Although reflux is the presumed inciting factor in the pathogenesis of Barrett’s oesophagus and antireflux therapy (medical or surgical) is effective in controlling the reflux symptoms, it usually does not result in reduction in length or grade of Barrett’s oesophagus. Patients with Barrett’s oesophagus with LGD are usually advised to undergo additional endoscopic biopsy surveillance. When intramucosal carcinoma is found, oesophagectomy is the treatment of choice. The treatment of patients with Barrett’s oesophagus with HGD is still controversial; both oesophagectomy and intensive endoscopic surveillance are treatment options. Alternatively, endoscopic ablation therapy, which is starting to become clinically applicable, could prove to be preferable. This chapter describes the current management of patients with Barrett’s oesophagus with HGD and summarizes the various methods and results of endoscopic ablation. In addition, the future direction of the management of Barrett’s oesophagus is discussed.

CURRENT MANAGEMENT OF HIGH-GRADE DYSPLASIA

The choice between surgery and endoscopic surveillance in the management of HGD in Barrett’s oesophagus is determined by three factors: first, by the natural history of the dysplasia; second, by the accuracy of the (preoperative) diagnosis; and third, by the risks of both strategies.

Unfortunately, little is known about the first factor, the natural history of HGD. In a prospective study with a mean follow-up time of 30 months, Levine et al. examined 58 patients with Barrett’s oesophagus with HGD. Of these, 26% progressed to intramucosal carcinoma, in 27% the grade of dysplasia degraded to a less severe histological diagnosis, and in 47% the HGD remained stable. Another study reported the follow-up of 22 patients with HGD, of whom 16 had been followed for several months. In two patients (13%) adenocarcinoma developed, in three patients (19%) HGD remained stable, and in 11 patients (69%) HGD could not be diagnosed in biopsies taken at consecutive exams. Therefore, the choice for...
surgical treatment must not be based only on the presumption that carcinoma will develop in all patients with HGD. Whether the results of both studies can be explained by real regression or progression of the dysplasia, by a "therapeutic" biopsy, or by a false-positive or a false-negative diagnosis is not clear. Apart from a few case reports, no other studies have investigated the natural history of HGD in Barrett’s oesophagus.

The second factor that determines the choice of therapy relates to the reliability of the endoscopic diagnosis of HGD in Barrett’s oesophagus. Studies that compare the endoscopic biopsy diagnosis and the surgical pathology findings have shown that areas of HGD are often small and cannot be distinguished macroscopically from Barrett’s oesophagus with no dysplasia. In only two studies was a correlation found between endoscopic biopsy results and surgical pathology findings of HGD in Barrett’s oesophagus. Others have reported that in 11% to 67% of patients with HGD on endoscopic biopsies, an adenocarcinoma was found in the resected specimen. In the English literature between 1990 and 1997, 10 retrospective reports of surgical therapy for Barrett’s oesophagus with HGD have been published. A total of 119 patients were analysed, of whom 56 (47%) were found to have adenocarcinoma in the resected specimen. In 41 (73%) this concerned a stage I carcinoma (T1N0M0, T1 = tumour that invades lamina propria or submucosa but not muscularis mucosae). These data explain why some authors advise an endoscopic biopsy surveillance for HGD, whereas others recommend resection of Barrett’s oesophagus containing HGD. The difference between pre- and postoperative diagnosis suggests that dysplastic areas are often missed by routine biopsies. Moreover, there is a considerable intra- and inter-observer variation in histological diagnosis of dysplasia. To increase the accuracy of the endoscopic biopsy, it is essential to take ample biopsies. A recommended protocol requires four biopsies, one from each quadrant, every 2 cm along the visible length of the Barrett’s mucosa, with additional biopsies from any abnormal-appearing areas. Furthermore, biopsies should be examined by a second experienced pathologist and, if available, compared with earlier biopsies from the same patient. Whenever the diagnosis of HGD remains uncertain, patients should undergo a repeat endoscopic examination within 1 month. As inflammation can make the diagnosis HGD difficult, these patients need to be treated with intensive acid suppression before the repeat endoscopic examination.

Finally, the risks of both strategies must be determined for each patient individually. The operative mortality of a resection for oesophageal carcinoma in our hospital was 3% between 1990 and 1995. Other studies report mortality rates of 3% to 10%. Short term morbidity of oesophagectomy in most studies has been described to be approximately 30%, However, complications in up to 78% have been reported. Quality of life after oesophagectomy is estimated to
be 0.8 on a quality-of-life scale from 0 to 1, with 1 being normal. In studies after 1990, 56 patients have been followed who underwent an oesophagectomy for Barrett’s oesophagus with HGD and in whom a carcinoma was detected in the resection specimen. The 5-year overall survival was 82%. In a prospective Dutch study including 112 patients who underwent an oesophageal resection for adenocarcinoma, a 5-year survival of 24% was found. In 12 patients the carcinoma was restricted to the (sub)mucosa; the 5-year survival in this subgroup was 63%. These results indicate that early detection of adenocarcinoma and subsequent oesophageal resection, preferable at the stage of HGD, is important in terms of survival. Therefore, in our opinion, endoscopic surveillance is indicated only in patients who can physically undergo an oesophagectomy when carcinoma is found. In the future this will probably change, as experimental studies focus on endoscopic ablation of Barrett’s epithelium and small, early stage adenocarcinomas, resulting in the regeneration of squamous epithelium in the oesophagus. The minimally invasive character of endoscopic ablation therapies make them well suited for non-operative candidates.

ENDOSCOPIC ABLATION THERAPIES

Endoscopic surgical ablation can be performed by either a thermal, chemical or mechanical method. Combination with antireflux medication (or possibly antireflux surgery) is recommended to provide optimal circumstances for regeneration of squamous epithelium.

Thermal ablation

Thermal ablation can be achieved using electrosurgery or laser coagulation. Multipolar electrocoagulation (MPEC) operates by passing a current between electrodes which are in contact with tissue. The main disadvantage of electrosurgery is the relatively uncontrolled depth of the ablation. Therefore, the skill and the experience of the performing physician will determine the effect of the treatment and the occurrence of side effects. The effect of electrosurgery can be enhanced by transferring the electrical energy to the tissue surface through electrically conductive plasma of argon gas (argon beam plasma coagulation, ABPC). This results in an evenly distributed ablation, and injury is controlled to a depth of 1 to 2 mm, because of insulating properties of the eschar formed after coagulation.

Thermal destruction of Barrett’s mucosa can also be achieved by contact or noncontact laser (photo)ablation, in which energy is rapidly transferred from the laser beam to the cell. Depth of the ablation depends on tissue optical properties.
and laser parameters. The absorption of optical radiation is strongly wavelength-dependent. Argon (514.5 nm) and potassium titanium phosphate (KTP, 532 nm) lasers penetrate tissue approximately 1 mm; neodymium:yttrium-aluminium-garnet (Nd:YAG, 1064 nm) lasers penetrate approximately 3 to 4 mm.

**Chemical ablation**

Photodynamic therapy (PDT) is a chemical ablation method. It is based on the accumulation of a photosensitizer in tissue. Subsequent illumination with light of an appropriate wavelength induces a photochemical reaction that results in tissue destruction (Chapter 3). The depth of treatment is dependent on the penetration of the laser light and localization of the photosensitizer in the oesophageal wall. The photosensitizers mainly used are haematoporphyrin derivative (HpD, a mixture of different porphyrins), and dihaematoporphyrin ester/ether (DHE, an enriched form of HpD marketed as Photofrin®). Problems associated with the administration of exogenous porphyrins are a low selectivity to accumulate in malignant tissues, skin photosensitivity for up to several months, and the occurrence of strictures in the oesophagus after PDT.27-30

A newer agent, 5-aminolevulinic acid (ALA), is a naturally occurring intermediate of the haem synthetic pathway and induces the endogenous production of photosensitive protoporphyrin IX, predominantly in epithelial layers.31,32 Consequently, tissue destruction is limited to the mucosa. In addition, skin photosensitivity lasts only 24 to 48 hours, because of the rapid metabolism of ALA via protoporphyrin IX into haem. Therefore, ALA seems highly suitable for treatment of Barrett’s oesophagus. However, incomplete ablation of the Barrett’s epithelium or the subsquamous persistence of Barrett’s mucosa have been reported.33,34 With respect to premalignancy, it is crucial to eliminate all Barrett’s cells. Furthermore, subsquamous Barrett’s mucosa (pseudoregression) is probably less accessible (or inaccessible) for endoscopic surveillance.35

**Mechanical ablation**

Endoscopic mucosal resection can be achieved by a "suck and cut" or "lift and cut" method using a diathermic snare.36 This technique has gained increasing popularity in the treatment of early oesophageal cancer. It is most suitable for the removal of polyps or possibly small flat lesions. However, the technique is less appropriate for the removal of larger flat areas like Barrett’s oesophagus. Surgical ultrasound, in which energy is concentrated very close to the tip of a transducer, is another method that causes primarily mechanical ablation by scrubbing, abrading or separating layers.25 An important characteristic of ultrasonic aspiration is its ability to fragment cellular tissue while leaving elastic connective tissue undamaged.37
RESULTS OF ENDOSCOPIc ABLATION THERAPIES

Numerous reviews concerning Barrett's oesophagus and the endoscopic treatment of this entity have been published over the last 5 years.\(^1\textsuperscript{,}^{38-44}\) They all describe one or more uncontrolled studies involving a few patients; however, no systematic overview including all reports of ablation therapy for Barrett's oesophagus exists.

Using the Index Medicus, the MEDLINE database, cross-referencing and abstracts of major meetings on endoscopic ablation therapy, we collected all of the case reports and patient studies on this topic up to May 1998. To complete the survey, results presented in abstracts are included in the tables in this chapter but will not be discussed in the text. Unfortunately, not all authors describe the grade of dysplasia before and after treatment, nor their exact definition of complete remission, nor whether Barrett's epithelium was completely ablated. Furthermore, the duration and thoroughness of the follow-up varied considerably.

Thermal ablation

Twenty-eight reports have been published on thermal ablation of Barrett's oesophagus, 13 as articles and 15 as abstracts (Table 2.1).\(^45\textsuperscript{,}^{72}\) These reports include a total of 284 patients, of which only seven were reported to have HGD (all patients with HGD are presented in abstracts).

Electrocoagulation (MPEC and ABPC) was used in five studies including 15 patients.\(^43\textsuperscript{,}^{48}\textsuperscript{,}^{52,53}\) MPEC was used in 12 patients. After 10 to 18 months follow-up, residual Barrett's oesophagus was found in none.\(^45\textsuperscript{,}^{48}\) Treatment took, on average, five sessions; complications were mild and occurred in 7%.\(^46\) ABPC was used in only three patients; all showed residual Barrett's oesophagus after treatment, and in one patient LGD progressed to HGD under treatment.\(^52,53\)

In contrast to electrocoagulation, the results of laser ablation are reported mainly in published articles. Laser ablation was used in 73 patients, of whom 50 were reported in articles and 23 in abstracts.\(^63\textsuperscript{,}^{72}\) Of the 50 patients, 33 were treated with argon or KTP laser and 17 with Nd:YAG laser. After laser ablation, residual Barrett's oesophagus was reported in 31 of 50 patients (62%). Using argon or KTP laser, residual Barrett's oesophagus was reported in 80% (26 of 33) of patients after 3 to 18 months of follow-up. With a minimal follow-up of 2 months after Nd:YAG laser treatment, residual Barrett's oesophagus was found in 29% (5 of 17). Laser ablation therapy usually took 1 to 8 sessions and, apart from minor retrosternal discomfort for 2 to 3 days, no complications were reported.
Table 2.1 Results of Studies Performing Thermal Ablation Therapy for Barrett's Oesophagus (for legend see page 39).

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Treatment</th>
<th>Patients (n), Grade of Dysplasia</th>
<th>FU (months)</th>
<th>residual HGD</th>
<th>Residual BO</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>McBride*</td>
<td>1995</td>
<td>MPEC</td>
<td>1 ND</td>
<td>15</td>
<td>0</td>
<td></td>
<td>6 sessions, not intestinal metaplasia but &quot;heterotopic gastric mucosa&quot;</td>
</tr>
<tr>
<td>Sampliner*</td>
<td>1996</td>
<td>MPEC</td>
<td>10 ND</td>
<td>10-18</td>
<td>0</td>
<td></td>
<td>2-4 sessions; only half the circumference was treated; residual BO in that half in 0 patients, untreated half served as internal control and was unchanged; BO in that half in 10 patients</td>
</tr>
<tr>
<td>Garcia Montes*</td>
<td>1998</td>
<td>MPEC</td>
<td>1</td>
<td>15</td>
<td>0</td>
<td></td>
<td>patient was a 13-year-old girl</td>
</tr>
<tr>
<td>Guelrud*</td>
<td>1997</td>
<td>MPEC</td>
<td>21</td>
<td>12</td>
<td>?</td>
<td>2</td>
<td>1-6 sessions</td>
</tr>
<tr>
<td>Jackson*</td>
<td>1997</td>
<td>MPEC</td>
<td>22</td>
<td>6</td>
<td>?</td>
<td>1</td>
<td>the 1 patient with residual BO had inadequate acid suppression</td>
</tr>
<tr>
<td>Kovacs*</td>
<td>1997</td>
<td>MPEC</td>
<td>20 ND</td>
<td>4.5</td>
<td>5</td>
<td></td>
<td>7 of 15 patients with CR had persistently abnormal pH studies</td>
</tr>
<tr>
<td>Michopoulos*</td>
<td>1997</td>
<td>MPEC</td>
<td>10</td>
<td>6</td>
<td>2</td>
<td></td>
<td>1 pseudoc regression</td>
</tr>
<tr>
<td>Dumoulin*</td>
<td>1997</td>
<td>ABPC</td>
<td>2 ND</td>
<td>?</td>
<td>2</td>
<td></td>
<td>5-7 sessions</td>
</tr>
<tr>
<td>Maass*</td>
<td>1998</td>
<td>ABPC</td>
<td>1 (LGD)</td>
<td>12</td>
<td>1</td>
<td>1</td>
<td>patient with LGD developed HGD under ABPC therapy</td>
</tr>
<tr>
<td>V. Laethem*</td>
<td>1997</td>
<td>ABPC</td>
<td>21 (17 ND, 4 LGD)</td>
<td>3</td>
<td>2</td>
<td></td>
<td>1 patient showed no effect</td>
</tr>
<tr>
<td>Bohnacker*, Porthun*</td>
<td>1997</td>
<td>ABPC</td>
<td>8 (1 MGD, 7 LGD/ND)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>4-9 sessions</td>
</tr>
<tr>
<td>Byrne*, Attwood*</td>
<td>1997</td>
<td>ABPC</td>
<td>35</td>
<td>1-12</td>
<td>0</td>
<td>10</td>
<td>1-7 sessions, 10 patients showed squamous regeneration with underlying intestinal metaplasia and additional 10 patients showed submucosal glands without goblet cells or intestinal metaplasia covered by squamous epithelium, 2 perforations</td>
</tr>
<tr>
<td>Boyer*</td>
<td>1997</td>
<td>ABPC</td>
<td>15 (no HGD)</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>1-9 sessions</td>
</tr>
</tbody>
</table>
Table 2.1 (continued) Results of Studies Performing Thermal Ablation Therapy for Barrett's Oesophagus.

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Treatment</th>
<th>Patients (n), Grade of Dysplasia</th>
<th>FU (months)</th>
<th>Residual HGD</th>
<th>Residual BO</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Martin</td>
<td>1998</td>
<td>ABPC</td>
<td>18 (no HGD)</td>
<td>?</td>
<td>10</td>
<td>3-10</td>
<td>3-10 sessions, 2 patients showed no response</td>
</tr>
<tr>
<td>Mörk</td>
<td>1998</td>
<td>ABPC</td>
<td>12 (10 ND, 2 LGD)</td>
<td>?</td>
<td>1</td>
<td>1-6</td>
<td>1-6 sessions, 1 pseudoregression</td>
</tr>
<tr>
<td>Stüker</td>
<td>1998</td>
<td>ABPC</td>
<td>15</td>
<td>8</td>
<td>0</td>
<td></td>
<td>1-7 sessions, results expressed per lesion and not per patient.</td>
</tr>
<tr>
<td>Berenson</td>
<td>1993</td>
<td>Argon</td>
<td>10 (8 ND, 2 LGD/MDG)</td>
<td>?</td>
<td>&quot;7&quot;</td>
<td></td>
<td>1-7 sessions, results expressed per lesion and not per patient.</td>
</tr>
<tr>
<td>Barham</td>
<td>1997</td>
<td>KTP</td>
<td>13 ND</td>
<td>3-18</td>
<td>10</td>
<td>1-6</td>
<td>1-6 sessions, no macroscopic BO left but 10 pseudoregression</td>
</tr>
<tr>
<td>Gossner</td>
<td>1997</td>
<td>KTP</td>
<td>8 (4 LGD, 4 HGD)</td>
<td>?-12</td>
<td>0</td>
<td>1</td>
<td>1 patient with residual BO showed pseudoregression</td>
</tr>
<tr>
<td>Biddlestone</td>
<td>1998</td>
<td>KTP</td>
<td>10 ND</td>
<td>60</td>
<td>9</td>
<td>9</td>
<td>9 pseudoregression</td>
</tr>
<tr>
<td>Brandt</td>
<td>1992</td>
<td>Nd:YAG</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td></td>
<td>CR 6 weeks after treatment, BO returned after 14 weeks, patient had insufficient anti reflux therapy (acid reflux documented by 24-hour pH monitoring)</td>
</tr>
<tr>
<td>Brandt</td>
<td>1995</td>
<td>Nd:YAG</td>
<td>1 (same patient)</td>
<td>12</td>
<td>0</td>
<td></td>
<td>second treatment under complete acid suppression documented by 24-hour pH recording</td>
</tr>
<tr>
<td>Sampliner</td>
<td>1993</td>
<td>Nd:YAG</td>
<td>1</td>
<td>11</td>
<td>1</td>
<td></td>
<td>only partially treated</td>
</tr>
<tr>
<td>Luman</td>
<td>1996</td>
<td>Nd:YAG</td>
<td>4 ND</td>
<td>6</td>
<td>4</td>
<td></td>
<td>4 maximal 3 sessions, prospective randomized trial, no effect seen on length of BO</td>
</tr>
<tr>
<td>Salo</td>
<td>1998</td>
<td>Nd:YAG</td>
<td>11 ND</td>
<td>6-52</td>
<td>0</td>
<td></td>
<td>1-8 sessions, combined with antireflux surgery</td>
</tr>
<tr>
<td>Krevsky</td>
<td>1998</td>
<td>Nd:YAG</td>
<td>15</td>
<td>2</td>
<td>4</td>
<td></td>
<td>3 patients showed no response, 1 stricture, 1 perforation</td>
</tr>
</tbody>
</table>
In conclusion, these studies show that thermal ablation therapies can reverse Barrett's oesophagus. However, multiple treatment sessions are required and because of the relatively uncontrollable depth of ablation, these methods are not without risk. Although in the manuscripts only minor complications were reported, in two abstracts perforation was said to occur after ABPC. Furthermore, the number of reported patients treated with electrocoagulation is small. The results of ongoing studies on electrocoagulation are therefore awaited.

From the present literature, it seems that electrocoagulation, and especially MPEC, results in more complete ablation compared with laser ablation, probably by producing deeper lesions. Also, from the results of laser ablation it seems that the more superficial the ablation (argon and KTP), the more often residual Barrett's epithelium is found. Finally, islands of residual columnar epithelium underneath regenerated squamous mucosa (pseudoregression) have been reported using MPEC, ABPC and KTP laser.

Chemical ablation

Nine authors have published their results of PDT treatment in 233 patients, 63 of them being reported in articles (Table 2.2). Exogenous porphyrins (HpD, DHE) were used in 45 patients and the endogenous sensitizer ALA was used in 18 patients. After PDT treatment with exogenous photosensitization, residual Barrett's oesophagus was reported to occur in 58% (26 of 45) of patients after 2 to 62 months follow-up. Besides skin photosensitivity, the main disadvantage of exogenous porphyrins was the formation of strictures in up to 45% of the patients, requiring endoscopic dilation in most cases. After PDT treatment with endogenous photosensitization, residual Barrett's epithelium was found in 94% (17 of 18) of patients after 1 to 44 months of follow-up. Four patients did not respond to ALA-PDT at all. Skin photosensitivity and strictures have not been reported after ALA-PDT. Chemical ablation was used in 47 patients with Barrett's oesophagus with HGD. Residual HGD was found in 10% (3 of 30) after PDT with exogenous photosensitisation and in 6% (1 of 17) after ALA-PDT.

Legend to Table 2.1

Abbreviations: a = abstract; l = letter; MPEC = multipolar electrocoagulation; ABPC = argon beam plasma coagulation; ND = no dysplasia; LGD = low-grade dysplasia; MGD = moderate-grade dysplasia; HGD = high-grade dysplasia; ? = not mentioned by the author; FU = follow-up (in months); BO = Barrett's oesophagus; CR = complete remission (no residual BO left). Sessions are expressed per patient.
Table 2.2 Results of Studies Performing Chemical Ablation Therapy (Photodynamic Therapy) for Barrett’s Oesophagus.

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Treatment/wavelength (nm)</th>
<th>Patients (n), Grade of Dysplasia</th>
<th>FU (months)</th>
<th>Residual HGD</th>
<th>Residual BO</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinelli</td>
<td>1992b</td>
<td>DHE/630</td>
<td>3 HGD</td>
<td>6-84</td>
<td>0</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>Laukka</td>
<td>1995</td>
<td>HpD/630</td>
<td>5 (1 HGD)</td>
<td>6</td>
<td>0</td>
<td>5</td>
<td>skin photosensitivity</td>
</tr>
<tr>
<td>Overholt</td>
<td>1995</td>
<td>DHE/630</td>
<td>7 (1 LGD, 6 HGD)</td>
<td>6-54</td>
<td>1</td>
<td>4</td>
<td>1 patient developed a T1 carcinoma during FU, skin photosensitivity</td>
</tr>
<tr>
<td>Overholt</td>
<td>1996</td>
<td>DHE/630</td>
<td>30 (10 LGD, 20 HGD)</td>
<td>6-62</td>
<td>2</td>
<td>17</td>
<td>in 58% stricture formation, in some patients additional Nd:YAG laser ablation of residual islands of BO</td>
</tr>
<tr>
<td>Overholt</td>
<td>1997a</td>
<td>DHE/630</td>
<td>62 (16 LGD, 46 HGD)</td>
<td>6-60</td>
<td>14*</td>
<td>39</td>
<td>2 pseudoregression, in 45% stricture formation</td>
</tr>
<tr>
<td>Wang</td>
<td>1997</td>
<td>HpD/630</td>
<td>64 (23ND,32LGD,9HGD)</td>
<td>25±2</td>
<td>5</td>
<td>57</td>
<td>BO with ND showed longer trajectory of regression than HGD</td>
</tr>
<tr>
<td>Wang</td>
<td>1997a</td>
<td>HpD/630</td>
<td>6 HGD</td>
<td>?</td>
<td>1</td>
<td>4</td>
<td>in 20% stricture formation</td>
</tr>
<tr>
<td>Barr</td>
<td>1996</td>
<td>ALA/630</td>
<td>5 HGD</td>
<td>26-44</td>
<td>0</td>
<td>4</td>
<td>2 pseudoregression</td>
</tr>
<tr>
<td>Gossner</td>
<td>1997</td>
<td>ALA/635</td>
<td>10 HGD</td>
<td>1-11</td>
<td>0</td>
<td>10</td>
<td>2 pseudoregression</td>
</tr>
<tr>
<td>Biddlestone</td>
<td>1998</td>
<td>ALA/630</td>
<td>3 (1 LGD, 2 HGD)</td>
<td>3-21</td>
<td>1</td>
<td>3</td>
<td>3 pseudoregression</td>
</tr>
<tr>
<td>Ackroyd</td>
<td>1997a</td>
<td>ALA/514</td>
<td>18 LGD</td>
<td>?</td>
<td>?</td>
<td>18</td>
<td>prospective randomized trial, 2 patients showed no response</td>
</tr>
<tr>
<td>Orner</td>
<td>1997a</td>
<td>ALA/632</td>
<td>9 (all kinds of dysplasia)</td>
<td>?</td>
<td>?</td>
<td>5</td>
<td>ALA application in spray, 2 patients showed no response</td>
</tr>
</tbody>
</table>
In conclusion, chemical ablation therapies can also reverse Barrett's oesophagus. However, complete remission is achieved only in a minority of patients. Probably, PDT with exogenous photosensitization induces deeper damage than ALA-PDT, which results in less complete remission after ALA-PDT. However, in contrast to HpD/DHE-PDT, skin photosensitivity and stricture formation did not occur after ALA-PDT. After PDT treatment of patients with Barrett's oesophagus and HGD, residual HGD was found in approximately 10% of patients. Pseudoregression has been described with exogenous (two patients) and endogenous (seven patients) photosensitization.78,33,34,66

Mechanical ablation

Results of endoscopic mucosal resection in Barrett's oesophagus have not yet been reported.83 Studies on oesophageal endoscopic mucosal resection focus on early oesophageal cancer.36 Surgical ultrasound has not yet been used in human studies. However, a study in pigs showed that endoscopic mucosal resection resulted in complete ablation of the squamous epithelium of the lower oesophagus in a single setting.24,64

CONCLUSION

Barrett's oesophagus is a premalignant condition. In particular, HGD in Barrett's oesophagus carries an increased risk for developing adenocarcinoma. The treatment of HGD in Barrett's oesophagus, surgery or an endoscopic surveillance program, is still controversial. An argument in favour of resection is that adenocarcinoma is frequently found in resection specimens of patients operated for HGD in Barrett's oesophagus. Moreover, survival increases with early detection. In contrast, mortality and morbidity of oesophageal resection is considered to be high in view of a premalignant disease. The natural course of HGD, the reliability of the endoscopic and histological diagnosis, the risks of both therapies, and the life expectancy and co-morbidity of each individual patient generally determine the choice of treatment.

Legend to Table 2.2

Abbreviations: a = abstract; b = chapter in book; l = letter; MPEC = multipolar electrocoagulation; ABPC = argon beam plasma coagulation; ND = no dysplasia; LGD = low-grade dysplasia; MGD = moderate-grade dysplasia; HGD = high-grade dysplasia; * = not specific HGD but dysplasia in general; ? = not mentioned by the author; FU = follow-up (in months); BO = Barrett's oesophagus; CR = complete remission (no residual BO left). Sessions are expressed per patient.
This review summarizes the results of 37 studies on experimental endoscopic therapies for Barrett's oesophagus. These therapies focus on the elimination of Barrett's epithelium with regeneration of squamous epithelium. All endoscopic surgical ablation techniques, in combination with antireflux treatment, can result in reversal of both HGD and Barrett's oesophagus. However, approximately 50% of patients treated by endoscopic ablation have residual Barrett's mucosa, which theoretically places them at risk for development of HGD and carcinoma. The deepest lesions and consequently lowest rate of residual Barrett's oesophagus (approximately 20%) are achieved by electrocoagulation. This method requires multiple sessions and is relatively prone to complications (specifically perforation).

In contrast, ALA-PDT causes a more superficial and controllable ablation; however, complete reversal of Barrett's epithelium has been achieved only in approximately 10% of patients. Optimization of the illumination parameters or repeated treatment regimens may improve these results. Furthermore, optimization of the laser parameters, using a low penetrating wavelength of laser light (e.g. green light and not red light), and/or using a 180° or 240° windowed cylindrical light distributor and not circumferential (360°) illumination, may prevent the occurrence of strictures, which are frequently found after PDT with the use of exogenous porphyrins.30,77,85

Estimating the real value of ablation therapies is not yet possible, as most reports are case reports, abstracts or non-randomized personal experiences. Only two prospective randomized trials have been published.70,82 The results of these two studies are not encouraging. In the first study, photoablation with Nd:YAG laser was performed in four patients.70 It was shown that the treatment had no effect on these patients compared with the four untreated patients. In the second study, which was a double blind, randomized, placebo-controlled study, ALA-PDT was performed in 18 patients with LGD; 18 other patients with LGD were illuminated without prior ALA administration (they received a placebo).82 In two patients treated with ALA-PDT no response was observed; 16 patients showed a mean decrease in the length of Barrett's oesophagus of 31%. No complete reversal of Barrett's oesophagus was achieved. In the placebo group, a small decrease in the length of Barrett's oesophagus was observed in two patients, which was significantly less than in the ALA group.

Interpretation of the results summarized in the present review is even more difficult, as different treatment schemes were used, the definitions of complete response were not uniform, and histological confirmation of the response was not achieved in all studies. Furthermore, some reports either did not indicate the time of follow-up, or described the results after only a very short follow-up. This will lead to relatively good initial results; however, Barrett's epithelium recurs after a
longer follow-up. Krevsky et al. found, in 11 patients treated with Nd:YAG photoablation, no evidence of Barrett’s oesophagus at 2 months of follow-up. At 6 months, however, in three of five evaluable patients, Barrett’s oesophagus was found and, at 12 months, the only evaluable patient had a recurrence of Barrett’s oesophagus.

In conclusion, the treatment parameters of the various ablation therapies should be optimized and the long term benefits of the therapies should be carefully evaluated in prospective randomized trials. Until then, the use of ablation therapy for Barrett’s oesophagus with HGD should remain investigational.
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CHAPTER 3

FUNDAMENTALS OF
5-AMINOLEVULINIC ACID-BASED
PHOTODYNAMIC THERAPY
INTRODUCTION

The observation that normal tissue reacts in a pathological fashion when saturated with porphyrins and exposed to light (porphyrias), has led to the idea to employ this photoreaction in the destruction of pathological tissue. This idea was aided by the early descriptions around 1930 of the uptake of porphyrins by tumour cells, and of the preferential uptake of haematoporphyrin by rat sarcoma. In the following years, porphyrins and haematoporphyrin derivative (and its more purified form Photofrin®) in particular, were investigated as possible photosensitive agents (photosensitizers). Indeed, activation of the photosensitizer by illumination with light of an appropriate wavelength created a photochemical reaction causing photodamage to the tumour. The term photodynamic therapy (PDT) was born and especially the alleged tumour selectivity of photosensitization and the subsequent development of suitable lasers made PDT an area of major investigation since 1970.

PHOTOPHYSICS AND PHOTOCHEMISTRY OF PDT

The foundation of PDT depends on visible light, photosensitizer and oxygen. When photosensitizers absorb light energy (porphyrins absorb light of wavelength around 400 nm and, in addition, the more penetrating light of longer wavelength subsidiary peaks between 500 and 635 nm), orbital electrons are excited and "promoted" to a higher orbital (Figure 3.1).

If such promotion occurs without alteration of the ground-state electron 'spin',

![Figure 3.1 Schematic representation of porphyrin energetic transactions. The type II photochemical reaction is thought to be the most important mechanism of photodynamic therapy cytotoxicity.](image-url)
it is said to be in a singlet excited state. If its spin is reversed so that it is parallel to the ground-state spin, it is said to have achieved a triplet excited state. The latter state is termed metastable, because of its relatively longer half-life than the singlet state, which has an extremely short half-life. Three types of events can occur with porphyrins in excited state (Figure 3.1). First, excited electrons can fall back to the ground state producing fluorescence or phosphorescence. Second, conversion of singlet to triplet state can occur (intersystem crossing). This occurs infrequently because of the instability of the singlet state. Finally, the excited porphyrins can undergo a photoreaction.

Two types of photoreaction can occur. Excited porphyrins in the singlet state can react directly with tissues or molecules (type I reaction) to cause electron transfer and produce radicals. These radicals can react with oxygen to give oxidized products (not only superoxide radicals but also highly reactive hydroxyl radicals). The triplet excited porphyrin state allows transfer of the excitation energy to molecular oxygen (type II reaction) thereby producing the highly reactive singlet oxygen (oxygen radical). This is an extremely powerful oxidizing agent, which acts not only on molecular species within the cell, but also with cellular structures such as membranes.2,12,13 Probably singlet oxygen is the most important cytotoxic agent responsible for the destruction of tumours.14 At low oxygen concentrations, type I mechanisms may significantly contribute to the photo-oxidation of membrane components and amino acids.15 Furthermore, the mechanism of action may change during PDT from a type II to a type I process as a result of PDT-induced changes in the oxygenation within the tumour tissue.16

Owing to the short intracellular lifetime of the biochemical active singlet oxygen species, their diffusion range is predicted to be limited to approximately 45 nm in cellular media.17,18 Because the diameter of human cells ranges from approximately 10 to 100 μm, the site of the primary generation of singlet oxygen determines which subcellular structures may be damaged. Therefore, the subcellular localization, which will be different using exogenously administered or endogenously produced porphyrins, partly determines the effect of PDT.

PHOTOBIOLOGY OF PDT

The necrotic processes which trigger the degradation of normal and malignant cells after PDT seem to be mediated by similar chemical reactions. The major biological targets of singlet oxygen and of most toxicologically relevant radical intermediates are nucleic acids, which are broken down into fragments.16 However, photosensitizers tend not to localize in the nucleus of the cell. As a consequence, DNA is not directly subjected to PDT-induced photodamage.
It is more likely that the function of proteins and enzymes are strongly impaired by photochemical modifications at active and/or binding sites. Transmembrane ion pumps, sugar and amino acid transport systems are inhibited and enzymes lose their biocatalytic activity.\textsuperscript{19} Cellular membranes, including plasma, mitochondrial and nuclear membranes, are severely damaged by oxidation of susceptible, unsaturated fatty acid residues and of cholesterol.\textsuperscript{20,21} Following PDT, electron micrographs show a disintegration of membranes and physicochemical measurements show a continuing depletion of the intracellular adenosine triphosphate (ATP) pool, indicating a reduced activity of the mitochondria.\textsuperscript{22,23} The decrease in intracellular ATP concentration has drastic consequences for all energy-consuming processes and causes a reduced rate of replication and repair. As the driving force for the transport of amino acids into the cell, dysfunction of $\text{Na}^+ / \text{K}^+$-ATPase leads to a decreased absorption of amino acids.\textsuperscript{24}

The breakdown of cellular membranes causes a release of phospholipids, which are readily attacked by phospholipases and degraded to free fatty acids.\textsuperscript{25} Of major importance is the release of arachidonic acid which initiates an acute inflammatory reaction. Prostaglandines primarily cause a vasodilatation of terminal arterioles and damage endothelial and smooth muscle cells which coat the inner surface of blood vessels.\textsuperscript{26} The gaps created between endothelial cells lead to local oedema by an enhanced efflux of water, macromolecules and blood cells into the tumour tissue.\textsuperscript{27} Moreover, thromboxanes promote the aggregation of platelets and trigger the formation of intravasal thrombi.\textsuperscript{28} The stasis of blood flow finally creates areas of local hypoxia and initiates necrotic processes by nutrient deprivation.

The acute inflammatory reaction is followed by a delayed inflammatory reaction, characterized by activation of the complement system and subsequent generation of a wide variety of products with chemotactic, inflammatory and cytotoxic properties. Histologically the delayed phase of inflammation begins with the infiltration of neutrophils.\textsuperscript{29} Attracted by chemotactic complement factors and by products of damaged tissues, neutrophils pass through gaps in the capillary endothelium. The infiltration of neutrophils is immediately followed by an invasion of leukocytes and macrophages, which act synergistically in a complicated sequence. Thus, the release of a wide variety of pathophysiologically highly reactive products, such as prostaglandines and thromboxanes, together with the activation of the complement system and infiltration of immunologically active blood cells ultimately initiates tumour necrosis.

Although the mechanism of PDT-induced cell death is largely attributable to necrosis, recently an apoptotic mechanism has been implicated also.\textsuperscript{30,31} The apoptotic program is a part of the genetic apparatus of the cell, such that certain signals lead to a series of events resulting in DNA and cellular fragmentation. At present, the contribution of apoptosis to PDT-induced damage is not well understood.
PROBLEMS WITH EXOGENOUS PHOTOSENSITIZATION

It is becoming more and more clear that porphyrins (Photofrin®) have a low selectivity with regard to uptake and retention by tumour versus normal cells. Other metabolically active tissues such as liver and kidney also accumulate porphyrins, but being remote from light sources, these are relatively safe from the photodestructive effects of therapy. However, normal skin also accumulates porphyrins, and a serious side effect of PDT using Photofrin® is continued light photosensitivity of exposed skin for several weeks or months after treatment.11 Further drawbacks of Photofrin® are the lack of chemical homogeneity and stability and its unfavourable physicochemical properties. New, so-called second and third generation photosensitizers are therefore under investigation.

In this thesis the third generation photosensitizer 5-aminolevulinic acid (ALA) is used. The following paragraphs describe the fundamentals of ALA-based PDT.

INTRODUCTION TO ALA-PDT

As outlined in the previous paragraphs, porphyrins in general are photosensitive agents. A naturally occurring particularly photosensitive form of porphyrin is protoporphyrin IX (PpIX). The iron complex of PpIX is called haem. Haem may bind to different proteins to form key biomolecules such as haemoglobin, myoglobin, cytochromes, catalase, peroxidase and tryptophan pyrrolase. Metabolism of porphyrins is therefore of crucial importance to maintain homeostasis. Almost all types of cells of the human body, with the exception of mature red blood cells, are equipped with a machinery to synthesize haem, and thus PpIX. PpIX has been used for photosensitization by modulating the haem synthetic pathway.

REGULATION OF HAEM SYNTHESIS

The initial step in the haem biosynthetic pathway is the formation of 5-aminolevulinic acid (ALA) (Figure 3.2).

In mammals and photosynthetic bacteria, ALA is formed from glycine and succinyl-CoA by the enzyme ALA synthase, located at the matrix side of the inner mitochondrial membrane.32 This is the main regulatory enzyme of the haem synthetic pathway. The next enzyme in the pathway, ALA dehydratase is located in the cytosol and induces the condensation of two molecules of ALA to yield porphobilinogen (PBG).33 The concerted action of PBG deaminase and
Uroporphyrinogen III (co)synthase condense four molecules of PBG and cyclize the tetrapyrrole chain to form uroporphyrinogen III. Both enzymes are located in the cytosol and the action of PBG deaminase is the rate limiting step. Uroporphyrinogen decarboxylase removes four acetic acid carboxyl groups from uroporphyrinogen to form the tetracarboxylic coproporphyrinogen III. Coproporphyrinogen III is decarboxylated and oxidized to protoporphyrinogen IX by coproporphyrinogen oxidase, which is situated in the intermembrane space of the mitochondria. The final step in the synthesis of PpIX is the oxidation of the tetrapyrrole ring by removal of six hydrogens, catalysed by protoporphyrinogen oxidase. The enzyme is embedded in the inner mitochondrial membrane with its active site on the matrix side of the membrane. It is an oxygen-dependent enzyme with high substrate-specificity. Protoporphyrinogen IX may also spontaneously oxidise to PpIX, but this is less likely to occur in the mitochondria where protoporphyrinogen IX is exposed to a rather anaerobic and reducing environment. The tetrapyrrole structure is now ready for incorporation of iron to form haem, which is catalyzed by ferrochelatase. Ferrochelatase is located in the inner mitochondrial membrane.

![Figure 3.2 Schematic representation of the haem biosynthetic pathway.](image-url)
PHOTOBIOLOGY OF ALA-PDT

The haem biosynthetic pathway is mainly regulated by the substrate availability and feedback inhibition of ALA synthase. Haem can inhibit this enzyme directly as well as its transcription, translation, and transport of the protein into mitochondria.\textsuperscript{36} The administration of exogenous ALA bypasses the rate limiting enzyme ALA synthase and the feedback control, and may thus induce intracellular accumulation of photosensitizing concentrations of PpIX.

In 1987 ALA-based photosensitization of cells \textit{in vitro} was pioneered.\textsuperscript{37} In the same year the first \textit{in vivo} animal study on ALA photosensitization was performed.\textsuperscript{38} In 1990 ALA has been introduced as a drug for clinical photodynamic therapy (PDT).\textsuperscript{39}

In most cell lines treated with ALA the main photosensitizer that accumulates is PpIX, indicating that the enzymes converting uroporphyrinogen III to PpIX are not rate limiting in the pathway.\textsuperscript{40-43} Because PpIX is formed in the mitochondria, photosensitivity following ALA administration is probably located in the mitochondria. ALA-PDT mainly exerts its effect through formation of singlet oxygen (type II photoreaction) although other reactive oxygen species may also be involved.\textsuperscript{42,44} As mentioned in the second paragraph of the present chapter, the range of action of singlet oxygen is very short.\textsuperscript{45} Therefore, the primary sites of action of ALA-PDT may be restricted to the sites of PpIX production and/or accumulation. Owing to the mitochondrial localization of PpIX, mitochondria have been shown to be damaged after ALA-PDT and most likely, they are the most important targets in the cytotoxicity of ALA-PDT.\textsuperscript{43,46} Additionally, other compartments are damaged by ALA-PDT. This is indicated by the observed damage to endoplasmatic reticulum, increased level of intracellular free Ca\textsuperscript{2+} and Na\textsuperscript{+} and cellular loss of K\textsuperscript{+}.\textsuperscript{42,46,47}

Because photosensitizers that "bound to mitochondria" are especially able to induce apoptosis upon photo-irradiation, ALA-based PDT in particular may be able to inactivate cells \textit{via} apoptosis.\textsuperscript{48}

Although vascular damage induced by PDT with exogenous porphyrins plays a crucial role in tumour eradication, the therapeutic effects of ALA-PDT are most likely due to direct cytotoxicity and acute inflammatory reactions with little damage to vascular structures.\textsuperscript{33}
ADVANTAGES OF ALA-PDT

As mentioned earlier in this chapter, the main drawbacks of exogenous porphyrin-mediated PDT are low tumour selectivity and prolonged skin photosensitivity. In contrast, skin photosensitivity with use of ALA lasts only 24 to 48 hours.\textsuperscript{35,49,50} Concerning tumour selectivity, the rate of ALA-derived PpIX synthesis has been shown to be higher in neoplastic cells than in untransformed cells.\textsuperscript{51,52} Several \textit{in vivo} animal studies have shown semi-selective ALA-induced PpIX accumulation in tumours compared to normal tissue.\textsuperscript{50,53-57} The tumour selectivity of ALA-derived PpIX accumulation appears to be related to tumour model used, time, ALA dose and route of administration. In general, the activities of ALA synthase and ferrochelatase were found to be lower in tumour cell lines than in normal cell lines, whereas those of ALA dehydratase and PBG deaminase were higher in malignant cell lines.\textsuperscript{33,58-61} Besides in tumours, ALA-induced PpIX seems to accumulate primarily in glands and tissues that line surfaces (epidermis, respiratory and gastrointestinal mucosa, endometrium, urothelium) and only little in the underlying submucosal and muscle layers.\textsuperscript{33,59,62-65} Therefore, ALA-PDT seems most suitable for premalignant and malignant lesions of epithelial origin, such as Barrett's oesophagus.
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PART II

ANIMAL MODEL
CHAPTER 4

ANIMAL MODEL FOR BARRETT'S OESOPHAGUS IN THE RAT AND OPOSSUM

This chapter has been adapted from Van den Boogert et al. Gastroenterology (submitted).
INTRODUCTION

Barrett’s oesophagus is defined as the presence of metaplastic columnar mucosa with goblet cells in the oesophagus (Chapter 1). The pathogenetic stimulus that causes Barrett’s metaplasia is still subject of research. Acid reflux has long been thought to be the only pathogenetic factor. Recently however, it was hypothesized that reflux of alkaline duodenal content might be involved in the pathogenesis of Barrett’s oesophagus, also. The risk of oesophageal adenocarcinoma in patients with Barrett’s oesophagus appears to be about 30- to 50-fold above that in general population, with an estimated incidence of about 1 in 150 patient years. Patients with Barrett’s oesophagus are treated with antireflux medication or antireflux surgery. However, these therapies have no effect on the Barrett’s oesophagus itself and malignant degeneration can still proceed. New forms of endoscopic ablation therapy (e.g. photodynamic therapy) intending to ablate the Barrett’s epithelium with regeneration of squamous epithelium are under investigation. To evaluate the various endoscopic ablation therapies and to study the pathogenesis of Barrett’s oesophagus, an animal model for Barrett’s oesophagus is needed. At present, the animal models for Barrett’s oesophagus that have been reported describe a small area (less than one millimeter) or small islands of Barrett’s epithelium, some even beneath the squamous epithelium, making them useless for evaluation of new forms of therapy for Barrett’s oesophagus.

Defining Barrett’s oesophagus in studies that have performed an oesophago-duodenostomy or oesophagojejunostomy to induce duodenal reflux is difficult as the oesophagus is in continuum with the small intestine. To differentiate between normal duodenal or jejunal mucosa (including goblet cells) and Barrett’s mucosa (intestinal metaplasia), especially adjacent to the anastomosis, can be very difficult. Therefore, defining Barrett’s oesophagus as the presence of any unequivocal columnar epithelium above the anastomosis remains controversial.

Another difficulty in interpreting the results of rat studies is caused by the difference in the anatomy and histology of the upper gastrointestinal tract compared to man. First, the rat oesophagus is lined by keratinized squamous epithelium whereas the human oesophagus is lined by non-keratinized squamous epithelium. Second, the rat has a squamous forestomach, so that the squamocolumnar junction is located within the stomach. Third, the rat oesophagus lacks submucosal glands that are found in the human oesophagus. This is of particular interest as Barrett’s oesophagus is thought to originate from a multipotent undifferentiated stem cell of oesophageal origin, probably located in oesophageal gland ducts, and not from proximal migration of cardiac columnar epithelium, as formerly thought. Finally, rats do not have a gall-bladder which results in a continuous flow of bile through the common bile duct into the small intestine.
In contrast to the rat, the upper gastrointestinal tract of the opossum, a marsupial, is very similar to man. The opossum therefore, has been used as an animal model for physiological oesophageal studies.\textsuperscript{16,17} To date, however, no studies on Barrett’s oesophagus have been reported in this species.

The present study aimed to develop a reliable and practical animal model for Barrett’s oesophagus. For that purpose, the development of Barrett’s epithelium by induction of duodeno-oesophageal reflux was studied both in the rat and in the opossum.

MATERIALS AND METHODS

Rats

Twenty-three male inbred Wag/Rij rats (Harlan CPB, Austerlitz, The Netherlands), weighing 200-275 grams were used. The animals had free access to rat chow (AM II, Hope Farms, Woerden, The Netherlands) and tap water acidified to pH 4.0.

Opossums

Twenty-three female gray short-tailed opossums (\textit{Monodelphis domestica}), weighing approximately 90 grams, were used. They were kindly provided by Professor A.P. Payne (University Glasgow, United Kingdom), who obtained the animals from the Southwest Foundation for Biomedical Research, San Antonio, Texas, USA. The animals were housed individually in plastic rat cages containing wooden shavings and nesting material. Animals were fed commercial wet and dry cat food (Rokus meat chunks, De Haan, Nieuwkoop, The Netherlands and Catfood 2572, Hope Farms, Woerden, The Netherlands) and water ad libitum.

Surgical technique

Ether anaesthesia was used for the rats. The opossums were anaesthetized by a combination of the following intramuscular anaesthesia: ketamine (4 mg/animal), xylazine (0.2 mg/animal) and midazolam (0.2 mg/animal). If necessary, additional ketamine (maximal 4 mg/animal) was given. An oesophagojejunostomy according to the model of Levrat was performed through a median laparotomy.\textsuperscript{18} The oesophagus was dissected from the stomach and anastomized end to site (interrupted 7/0 prolene sutures) to the jejunum approximately 3 cm distal from the entry of the common bile duct. The abdominal wall of the rats was closed in one layer, the abdominal wall of the opossums in two layers. Additionally, the opossum abdominal wall was tightly packed with plaster until day 5 postoperatively, as these animals would otherwise remove their sutures by gnawing through them. After
surgery the animals received 2 ml glucose 10% subcutaneously and water ad libitum. Chow was provided after 24 hours.

**Design of the study**

Three rats and three opossums served as unoperated controls. The remaining animals underwent an oesophagojejunostomy with gastric preservation to induce reflux of gastric and duodenal (pancreatic and biliary) juice. Ten rats and 10 opossums were sacrificed at 8 months after surgery and 10 animals of both species at 12 months after surgery.

**Handling of specimens**

Animals were weighed just before sacrifice. The thoracic and abdominal cavities were inspected and the oesophagus, the anastomosis, and the proximal jejunum were excised en bloc. The oesophagus was opened longitudinally, the circumference was measured, and the specimen was curled up from distal to proximal (Swiss roll) and fixed in formalin.

**Histological examination**

Specimens were embedded in paraffin wax, sectioned and stained with haematoxylin and eosin (H&E), periodic acid-Schiff (PAS) and alcian blue. The sections were microscopically evaluated by a histopathologist blinded for the species and the treatment. The oesophagus was examined for hyperkeratosis, squamous hyperplasia, erosions, metaplastic columnar epithelium, dysplasia, benign papillomas, and carcinoma. A specimen was defined as having squamous hyperplasia when there was papillary elongation or basal cell hyperplasia, occasionally resulting in the formation of intramural cysts. Dysplasia was diagnosed according to standard criteria and included dysplasia arising in either glandular or squamous epithelium. Barrett's oesophagus was defined as oesophageal mucosa lined by columnar and goblet cells.

**Composition of bile**

Bile was collected in five opossums by aspirating bile from the gall bladder after sacrifice. In five rats the common bile duct was cannulated and bile was collected for approximately one hour. Thereafter, rats were sacrificed.

Total bile acids were analysed enzymatically according to Mashige. The quantitation is based on enzymatic dehydroxylation of the 3α-OH group of the steroid moiety which is present on all bile acids.

Individual bile acids concentration in bile samples was then determined gaschromatographically. 23-Nordeoxycholic acid (Steraloids Inc. Wilton, N.H. USA) was added as internal standard to individual bile samples.
standard (5.3 mmol l⁻¹) was added to 100 µl of rat bile or 25 µl of opossum bile. The bile acids were enzymatically hydrolysed with cholyglycinehydrolase (Sigma Aldrich Chemie, Zwijndrecht, The Netherlands) during 16 hours at 37°C. Thereafter, the bile acids were extracted twice with chloroform and the extract dried under nitrogen. The unconjugated bile acids were then derivatised to their methylesteracetate derivatives according to Lillington et al.²² Gaschromatography was performed on a HP 5880 equipped with a Split/Splitless injector (Hewlett Packard, Amsterdam, The Netherlands) and a 15 m, 0.22 mm ID fused silica capillary column coated with 0.2 µm CP-Sil19CB (Chrompack, Middelburg, The Netherlands). Gaschromatographic conditions were as follows: carrier gas (Helium) 2 ml min⁻¹; injector and detector temperature 300°C; oven temperature 250°C during 2 minutes and raised to 290°C at 8°C min⁻¹. One µl split injection was done with a split ratio of 30:1.

The ratio glycine:taurine was determined according to Blom et al.²³ After enzymatic deconjugation the bile acids were extracted using chloroform. The remaining chloroform in the hydrolysate was removed by aeration with nitrogen gas and 0.2 ml hydrolysate was mixed with 0.2 ml sulfosalicylic acid (6% wt./vol.) and centrifuged for 8 min at 14000g. Depending on the bile acid concentration, the supernatant was diluted with water (estimated end bile acid concentration 500 µmol l⁻¹) and mixed with 1 part of sulfosalicylic acid (6% wt./vol.) containing norleucine as internal standard. Glycine and taurine concentrations were determined in the hydrolysate by ion-exchange chromatography on a Biochrom 20 Amino Acid Analyser (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

Statistical analysis

The length of the Barrett’s oesophagus and the weight of the animals are expressed as mean ± standard error of the mean (SEM). Comparisons were made using Student’s t-test. A difference was considered to be significant at P values of <0.05.

RESULTS

General observations

Two of the opossums were found dead at the day after surgery, due to opening of the abdominal wound by gnawing through plaster and sutures. Another two animals died at week 5 after surgery due to stenosis of the anastomosis resulting in a dilated oesophagus and weight loss. One opossum was found dead at week 10 due to an abces near the anastomosis.

The 15 remaining animals recovered quickly from surgery and were very active.
until sacrifice. They ate and drank normally and did not lose weight (Table 4.1). Seven opossums were sacrificed at 8 months, and eight animals at 1 year after surgery.

In contrast to the opossums, the rats did not thrive well and lost weight following the oesophagojejunostomy. Two animals died of anastomotic stenosis and seven of complications from reflux oesophagitis such as malnutrition and pneumonia.

In the postoperative period, all operated rats lost weight or gained less weight than control animals ($P<0.01$) (Table 4.1). Seven rats were sacrificed at 8 months and four animals at 12 months after surgery.

Table 4.1 Incidence and length of Barrett’s oesophagus (BO) and weight of opossums and rats.

<table>
<thead>
<tr>
<th>groups (n)</th>
<th>BO (n)</th>
<th>length of BO (mm)</th>
<th>weight (grams)</th>
</tr>
</thead>
<tbody>
<tr>
<td>opossums</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>controls (3)</td>
<td>0</td>
<td>0</td>
<td>96 ± 4</td>
</tr>
<tr>
<td>8 months (7)</td>
<td>0</td>
<td>0</td>
<td>93 ± 4</td>
</tr>
<tr>
<td>12 months (8)</td>
<td>2</td>
<td>0.4 ± 0.3</td>
<td>97 ± 5</td>
</tr>
<tr>
<td>rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>controls (3)</td>
<td>0</td>
<td>0</td>
<td>386 ± 21</td>
</tr>
<tr>
<td>8 months (7)</td>
<td>6</td>
<td>6.1 ± 1.4</td>
<td>274 ± 46*</td>
</tr>
<tr>
<td>12 months (4)</td>
<td>4</td>
<td>16.5 ± 1.6</td>
<td>255 ± 34*</td>
</tr>
</tbody>
</table>

* $P<0.01$ compared to the control group.

Histology

Macroscopically, marginal thickening of the longitudinal white folds could be observed in the distal oesophagus of operated opossums. No other abnormalities were noticed. In operated rats, the distal and middle oesophagus was thickened and dilated. The distal part had a smooth pink aspect, in most cases separated from the white squamous lining of the proximal oesophagus by a zone with an uneven surface with erosions.

Microscopically, in control animals, the differences between the opossum and rat oesophagus were clearly visible (Figures 4.1 and 4.2).
Figure 4.1 Section of the distal oesophagus of a control opossum, lined by non-keratinized squamous epithelium. Oesophageal glands, staining positively with PAS and alcian blue, are visible in the submucosa (x40, H&E staining).

E=epithelium, G=submucosal gland, K=keratin layer, M=muscularis propria, SM=submucosa.

The opossum oesophagus is lined by non-keratinized squamous epithelium and has submucosal glands, whereas rats lack these glands and have keratinized squamous epithelium. In control opossums, the epithelial layer was slightly thicker and the papillae more pronounced in the distal oesophagus than in the proximal oesophagus. In operated opossums, the junction between columnar jejunal epithelium and squamous oesophageal epithelium was located exactly at the site of the anastomosis (marked with the sutures) in all seven animals in the 8-months group and in six out of eight animals in the 12-months group (Table 4.1).

One opossum in the latter group showed microscopic foci of specialized intestinal metaplasia of 1 mm and one of 2 mm length (Figure 4.3). The squamous epithelium showed very mild regenerative thickening and was very sparsely infiltrated by neutrophils.

Figure 4.3 Distal opossum oesophagus 1 year after oesophago-jejunostomy. The section shows microscopic foci of specialized intestinal metaplasia (length 1 mm) (x20, PAS staining).

J=jejunum, M=foci of specialized intestinal metaplasia, S=squamous epithelium.
In rats, Barrett’s oesophagus was found in the distal oesophagus from the anastomosis to 6.1 ± 1.4 mm (8 months) and 16.5 ± 1.6 mm (12 months) cranial from the sutures (Table 4.1, Figure 4.4).

Figure 4.4 Rat oesophagus after 1 year of gastroduodenal-oesophageal reflux, showing macroscopic Barrett's oesophagus of almost 2 cm length. The oesophagus with a small piece of jejunum was opened longitudinally and curled up from distal to proximal (x10, H&E staining). A=site of anastomosis, BO=Barrett's oesophagus, E=erosion, J=jejenum, S=squamous epithelium.

On haematoxylin and eosin staining, Barrett’s oesophagus consisted of columnar epithelium with intestinal metaplasia. This glandular epithelium was quite different from the adjacent jejunal epithelium (Figure 4.5). The villi were less pronounced and the mucosa resembled gastric mucosa (with intestinal metaplasia), with glands and foveolae instead of crypts and villi as found in the jejunum.

Also on PAS and alcian blue staining the difference between Barrett’s oesophagus and jejunum was clear. No dysplasia was found in the columnar lining. Proximal from the Barrett’s oesophagus an area of erosion and inflammation with cellular infiltration and vasodilatation was apparent in all animals. Proximal from this area a rather small zone of hyperplastic squamous epithelium with some hyperkeratosis was found, after which the oesophagus was lined by normal squamous epithelium.
Figure 4.5 Distal rat oesophagus exposed to gastroduodenal reflux during 1 year. Cranial from the sutures of the oesophagojejunal anastomosis, the oesophagus was lined by columnar epithelium with intestinal metaplasia and characteristic goblet cells (arrow) (A). This glandular epithelium is quite different from the adjacent jejunal epithelium (B). It resembles gastric mucosa, with glands and foveolae instead of crypts and villi as found in the jejunum (x40, H&E staining).

**Composition of bile**

Total bile acid concentration in bile in the gall bladder of opossums was comparable to the bile acids concentration in human gall bladder bile. Total bile acid concentration in opossums was 4 to 5 times higher than the concentration in bile in the common bile duct in rats (Table 4.2).

**Table 4.2** Total bile acid concentration and percentage of individual bile acids in rat bile (collected in the common bile duct) and opossum bile (collected in the gall bladder). Values are expressed as mean ± standard error of the mean (n=5 in both groups). The data on human bile (collected in the gall bladder) are taken from the literature.

<table>
<thead>
<tr>
<th></th>
<th>rats</th>
<th>opossums</th>
<th>man</th>
</tr>
</thead>
<tbody>
<tr>
<td>total bile acid concentration (mmol L⁻¹)</td>
<td>39±3</td>
<td>166±28</td>
<td>50-200</td>
</tr>
<tr>
<td>cholic acid (%)</td>
<td>86±2</td>
<td>42±7</td>
<td>35</td>
</tr>
<tr>
<td>chenodeoxycholic acid (%)</td>
<td>10±2</td>
<td>27±4</td>
<td>35</td>
</tr>
<tr>
<td>deoxycholic acid (%)</td>
<td>4±2</td>
<td>28±7</td>
<td>24</td>
</tr>
<tr>
<td>lithocholic acid (%)</td>
<td>0</td>
<td>4±4</td>
<td>6</td>
</tr>
<tr>
<td>glycine:taurine</td>
<td>1:2 to 1:5</td>
<td>1:48 to 1:118</td>
<td>2:1 to 3:1</td>
</tr>
</tbody>
</table>
Opossum bile is best comparable with gall bladder bile and rat bile aspirates with hepatic bile. The chemical composition of opossum bile resembled the human situation whereas rat bile contained more cholic acid (86% compared to 42%) and less chenodeoxycholic and deoxycholic acid (10% and 4% compared to 27% and 28%, respectively).

The ratio glycine:taurine was higher in man (2:1 to 3:1) than in rats (1:2 to 1:5). In opossums the ratio glycine:taurine was very low (1:48 to 1:117) (Table 4.2).

**DISCUSSION**

From our results it can be concluded that chronic reflux of duodenal juice induces macroscopic Barrett’s oesophagus in rats. A variety of stainings demonstrated the similarity of rat Barrett’s oesophagus to that found in man, and proved it to be of another entity than migrated jejunal mucosa. The duration of the period of reflux correlated well with the length of the Barrett’s oesophagus. Thus, an oesophagojejunostomy in the rat provides a unique, reliable animal model for Barrett’s oesophagus.

Great efforts have been made to develop an animal model for oesophageal adenocarcinoma and to define the pathogenetic stimulus of its precursor Barrett’s oesophagus.25 However, both animal and human studies show conflicting results.1,6-14,26-28 From rat studies, there seems little doubt that duodenogastric reflux is important in the pathogenesis of gastric cancer and a similar picture has emerged with regard to oesophageal adenocarcinoma.2,5,11,26,27 The issue is more complex with regard to animal models for the development of Barrett’s oesophagus. Two studies have demonstrated that columnar re-epithelialization of the dog denuded lower oesophagus occurs in the presence of acid reflux and acid and bile reflux.6,7 In the absence of reflux or with bile reflux alone the oesophagus regenerated with squamous epithelium. In rat studies, five authors reported the development of columnar metaplasia of the oesophagus.6,12 In all, Barrett’s oesophagus was induced by reflux of duodenal juice into the oesophagus. However, Barrett’s oesophagus was induced only in a minority of animals or was confined to a small area or small islands near the anastomosis. These models can be used to study the pathogenesis of Barrett’s oesophagus but not for evaluating the effects of endoscopic ablation therapies. For this, an animal model inducing long segments of Barrett’s oesophagus in a vast majority of animals is needed.

Two studies have investigated gastro-oesophageal reflux as causal factor of oesophagitis in the opossum. In one study a cardioplasty was performed in five animals.29 After 10 weeks, however, lower oesophageal sphincter activity returned
Animal model for Barrett's oesophagus

to normal and at this time, gastro-oesophageal reflux ceased, and oesophagitis healed. In the other study, the cardia of eight opossums was resected whereafter six animals survived for 31 to 72 weeks. Hyperplasia of the squamous basal cells (called chronic oesophagitis by the authors) was found in these animals.

The difference between the gastrointestinal tract of the opossum and the rat could explain the fact that opossums did not develop macroscopic Barrett's oesophagus in the present study. Like man, the opossum has a gall bladder, that stores and concentrates bile and releases it into the small intestine when food, and fat in particular, is present in the small bowel. Furthermore, the extent of the bile flow and the flow of pancreatic secretions into the duodenum in the opossum and in man depends on the activity of the sphincter of Oddi. The tone of the sphincter of Oddi between meals is high, and pancreaticobiliary reflux consequently will be confined to only several time intervals per day. As mentioned before, the opossums showed no discomfort following oesophagojejunostomy and did not loose weight. The rat in contrast, lacks a gall-bladder and although it has a true sphincter of Oddi, the tonic activity of this sphincter is not very high so that fluid bile and pancreatic juice continuously flow into the duodenum. Following surgery rats but not opossums showed considerable discomfort and lost weight. Furthermore, the in vivo production of bile acids in rat liver is 3- to 5-fold higher than that in human liver; the data for opossums are not known. From the present study, reflux of bile acids seems to be the most important factor in inducing Barrett's oesophagus as the absence of a gall bladder, leading to continuous bile reflux, is the main difference between rats and opossums.

Of the components of duodenal juice that may play a role in the pathophysicsology of Barrett's oesophagus, bile salts and their bacterial enzymatic degradation products are indeed considered most important because of their possible role in carcinogenesis in the colon. In the present study bacterial degradation of bile acids is likely to occur in the oesophagus as the oesophagus is in continuum with the small bowel after oesophagojejunostomy. Studies have demonstrated that cell toxicity of (un)conjugated bile acids is related to their relative hydrophobicity, and thus to their capacity of passive diffusion. In the present study the percentage of the more hydrophobic (cheneo)deoxycholic acids was higher in opossums and man than in rats, whereas rat bile contained more hydrophylic cholic acids. Bile acids in gall bladder as well as hepatic bile are normally found conjugated with glycine or taurine. These conjugated forms contribute to an increase in hydrophilic character. However, the pK values of glycine and taurine conjugates differ greatly (pK_{taurine conjugates} = 1.85-1.95, pK_{glycine conjugates} = 3.95-4.84), making taurine conjugates more hydrophilic and less toxic. Consistent with data from others, in the present study opossum bile acids were found to be almost exclusively conjugated with taurine. These findings support the concept that taurine conjugated bile acids
protect against loss of surface epithelium and initiation of subsequent proliferative response induced by hydrophobic bile salts. 39, 40

The conflicting results of duodenal reflux in animal and human studies and the observation that previous gastric surgery, inducing duodenal reflux, does not lead to a higher prevalence of Barrett's oesophagus, has led to the hypothesis that other factors are involved in the pathogenesis of Barrett's oesophagus. 41 Oxygen radicals produced by infiltrating neutrophils and macrophages could be a pathogenetic factor in Barrett's oesophagus and oesophageal carcinomas. 42 Reactive oxygen species are mutagenic and are involved in tumour promotion. In all experimental rats the distal segment of the oesophagus lined by columnar epithelium showed marginal infiltration. Proximal from the Barrett's segment an area of erosion and severe inflammation with extensive infiltration of neutrophils was always seen proximal from which the oesophagus was lined by squamous epithelium. In contrast, the opossum oesophagus showed no signs of erosion and the oesophageal wall was only very sparsely infiltrated by neutrophils.

Barrett's oesophagus is thought to originate from a multipotent undifferentiated stem cell of oesophageal origin, rather than from proximal migration of cardiac columnar epithelium, as formerly thought. 6, 13, 14, 43 The present study confirms that Barrett's oesophagus does not originate from proximal migration of gastric columnar cells. Proximal migration of jejunal cells seems unlikely as several stainings show that the columnar epithelium proximal from the anastomosis can be differentiated easily from the adjacent jejunal mucosa. Furthermore, it can be concluded from the present study that, assuming that Barrett's oesophagus originates from multipotent undifferentiated stem cells, these stem cells are not located in oesophageal glands or oesophageal gland ducts, as the rat lacks these structures. In a situation of prolonged gastroduodenal reflux into the rat oesophagus, some gland-like structures develop within the oesophageal mucosa. These structures resemble the ulcer-associated cell lineage (groups of near acinar structures within the lamina propria associated with a duct draining to the villus surface) as described by Hanby et al. 43 Probably, this cell lineage and Barrett's epithelium originate from stem cells in the basal cell layer of the surface squamous epithelium. The opossum oesophageal glands and gland ducts histologically show no change under the influence of duodenogastro-oesophageal reflux.

In conclusion, this is the first report of an animal model with macroscopic Barrett's oesophagus over a length of 2 cm. This animal model can be used in studies to evaluate the short- and long-term effects of the various endoscopic ablation therapies for Barrett's oesophagus. After one year of duodenal-oesophageal reflux, macroscopic Barrett's oesophagus was induced in rats whereas approximately 25% of opossums developed only microscopic foci of specialized intestinal metaplasia. Barrett's oesophagus in rats does not originate from
oesophageal glands but likely from epithelial stem cells stimulated by contents of duodenal refluxate and, possibly, inflammatory mediators. Thorough investigation of the pattern of various stainings and the long trajectory of columnar lining of the oesophagus in the present long-term study prove the similarity with human Barrett's oesophagus. It would be of great interest to investigate whether opossums that have undergone an oesophagojejunostomy combined with cholecystectomy and papillotomy will develop macroscopic Barrett's oesophagus. Combined with studies on the effect of the individual components of duodenal reflux material and the role of infiltrating neutrophils, further steps can be made to elucidate the pathogenesis and etiology of Barrett's oesophagus.
REFERENCES


40. Heuman DM, Pandak WM, Hylemon PB, Vlahcevic ZR. Conjugates of ursodeoxycholate


CHAPTER 5

OESOPHAGOJEJUNOSTOMY PROVIDES A UNIQUE, REPRODUCIBLE MODEL FOR BARRETT'S OESOPHAGUS

This chapter has been adapted from Van den Boogert et al. Journal of Surgical Oncology (submitted).
INTRODUCTION

In many western countries the incidence of adenocarcinoma of the oesophagus is rising. The majority of these adenocarcinoma arise in a Barrett’s oesophagus. Barrett’s oesophagus is defined as the presence of metaplastic columnar mucosa with goblet cells in the oesophagus (Chapter 1). The pathogenesis of Barrett’s metaplasia and malignant degeneration of this entity are not clearly understood. Acid reflux has long been thought to be the only pathogenetic factor. Patients with Barrett’s oesophagus are usually treated with antireflux therapy. Although very effective in the treatment of reflux, antireflux medication and antireflux surgery have no effect on the Barrett’s epithelium itself.

Photodynamic therapy (PDT) with 5-aminolevulinic acid (ALA)-induced sensibilisation may be used to selectively eliminate Barrett’s oesophagus. An animal model for Barrett’s oesophagus is needed to investigate the pharmacokinetics of ALA-induced photosensitization, to find optimal treatment parameters and evaluate the short- and long-term effects of PDT for Barrett’s oesophagus.

Several studies have been published on rat models for the development of adenocarcinoma of the oesophagus. Most models used carcinogen (nitrosamines) but recently oesophageal adenocarcinoma were produced in rats undergoing various operations without the use of carcinogen. Five authors report the development of columnar metaplasia of the oesophagus. In all, this was induced by reflux of duodenal juice into the oesophagus. In the study of Miwa et al., the majority of rats developed Barrett’s oesophagus after a period of 50 weeks of gastroduodenoeosophageal reflux or duodeno-oesophageal reflux, whereas gastro-oesophageal reflux alone induced Barrett’s oesophagus in only 13%. Seto et al. reported that acidified water had a synergistic effect on the development of Barrett’s oesophagus induced by reflux of duodenal contents. In contrast, Ireland et al. found that the presence of gastric juice in refluxed duodenal juice protected against the development of Barrett’s oesophagus and adenocarcinoma. Goldstein et al. found columnar epithelium in 10 out of 11 rats, 31 weeks after an oesophagoduodenostomy. In the denuded canine oesophagus columnar re-epithelialization occurred in the presence of acid reflux and acid and bile reflux, but not with bile reflux alone. As the rat oesophagus is lined by keratinized squamous epithelium, denudation in the present study was performed surgically or by locally applicated salicylic acid to bring about keratolysis.

The aim of the present study was to establish an animal model for Barrett’s oesophagus and investigate several pathogenetic factors and possible synergistic factors that could result in a faster induction of Barrett’s oesophagus.
MATERIALS AND METHODS

Rats

Forty-two male inbred Wistar rats (Harlan CPB, Austerlitz, The Netherlands), weighing 200-275 grams were used. The animals had free access to rat chow (AM II, Hope Farms, Woerden, The Netherlands) and tap water acidified to pH 4.0.

Surgical technique

Surgery was performed under ether anaesthesia. Through a median laparotomy an oesophagojejunostomy was performed.19 The oesophagus was dissected from the stomach and anastomosed end to side (interrupted 7/0 prolene sutures) to the jejunum approximately 5 cm distal from the ligament of Treitz. Additionally, a gastrectomy as described by Santini et al. was performed to determine the effect of acidified drinking water.20 The abdominal wall was closed in one layer (2/0 prolene sutures). After surgery the animals received 2 ml glucose 10% subcutaneously and water ad libitum. Chow was provided after 24 hours.

Design of the study

Forty-two rats were randomly allocated to seven groups of six animals each. One group served as control group: the animals were not operated and three of them received N-nitrosomethyl-benzylamine (NMBA) (Ash Stevens Inc., Detroit, USA) and acidified water. The carcinogen was analysed by reverse phase high performance liquid chromatography and found to be >95% pure. NMBA was given by subcutaneous injection at a dose of 0.5 mg kg⁻¹, 3 times per week, for 5 weeks.11

In the experimental groups an oesophagojejunostomy with gastrectomy was performed (Table 5.1). In one group this procedure was combined with acidified drinking water to induce duodenal reflux in an acidified environment.15 Acidification of the drinking water was achieved with HCl to a pH of 1.8. In the other groups the oesophagojejunostomy was combined with NMBA in a scheme as described above, starting at week 2 after surgery, both acidified drinking water and postoperative NMBA, keratinolysis, or denudation of the mucosa. Keratinolysis of the oesophageal lining was performed by application of salicylic acid (Bufa, Uitgeest, The Netherlands) in the distal oesophagus. After 20 minutes the remaining salicylic acid was washed out and the anastomosis was made. Denudation was achieved by surgically stripping the oesophageal mucosa over 2 cm in length for half the circumference before the anastomosis was made.17,18

The animals were weighed weekly and sacrificed at week 18 after surgery.
Table 5.1 Design of the study

<table>
<thead>
<tr>
<th>Groups (n=6)</th>
<th>procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>oesophagojejunostomy</td>
</tr>
<tr>
<td>2</td>
<td>+ acidified drinking water</td>
</tr>
<tr>
<td>3</td>
<td>+ NMBA</td>
</tr>
<tr>
<td>4</td>
<td>+ acidified drinking water + NMBA</td>
</tr>
<tr>
<td>5</td>
<td>+ salicylic acid</td>
</tr>
<tr>
<td>6</td>
<td>+ mucosal stripping</td>
</tr>
</tbody>
</table>

NMBA = N-nitrosomethyl-benzylamine; CB = common bile duct; D = duodenum; J = jejunum; O = oesophagus.

Handling of specimens

Just before sacrifice, blood was collected by cardiac puncture to determine white blood cell count, red blood cell count, haemoglobin, and haematocrit. The thoracic and abdominal cavities were inspected and the oesophagus, the anastomosis, and the proximal jejunum were excised en bloc. The oesophagus was opened longitudinally, the circumference was measured, and the specimen was curled up from distal to proximal (Swiss roll) and fixed in formalin.

Histological examination

Histological examination was performed by a histopathologist blinded for the treatment. Specimens were embedded in paraffin wax, sectioned and stained with haematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and alcian blue. The oesophagus was examined histologically for squamous hyperplasia, hyperkeratosis, erosions, Barrett's oesophagus, benign papillomas, dysplasia, and carcinoma. Squamous hyperplasia was defined as papillary elongation or basal cell hyperplasia, occasionally resulting in the formation of intramural cysts. A specimen was defined as having Barrett's oesophagus when there was oesophageal mucosa lined by columnar epithelium with intestinal metaplasia and characteristic goblet cells. Dysplasia was diagnosed according to standard criteria and included dysplasia arising in either glandular or squamous epithelium. Carcinomas were classified into three histological groups: pure squamous cell carcinoma, carcinoma with both squamous and mucinous differentiation (adenosquamous carcinoma) and pure adenocarcinoma.
Statistical analysis

The values of the blood indices and oesophageal circumference are expressed as mean ± standard error of the mean (SEM). Comparisons were made using Student's t-test. A difference was considered to be significant at P values of <0.05.

RESULTS

General observations

Five animals (four in the denudation group) died before the end of the experiment. The cause of death was an anastomotic stenosis of the denuded oesophagus in four, and unknown in one. The rats in the experimental groups lost weight until week 5 after surgery, thereafter weight increased very slowly. The weight of the control animals increased until the end of the experiment and was significantly higher than in the experimental groups from week 2 to the end of the experiment (Figure 5.1).

![Figure 5.1](image)

**Figure 5.1** Time course of weight changes in control animal and experimental groups. Points represent the mean weight of each group. Control = no oesophagojejunostomy (3 animals with and 3 animals without acidified drinking water and N-nitrosomethyl-benzylamine [NMBA]), group 1 = oesophagojejunostomy, 2 = oesophagojejunostomy + acidified drinking water, 3 = oesophagojejunostomy + NMBA, 4 = oesophagojejunostomy + acidified drinking water + NMBA, 5 = oesophagojejunostomy + salicylic acid, 6 = oesophagojejunostomy + mucosal stripping. a = P < 0.05 (control group versus experimental groups).
White blood cell and red blood cell counts at the end of the experiment did not differ significantly between the study groups and the control animals. A trend though was noticed for white blood cell count to be higher and red blood cell count to be lower in the study groups (Table 5.2). Haemoglobin and haematocrit were significantly lower in the study groups compared to the control group ($P < 0.001$). The distal oesophagus was significantly dilated and thickened in the operated groups compared to the control group ($P < 0.01$) (Table 5.2). There were no obvious macroscopic differences among the six subgroups. The mucosa of the lower oesophagus was grossly irregular and showed various abnormalities in most animals: multiple white plaques, erosions, glandular-like pink areas and in some animals papillomas. No macroscopic tumours were found.

Table 5.2 Blood indices and oesophageal circumference in rats 18 weeks after oesophagojejunostomy.

<table>
<thead>
<tr>
<th>groups</th>
<th>WBC ($\times 10^9$ l$^{-1}$)</th>
<th>RBC ($\times 10^{12}$ l$^{-1}$)</th>
<th>HGB (nmol l$^{-1}$)</th>
<th>HCT l$^{-1}$</th>
<th>circumference (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>7.4 ± 1.8</td>
<td>7.7 ± 0.4</td>
<td>8.2 ± 0.3</td>
<td>0.44 ± 0.01</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>1</td>
<td>10.5 ± 1.1</td>
<td>6.7 ± 0.8</td>
<td>2.8 ± 0.3 *</td>
<td>0.21 ± 0.02 *</td>
<td>8.7 ± 0.8 *</td>
</tr>
<tr>
<td>2</td>
<td>13.6 ± 2.1</td>
<td>6.1 ± 0.9</td>
<td>2.8 ± 0.3 *</td>
<td>0.21 ± 0.02 *</td>
<td>10.0 ± 0.9 *</td>
</tr>
<tr>
<td>3</td>
<td>12.4 ± 1.8</td>
<td>5.8 ± 0.9</td>
<td>2.6 ± 0.3 *</td>
<td>0.20 ± 0.02 *</td>
<td>10.5 ± 0.6 *</td>
</tr>
<tr>
<td>4</td>
<td>13.0 ± 2.3</td>
<td>5.7 ± 0.6</td>
<td>3.0 ± 0.5 *</td>
<td>0.19 ± 0.02 *</td>
<td>9.0 ± 0.5 *</td>
</tr>
<tr>
<td>5</td>
<td>15.6 ± 5.2</td>
<td>5.8 ± 1.0</td>
<td>2.7 ± 0.3 *</td>
<td>0.20 ± 0.02 *</td>
<td>11.3 ± 1.3 *</td>
</tr>
<tr>
<td>6</td>
<td>4.5 ± 2.0</td>
<td>7.7 ± 0.7</td>
<td>3.9 ± 0.7 *</td>
<td>0.27 ± 0.05 *</td>
<td>17.5 ± 7.5 *</td>
</tr>
</tbody>
</table>

WBC=white blood cell count; RBC=red blood cell count; HGB=haemoglobin; HCT=haematocrit, * $P < 0.001$ (control group versus experimental groups). Control=no oesophago­jejunostomy (3 animals with and 3 animals without acidified drinking water and N-nitrosomethylbenzylamine [NMBA]), group 1=oesophagojejunostomy, 2=oesophagojejunostomy + acidified drinking water, 3=oesophagojejunostomy + NMBA, 4=oesophagojejunostomy + acidified drinking water + NMBA, 5=oesophagojejunostomy + salicylic acid, 6=oesophagojejunostomy + mucosal stripping.

Histological changes

The oesophageal mucosa of control animals, including those that had received acidified water and NMBA, showed no abnormalities. The mucosal changes in the distal oesophagus of all experimental animals were confirmed histologically to be those of severe reflux oesophagitis. There was no difference in type or severity of histological signs of oesophagitis among the experimental groups (Table 5.3).

Columnar lining above the anastomosis in the H&E sections was found in 50% to 100% of the animals in each group, confined to a small area of 1 to 9 mm with
Table 5.3 Number of animals with histological changes after the various regimens.

<table>
<thead>
<tr>
<th>Groups (n)</th>
<th>hyper-keratosis</th>
<th>squamous hyperplasia</th>
<th>erosion</th>
<th>BO dysplasia</th>
<th>papilloma</th>
<th>carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>control (6)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 (6)</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>5</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>2 (6)</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>3 (6)</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>4 (5)</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>5 (6)</td>
<td>6</td>
<td>6</td>
<td>5</td>
<td>6</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>6 (2)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

BO = Barrett’s oesophagus; scc = squamous cell carcinoma; asc = adenosquamous carcinoma.

Groups: control = no oesophagojejunostomy (3 animals with and 3 animals without acidified drinking water and N-nitrosomethyl-benzylamine [NMBA]); 1 = oesophagojejunostomy; 2 = oesophagojejunostomy + acidified drinking water; 3 = oesophagojejunostomy + NMBA; 4 = oesophagojejunostomy + acidified drinking water + NMBA; 5 = oesophagojejunostomy + salicylic acid; 6 = oesophagojejunostomy + mucosal stripping.

A mean of 2 mm (Figure 5.2). The columnar epithelium showed intestinal metaplasia with PAS positive goblet cells but no dysplasia. In animals with duodenal reflux alone or combined with NMBA, 11 out of 12 animals showed columnar lining of the distal oesophagus, in animals with additionally acidified drinking water Barrett’s oesophagus was noticed in 9 out of 11. In all animals with keratinolysis glandular epithelium was found. In animals with duodenal reflux and NMBA in four out of six animals, and in the group with reflux, NMBA and acidified water in three out of five animals a squamous cell carcinoma was found, histologically characterized by infiltrating, atypical, squamous epithelial cells (Figure 5.3).

Figure 5.2 Distal rat oesophagus exposed to duodenal reflux during 18 weeks. The oesophagus was lined by columnar epithelium with intestinal metaplasia and characteristic goblet cells (arrow) cranial from the sutures of the oesophagojejunal anastomosis over a length of 1 to 9 mm (x40, H&E staining).
Additional stainings with anti-smooth muscle actin showed that some carcinomas penetrated the muscularis mucosae and invaded the submucosa. In both groups, one animal had developed adenosquamous carcinoma, with foci of mucous epithelial cells with abundant cytoplasm or atypical glandular formation, staining positively with alcian blue and PAS (Figure 5.4). No pure adenocarcinomas were found. In animals receiving NMBA without an oesophagojunostomy no abnormalities, especially no carcinomas, were found.

**Figure 5.3** Oesophageal squamous cell carcinoma histologically characterized by infiltrating, atypical, squamous epithelial cells after 18 weeks of duodeno-oesophageal reflux in combination with carcinogen (N-nitrosomethyl-benzylamine). The section stained negatively with PAS and alcian blue (x40, H&E staining).

**Figure 5.4** Adenosquamous carcinoma in a rat 18 weeks after an oesophagojunostomy combined with N-nitrosomethyl-benzylamine. Histology showed foci of keratinization (K) and foci of mucous epithelial cells (arrow) staining positively with PAS and alcian blue (x40, PAS staining).

**DISCUSSION**

From the present study it can be concluded that reflux of duodenal contents alone induces Barrett's oesophagus in rats. In combination with a carcinogen, duodeno-oesophageal reflux induces oesophageal carcinoma. Neither acidification of the drinking water, nor the administration of a carcinogen, a combination of both, keratinolysis or denudation hastened the induction of Barrett's oesophagus.
Great efforts have been made to define the pathogenetic stimulus of Barrett's oesophagus. Usually, Barrett's oesophagus is found in patients who have a marked degree of acid gastro-oesophageal reflux, considerably exceeding that seen in controls or in patients with oesophagitis without columnar metaplasia. Therefore, acid reflux has long been thought to be the only pathogenetic factor. Two studies in dogs and one in rats confirm a pathogenetic role for acid reflux. In other animal studies reflux of gastric contents alone does not induce Barrett's oesophagus or even protects against the development of oesophageal adenocarcinoma. In the present study administering acidified water had no effect on the epithelium in the control group and no influence on the induction of Barrett's oesophagus by duodenal reflux and no co-carcinogenic effect on the development of oesophageal carcinoma by NMBA and duodenal reflux.

The results of animal studies, the fact that oesophagitis is seen in patients who have undergone total gastrectomy and in those with achlorhydria, and the observation that the increase of oesophageal adenocarcinoma parallels the introduction of H-2 blockers and proton pump inhibitors has led to the concept of alkaline (bile) duodenal reflux as causal factor of Barrett's oesophagus. In man, this hypothesis has been evaluated in several studies. An analysis of frequency and duration of reflux and reflux material in patients with Barrett's oesophagus, gastro-oesophageal reflux disease without Barrett's oesophagus, and controls shows conflicting results, partly due to inaccurate detection methods. The results of studies that employ oesophageal pH monitoring to investigate duodenogastro-oesophageal reflux should be interpreted with caution as the presence and amount of duodenal juice and the pH of the refluxate is not clearly correlated. Studies that determine bile acids, trypsin or bilirubine in the oesophagus or in aspirated refluxate probably give more reliable information on duodenal reflux. Three studies have reported that bile acid concentrations are probably not toxic, or did not find a difference in quantity of bile reflux between patients with Barrett's oesophagus and reflux patients without Barrett's oesophagus or between patients with Barrett's oesophagus and controls. Others, however, have reported increased bile reflux in the oesophagus in patients with Barrett's oesophagus compared to controls or oesophagitis patients without Barrett's oesophagus. Additionally, in three studies a greater bile reflux was detected in patients with complicated Barrett's oesophagus compared to subjects with non-complicated Barrett's oesophagus. From the results of the present study it appears that reflux of duodenal contents alone induces Barrett's oesophagus and exerts a co-carcinogenic effect on the rats oesophagus. The presence of duodenal refluxate (bile and/or pancreas) was crucial as NMBA alone did not cause any oesophageal lesions.

Only one animal study has investigated the influence of biliary and pancreatic reflux on the induction of oesophageal carcinoma. In combination with the
administration of a carcinogen, pancreatic or pancreatic and biliary reflux induced carcinoma of the oesophagus in 13% and 33% of rats, while the carcinogen in combination with bile reflux alone did not induce carcinomas. It was concluded that pancreatic secretions are crucial in inducing oesophageal carcinoma and that biliary reflux appears to exert a co-carcinogenic effect. Furthermore, it has been suggested that the depth of mucosal injury determines either squamous or columnar re-epithelialization. Deep injury, including damage to both surface mucosa and gland ducts, will be repaired by columnar epithelium alone. This deep injury can be caused by enzymes with proteo- and lipolytic activity like gastric and pancreatic lipase. In the present study neither superficial injury (keratolysis) nor deep injury (denudation) hastened the induction of Barrett’s oesophagus. Furthermore, it appeared that one week of duodenal reflux alone already induced deep injury to the oesophageal mucosa. Histology at that time point showed erosion, ulceration and inflammation with cellular infiltration and vasodilatation.

In conclusion, an oesophagojejunostomy in rats provides a unique, reproducible model for the induction of Barrett’s oesophagus. The presented animal model can be used to investigate the possibilities of photodynamic therapy (PDT) for Barrett’s oesophagus by studying the pharmacokinetics of the photosensitizer, find optimal treatment parameters and evaluate the short- and long-term effects of PDT for Barrett’s oesophagus. Furthermore, it gives us the opportunity to investigate whether PDT-treated Barrett’s oesophagus regenerates with squamous epithelium when there still is duodeno-oesophageal reflux. In other words: must PDT treatment be followed or even preceded by antireflux surgery? For this, rats with reflux-induced Barrett’s oesophagus as described in the present study will have to undergo oesophageal PDT with or without previous Roux-en-Y procedure to discontinue duodeno-oesophageal reflux.
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CHAPTER 6

GASTRIC PERFORATION IN RATS AFTER OESOPHAGOJEJUNOSTOMY

This chapter has been adapted from Van den Boogert et al. Laboratory Animal Science 1999;38:49-52.
INTRODUCTION

Barrett's oesophagus is a premalignant lesion of the oesophagus defined as the presence of columnar epithelium with intestinal metaplasia in the oesophagus, probably caused by longstanding (duodeno)gastro-oesophageal reflux (Chapter 1). To study the development of Barrett's oesophagus in rats, animal models were used to induce duodeno-oesophageal reflux. Chapters 4 and 5 describe such models. Before these animal models could be applied a small number of pilot studies has been carried out. In one of these studies many animals became ill and some died. This chapter describes this particular pilot study and discusses the encountered problem. The experimental protocol was approved by "The Committee on Animal Research" of the Erasmus University Rotterdam. Forty inbred male WAG/Rij rats (Harlan CPB, Austerlitz, The Netherlands), weighing approximately 225 grams, were studied. Animals were bred under specific-pathogen-free conditions and were kept under conventional conditions from 8 weeks after birth. The animals were kept two in a cage on wooden bedding in a climate-controlled room (12 hours light/dark cycle) and had libitum access to rat chow (AM II, Hope Farms, Woerden, The Netherlands) and tap water. All animal handling was carried out by experienced biotechnicians according to the latest developments in husbandry, nutrition and animal housing. Before use, the animals acclimated for at least one week. In all experiments anaesthesia was maintained throughout the operative procedure by placing a glass beaker containing cotton-wool soaked in ether over their nostrils. All animals underwent an oesophagojejunostomy with gastric preservation according to the Levrat model, which induced jejuno-oesophageal reflux.1 Median laparotomy was performed, the oesophagus was dissected 2 mm above its entry in the stomach, the stomach was closed and the oesophagus was anastomosed end-to-side (7/0 silk, B Braun, Melsungen, Germany) to the jejunum, approximately 5 cm distal to where the transverse colon crosses the small intestine. The abdomen was closed in one layer (2/0 silk, B Braun, Melsungen, Germany). After surgery, rats received 2 ml of glucose 10% subcutaneously and water ad libitum. Chow was provided after 24 h. Despite some weight loss, the first 4 weeks after surgery, rats did well. From day 26 until day 39 after surgery, many rats became ill and, if untreated, died within 2 days.

PATHOLOGIC FEATURES

Necroscopy was performed on the diseased animals, at which the cause of death was clearly evident: gastric perforation on the greater curvature, followed by peritonitis (Figure 6.1).
Gastric perforation

Gastrectomy was performed in the remaining animals; some, however, already suffered from gastric perforation and peritonitis and had to be euthanised.

The site of perforation in the presented animals was sharply limited to the squamous epithelium at the region of transition, where the forestomach joins the glandular epithelium, and was located exclusively on the great curvature. Macroscopically, the stomach further did not contain any gross abnormalities. Besides oesophagitis, other lesions could not be observed. In animals in which the stomach had not been perforated, the stomach was filled with a clear yellowish fluid but was not abnormally distended. Gastric content was analysed for the presence of bacteria, and the pH was measured. Besides *Escherichia coli* in some animals, other bacteria could not be isolated by culture. The pH of the liquid gastric content ranged from 4.5 to 7.0. The stomach was excised, opened and fixed in formalin.

**Figure 6.1** Photograph of the open rat abdomen. Arrow 1 points to the gastric perforation in the forestomach next to its junction with the glandular mucosa, central at the great curvature. Arrow 2 indicates the site of the oesophagojejunal anastomosis, 5 weeks after end-to-side oesophagojejunostomy.

Sections were stained with haematoxylin and eosin (H&E), periodic acid-Schiff, Alcian blue, Gram, Giemsa (modified for *Helicobacter* sp.) and Grocott stains. Macroscopically, changes in the glandular mucosa were not observed, the forestomach contained hyperplastic lesions, and ulceration was apparent on the greater curvature.

Histological examination of the distal half of the stomach revealed no abnormalities (Figure 6.2). Microscopically, the proximal half of the stomach had hyperkeratosis, hyperplasia of the squamous epithelium, papillomas, inflammatory reaction, haemorrhage, and ulceration (Figures 6.2 and 6.3).

In the basal cell layer of the squamous epithelium, the cell nuclei were prominent but not hyperchromatic; beneath the muscularis mucosae, the connective
tissue was frequently oedematous and contained leucocytes (especially eosinophils). Bacteria or fungi could not be identified on the gastric surface or in the gastric wall.

**Figure 6.2** Photomicrograph of a section of the squamo-columnar junction of the rat stomach 5 weeks after oesophagojejunostomy. The forestomach (A) is lined by squamous epithelium, and the glandular stomach (B) is lined by columnar epithelium. Hyperplasia, inflammation, and ulceration were found in the forestomach. The glandular stomach did not contain any abnormalities (x10, H&E stain).

**Figure 6.3** Photomicrograph of a section near the site of perforation in the forestomach, showing hyperkeratosis, and hyperplasia of the squamous epithelium (arrow 1), oedema and inflammation mainly consisting of eosinophilic granulocytes (arrow 2), haemorrhage (arrow 3), and necrosis of the muscularis propria (arrow 4). Bacteria or fungi could not be identified (x40, H&E stain).

**QUESTION**

What are the various causes of gastric perforation in rats? Combining our findings, what is the most plausible cause in the presented rats?

**DIAGNOSIS AND DISCUSSION**

The differential diagnosis of the gastric ulcers described, includes spontaneously developing ulcers and iatrogenic ulcers.

Evaluation of laboratory mice and rats indicated that infections with
Helicobacter bilis and H. muridarum are widespread but that infections are usually subclinical. However, under experimental circumstances (e.g., ischemic stomach, pre-existing ulcer, mucosal disruption) H. pylori and Gastrospirillum suis have an ulcerogenic action. In the aforementioned rats, the stomach was filled with an odourless clear fluid and, if present, peritonitis had the aspect of a sterile infection. Bacteriologic and histologic examinations confirmed this observation. Besides some non-pathogenic E. coli, other bacteria could not be isolated by culture and spiral bacterial organisms were not found on H&E, Gram, or modified Giemsa staining. Furthermore, Helicobacter and Gastrospirillum infections cause inflammation of the glandular stomach. In rats, the proximal half of the stomach, called the forestomach or non-glandular portion, is lined by stratified epithelium continuous with that lining the oesophagus; along its distal edge it forms a ridge at the junction with the glandular stomach, which is divided, like the human stomach, into fundic and antral areas. Unlike Helicobacter sp., Lactobacillus and Bacillus spp. may adhere to keratinized epithelium of the forestomach. In pigs mono-infected with these species and fed a carbohydrate-enriched liquid diet, ulcers of the forestomach developed. In contrast, piglets fed the same diet and inoculated with Gastrospirillum sp. did not develop forestomach ulcers. In the study reported here, no Lactobacillus or Bacillus (both Gram-positive) species could be identified by use of Gram staining.

The striking finding of the present work was limitation of the gastric perforations to the squamous epithelium and, in particular, the involvement of the epithelial fold immediately adjacent to the glandular stomach. To our knowledge, only one other report of the course of Candida albicans colonization and infection of the alimentary tract of rats treated with antibiotics described lesions limited to this precise location. The authors suggest that localization of infection at the squamo-columnar junction may conceivably be related to the susceptibility of cornified epithelium in general, increased thickness of the cornified epithelium at this point, or interruption of the smooth mucosal contour with pooling of juices that contain neutral cardiac gland secretions. In the present study, Grocott staining did not reveal fungal invasion.

Experimentally induced gastric ulcers may be grouped under several general headings: resulting from a deficient blood supply to the gastric mucosa; caused by the action of bacteria; resulting from the action of toxic agents; caused by the action of digestive juices, and resulting from trophic and glandular disturbances.

Deficient blood supply could be the result of surgery, because the oesophagus had to be dissected from the stomach; care, however, was taken not to damage the left gastric artery. Careful histologic examination did not reveal any signs of
ischaemia (e.g. thrombi, loss of epithelial cells on top of the mucosa). The long interval between surgery and gastric perforation does also not favour an ischaemic cause.

Also, perforation could be the result of infection, especially in rats after oesophagojejunostomy in which the stomach is transformed into a blind loop that predisposes the animal to bacterial overgrowth. Additionally, the antibacterial action of the normally low pH in the stomach was undone by the procedure because dissection of the vagus nerves led to inhibition of acid secretion. This resulted in high intraluminal pH of 4.5 to 7.0, whereas normal pH in rats that did not undergo surgery was significantly lower, between 1.5 and 2.5 ($P<0.01$). A pH > 4 is no longer bactericidal. Although in man, *Helicobacter pylori* often is involved in gastric ulceration, as stated above *Helicobacter* sp. were not detected in the animals of this study.

In humans, the second most common cause of gastric ulceration is the use of drugs, more specifically, the use of non-steroidal anti-inflammatory drugs. In rats, acetylsalicylic acid, indomethacin, and ethanol are often used to induce gastric ulceration. In the rats of the present study drugs were not used.

In addition, action of digestive juices could lead to gastric ulceration. The damaging effects of bile on the stomach are widely discussed, but little understood. Moreover, results of animal studies are not unanimous. Implantation of the lower or upper bile duct into the glandular part of the stomach (influx of bile with or without pancreatic juice) induced gastric erosions and ulcers in the glandular part of the stomach. Combination of these procedures with total vagotomy did not increase the incidence of gastric lesions. Others reported forestomach lesions and non-glandular carcinoma after duodenogastric reflux, resembling those found in the oesophagus after longstanding duodeno- or jejuno-oesophageal reflux. In the present study no duodenogastric reflux inducing procedure was performed. Due to vagotomy and possibly some additional jejunal reflux, pH of the gastric content was significantly higher than that in control rats.

Finally, trophic and glandular disturbances could lead to ulceration and eventually perforation. After total bilateral vagotomy in rats, gastric secretory and motor functions were severely impaired, leading to a state of fixed complete closure of the cardia and contraction of the pylorus. Within several days after total vagotomy, many animals died with a distended stomach containing large masses of food that were also present in the oesophagus and oral cavity. In contrast, other studies could not find any lesions of the rat stomach after vagotomy. Vagal stimulation induces high gastric glandular ulcer incidence in rats. Stress ulcers, principally located in the glandular stomach, can be formed in rats by immobilization, cold, or water restraint stress. The presence of luminal acid and ischaemia, probably caused by vagal-mediated increased gastric motility, are
Gastric Perforation

necessary for the induction of stress ulcer. Another "stress" ulcer may be induced by use of a Shay procedure (ligation of the pylorus when the stomach is empty). After 7 to 8 h, non-glandular ulcers are formed due to high intraluminal pressure, which led to local circulatory disturbance. Ligation for a longer period resulted in gastric perforation. A certain degree of acidity (pH < 1.5) was indispensable for ulcer formation because ligation in combination with vagotomy did not cause ulcers, although the same high intraluminal pressure was reached. Absence of additional stressors and performed dissection of the vagus nerves make a stress ulcer in the presented rats unlikely.

Although other studies in which an oesophagojejunostomy with gastric preservation was performed according to the Levrat model did not describe gastric perforation, Dr. Levrat himself found that, in all his animals (sacrificed at day 30 after oesophagojejunostomy), hyperplastic and ulcerative lesions of the forestomach developed and resembled those found in the oesophagus. In their book, Lambert and Levrat describe a perforated ulcer in the by-passed forestomach on the greater curvature in 10 of 91 rats after oesophagojejunostomy. They hypothesized that acid secretion was responsible for this action. They further pointed out that the gastric consequences of withdrawal of food are particularly strange. When fasting is total, after 6 to 10 days, widespread lesions of the forestomach, including hyperplasia, ulceration and pseudo-papilloma formation, appear. The glandular part of the stomach has only minimal lesions. Morris et al. investigated the effect of various dietary deficiencies in formation of gastric lesions in rats. In a high percentage, lesions of the forestomach, leaving the glandular stomach more or less undamaged, were observed. Fasting and partial inanition were found to be very effective and more important in inducing papillomas of the forestomach than was the individual deficiency of protein, carbohydrate, or fat. The presence of small amounts of food and a high frequency of intermittent feeding prevented formation of non-glandular papillomas.

In conclusion, crucial in the present study might have been withdrawal of food away from the bypassed stomach. The pathogenesis remains unclear, but gastric acid was not the causal factor. Besides anacidity, vagotomy leads to a motor-impaired stomach unable to evacuate its alkaline contents (neutral cardiac gland secretions with probably some refluxed jejunal contents) leading to pooling at the junction between non-glandular and glandular stomach. The most important lesson from the present observation is that the gastric consequences of withdrawal of food should never be forgotten when experiments (for example on the ulcer-inducing or anti-ulcer effect of agents) are carried out on the rat stomach.

99
REFERENCES

PART III

PHARMACOKINETICS OF 5-AMINOLEVULINIC ACID
CHAPTER 7

PHARMACOKINETICS OF 5-AMINOLEVULINIC ACID-INDUCED PORPHYRIN PRODUCTION IN RATS

This chapter has been adapted from Van den Boogert et al. Journal of Photochemistry and Photobiology B Biology 1998;44:29-38.
INTRODUCTION

Photodynamic therapy (PDT) has developed during the past two decades into a treatment modality for several types of malignancies. PDT usually involves systemic administration of a photosensitizer to accumulate in the tumour. Subsequent illumination with light of a specific wavelength, absorbed by the photosensitizer, results in a photochemical reaction that destroys the tumour (Chapter 3). The photosensitizer at present most often used is porfimer sodium (Photofrin®), a mixture of various porphyrins. Prolonged cutaneous photosensitization to sunlight for at least four to six weeks is the major side effect of Photofrin®-based PDT. In addition, depending on tumour type and anatomical location, Photofrin® has poor tumour selectivity. In recent years, 5-aminolevulinic acid (ALA) has been advocated as a photosensitizing agent. ALA induces endogenous production of porphyrins. ALA is a naturally occurring intermediary in the haem biosynthetic pathway, which is metabolized at tissue level to the photo-active compound, protoporphyrin IX (PpIX) (Chapter 3). In most tissues the formation of ALA is rate limiting in the synthesis of PpIX and haem and ALA formation is regulated via a feedback control mechanism dependent on the concentration of free haem. Exogenous ALA bypasses both the rate-limiting step and the feedback control, thereby inducing intracellular accumulation of PpIX. ALA-induced porphyrin accumulation is found more tumour selective than Photofrin® with skin photosensitivity for less than 24 hours.

The pharmacokinetics of ALA are poorly understood. It has been suggested that several mechanisms result in ALA-induced porphyrin accumulation in tissue: (1) intracellular PpIX synthesis as documented in in vitro studies; (2) uptake of PpIX produced in the liver after either absorption in the gastrointestinal tract, or direct entrance into the systemic circulation; and (3) uptake of PpIX from production elsewhere. There is some evidence for an enterohepatic circulation of PpIX in humans. Other studies have shown that a large proportion of ALA is excreted unchanged in the urine and faeces within a few hours after administration and is metabolized, as can be concluded from labelling studies including the detection of labelled CO₂ in breath. ALA is poorly, if at all, reabsorbed by the tubules of the kidney and is eliminated by renal filtration only.

Previous studies show that ALA-induced PpIX accumulation occurs primarily in (pre)malignant tissues, tissues that line surfaces (for example, mucosa) or glands with ducts that drain onto such surfaces (for example, sebaceous glands, salivary glands). However, major tissues of mesodermal origin (muscle, connective tissue, cartilage) do not accumulate PpIX in vivo. Different results are reported from in vitro studies: ALA administration to fibroblasts and macrophages may lead to substantial accumulation of porphyrins.
Before ALA can be used routinely for PDT, it is necessary to study the pharmacokinetics of ALA after various routes of administration. This could lead to more efficient treatment regimens with optimal timing of illumination and fewer side effects. In a rat model we therefore analysed the ALA and porphyrin concentrations in various organs after either oral or intravenous administration of ALA. Porphyrin localization within tissues was determined using laser scanning microscopy. Liver and renal function tests were performed to determine toxicity. From analysis of the sequence of events that take place after ALA administration, we investigated whether, in addition to in situ PpIX synthesis, an enterohepatic circulation contributes to PpIX accumulation and whether there is storage of the prodrug ALA in relation to the re-appearance of fluorescence found after bleaching. Maximum and total ALA concentration were analysed for their predictive value of selective porphyrin accumulation.

**MATERIALS AND METHODS**

**Animals**

Forty-eight male WAG/Rij rats (Harlan CPB, Austerlitz, The Netherlands), weighing approximately 200 grams each were used. ALA was obtained from Sigma Chemical Company (Zwijndrecht, The Netherlands) and had a purity of 98%. 200 mg kg\(^{-1}\) ALA was dissolved in phosphate-buffered saline (pH 7.0). The solution was used immediately, either by intravenous injection (i.v.) into the penis vein under ether anaesthesia (21 rats) or by oral gavage (p.o.) without anaesthesia (21 rats). The rats were placed in a metabolic cage in the dark. At each time point (1, 2, 3, 4, 6, 12 or 24 h after administration of ALA) three rats in both groups were killed by aortic puncture under ether anaesthesia in subdued light. Six rats served as a control group; they received phosphate-buffered saline either i.v. under ether anaesthesia (n=3) or p.o. (n=3) and were sacrificed as mentioned above after 4 h. Blood was centrifugated at 4°C; erythrocytes and plasma were frozen separately at -80°C. Fifteen tissues were removed: bladder, 4 cm of the distal colon (cleaned with saline), heart, brain, 2 cm\(^2\) shaven abdominal skin, 6 cm jejunum just distal to the ligament of Treitz (cleaned with saline), two liver lobes, left lung, disc of the middle part of the stomach (cleaned with saline), spleen, left kidney, oesophagus, 1 cm\(^2\) abdominal wall muscle, intra-abdominal fat, and right and left ischiatic nerve. The tissues were immediately put on ice, kept in the dark and frozen at -80°C as soon as possible. Urine and duodenal aspirates were also stored at -80°C. Additionally, from three control rats and the rats sacrificed at 3 h after oral ALA administration, a small piece of every tissue was snap frozen and stored at -80°C for fluorescence microscopy.
Materials
The following reagents were purchased from Porphyrin Products, Logan, Utah, USA: PpIX, disodium salt and zinc PpIX. Tris HCl was purchased from Boehringer Mannheim (Mannheim, Germany); other chemicals were purchased from Merck, (Darmstadt, Germany).

Liver enzymes and renal function tests
Serum levels of alanine aminotransferase (ALAT) and aspartase aminotransferase (ASAT) were determined photometrically in 50 μl serum using routine methods based on the reference method of the International Federation of Clinical Chemistry (Granutest 25, Merck, Darmstadt, Germany). Urea and creatinine were determined using standard laboratory equipment. Urea was determined using the GIDH method, and creatinine using the Jaffé method without proteination (multi-test analyser systems, Merck, Darmstadt, Germany).

Measurement of protein content
Protein in tissues was measured according to Lowry et al. Tissues were thawed, cut into small pieces, suspended in sterile water (1:10 wt./vol.) and homogenized in a tissue grinder. To 20 μl of this homogenate 1.0 ml alkaline copper-citrate reagent was added. After 10 min Folin reagent was added, and minimally 1 h thereafter extinction was measured spectrophotometrically (Shimadzu UV-1202, s’Hertogenbosch, The Netherlands) at 725 nm.

Determination of ALA and porphobilinogen in plasma and tissue
ALA was extracted from plasma and tissue samples with a mixture of dimethylsulfoxide (DMSO) and methanol (MeOH) (30:70 vol./vol.). Tissue homogenates were prepared at 4°C in subdued light by homogenizing rat tissue at 10-20% wt./vol. water using a Potter Elvehjem homogenizer. 120 μl homogenized tissue or plasma was then mixed with 480 μl DMSO/MeOH, and centrifugated for 4 min at 16000g. The supernatant was diluted three times with NaCl (150 mmol l⁻¹) and was then used for enzymatic ALA detection. The ALA concentration in supernatant from tissue homogenates or plasma was quantified using a fluorimetric enzyme assay. The assay is based on the conversion of ALA into uroporphyrinogen I using an enzyme mixture of aminolevulinic acid dehydratase and porphobilinogen deaminase prepared from human red blood cells in part according to a procedure described by De Rooij et al. 200 μl ALA standard solution (0.12 nmol ml⁻¹), plasma or diluted tissue homogenate was mixed with 50 μl of the enzyme mixture (150 units porphobilinogen deaminase, 1000 units aminolevulinic acid dehydratase, 1 unit = 1 pmol uroporphyrinogen I per hour/37°C) in TrisHCl buffer (0.25 mol l⁻¹; pH 8.0, dithiothreitol 2.5 mmol l⁻¹ and MgCl₂ 20 mmol l⁻¹) or with 50 μl of
TrisHCl buffer for blank detection. The samples were incubated overnight (16 h) at 37°C and the reaction was stopped by addition of 750 µl stop buffer (one part TrisHCl (50 mmol l⁻¹) and two parts trichloroacetic acid (1.5 mol l⁻¹). Then subsequently the samples were placed on an ultraviolet light source (350 nm Woods light) to convert porphyrinogens into porphyrins and centrifuged for 30 min at 1500g. The porphyrin concentration in the supernatant was determined in a Perkin Elmer fluorimeter (LS 5B) using a red-sensitive photomultiplier. The ALA standard line was used to calculate the original ALA concentration in tissue concentration (nmol per mg protein).

**Determination of ALA, porphobilinogen and porphyrins in urine**

Porphobilinogen and ALA were separated from urine by ion-exchange chromatography using a column and procedure according to the instructions of the manufacturer (BioRad Laboratories, ALA Test Instruction Manual, 1967). This method is based on the procedure of Mauzrall and Granick. In summary: first 0.5 ml of urine was loaded on a mixed-bed ion-exchange column. Interfering substances were then removed by washing the column (three times with 10 ml of water). ALA is then eluted by loading the column with 3.5 ml of sodium acetate and porphobilinogen is subsequently eluted by loading the column with 4 ml of acetic acid. Both the ALA fraction and the porphobilinogen fraction are then converted into a red compound by adding Ehrling’s reagents. The product is then quantified using a spectrophotometer (553 nm). Porphyrins in urine were separated and quantified in a high-performance liquid-chromatography procedure according to Lim and coworkers using a reversed-phase column and a fluorescence detector (LC Analist system with LS40 detector, Perkin Elmer, Nieuwerkerk a/d IJssel, The Netherlands). This procedure results in complete separation of porphyrin isomers.

**Porphyrin analysis**

The analysis was carried out according to Chisolm and Brown with the following modifications: the tissues were suspended in sterile water (1:10 wt./vol.) and homogenized in a tissue grinder. To 100 µl of this homogenate the following were added: 700 µl HCL 2 mmol l⁻¹, and 800 µl ethyl-acetate/glacial acetic acid (3:1). After 10 min centrifugation at 1800g the ethyl-acetate phase with some protein is removed, and after a second centrifugation for 5 min at 1800g, the HCL phase is measured in a LS 5B fluorescence spectrometer using a red-sensitive photomultiplier (Perkin Elmer, Nieuwerkerk a/d IJssel, The Netherlands) at an excitation wavelength of 410 nm and an emission wavelength of 650 nm. Although porphyrins are commonly determined by emission at 601 nm, under the conditions described, PpIX has also a specific emission at 650 nm, which is about 80% of the emission at 601 nm and can be easily detected using a red-sensitive photomultiplier.
In our experience, bile acids in duodenal aspirates, and to a lesser extent other tissues, contain fluorophores with high emission at lower wavelength, which give rise to interference in porphyrin quantitation at 601 nm. Emission at 650 nm in a direct extraction assay as described above was found to be proportional to PpIX quantification, determined by a limited number of high-performance liquid-chromatography porphyrin separations. Porphyrin standards were analysed separately for their actual concentration using ultraviolet spectroscopy and the molar extinction coefficient ($\varepsilon_{407} = 0.275$ $\text{L mol}^{-1} \text{cm}^{-1}$). Recovery of porphyrins was checked by adding standard PpIX to the samples. A recovery between 90 and 100% was achieved.

**Fluorescence microscopy**

Tissue blocks were mounted on OCT medium (Tissue-Tek, Elkhart, USA), cut into sections of 10 μm thick (four sections per sample) using a cryostat microtome (Kryostat 1720, Leica, Rijswijk, The Netherlands) and stored at -80°C, only being allowed to thaw just prior to fluorescence microscopy. For the analysis of porphyrin localization a laser scanning microscope (Zeiss, Oberkochen, Germany) was used. Preparations were imaged using a helium/neon laser (633 nm) for fluorescence excitation. The emitted light was detected by a photomultiplier after passing a 665 nm long-pass filter. After fluorescence microscopy, sections were fixed in formalin and stained with haematoxylin and eosin for comparative histological analysis using conventional light-microscopy study.

**Statistical analysis**

The values of the liver enzymes and renal function tests are expressed as mean ± standard error of the mean (SEM). Statistical analysis of these tests was done with the Student’s $t$-test. Peak ALA and porphyrin concentrations in tissues after oral and intravenous ALA administration were compared using the Student’s $t$-test. Comparison of the total amount of porphyrins in tissues after oral and intravenous ALA administration was performed by an area under the curve analysis. $P$ values less than 0.05 were considered significant. A linear regression model was used to analyse the relation in each tissue between maximum ALA concentration, total amount of ALA, maximum porphyrin concentration and total concentration of porphyrins.

**RESULTS**

All animals survived until sacrifice and none showed signs of discomfort.
Liver enzymes and renal function tests

The values of ALAT and ASAT of the i.v. control rats (4 h after phosphate-buffered saline administration) were higher than those of the p.o. control rats (114.6±23.2 versus 58.5±9.4 IU l⁻¹ and 126.8±17.5 versus 83±10.2 IU l⁻¹) (Table 7.1).

Table 7.1 Liver enzymes and renal function tests after ALA administration.

<table>
<thead>
<tr>
<th>Hours after</th>
<th>ALAT (IU l⁻¹)</th>
<th>ASAT (IU l⁻¹)</th>
<th>Creatinine (mmol ml⁻¹)</th>
<th>Urea (µmol ml⁻¹)</th>
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<tr>
<td>0*</td>
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<tr>
<td>3</td>
<td>62.0±6.6</td>
<td>143.9±21.2</td>
<td>91.2±10.8</td>
<td>174.5±35.8</td>
</tr>
<tr>
<td>4</td>
<td>62.8±2.9</td>
<td>118.6±8.9</td>
<td>114.7±10.7</td>
<td>148.7±11.0</td>
</tr>
<tr>
<td>6</td>
<td>58.7±1.7</td>
<td>148.9±31.1</td>
<td>111.9±13.7</td>
<td>163.3±6.5</td>
</tr>
<tr>
<td>12</td>
<td>38.1±0.2</td>
<td>72.3±16.1</td>
<td>96.1±7.4</td>
<td>118.6±20.6</td>
</tr>
<tr>
<td>24</td>
<td>42.9±10.3</td>
<td>59.0±2.4</td>
<td>93.6±3.9</td>
<td>108.2±9.4</td>
</tr>
</tbody>
</table>

Mean±standard error of the mean of liver enzymes and renal function tests (alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), creatinine, urea) after administration of 5-aminolevulinic acid (ALA) orally (po) or intravenously (iv). Each value represents the mean of three rats.

* The values at 0 h are obtained from rats, 4 h after receiving phosphate-buffered saline po or iv.

In the p.o. group, serum ALAT and ASAT did not change after administration of ALA. In the i.v. group, liver functions returned to normal 12 h after ALA administration. Creatinine and urea of the p.o. and i.v. control rats did not differ (36.7±1.2 versus 36.7±1.8 µmol l⁻¹ and 5.7±0.5 versus 5.9±0.4 mmol l⁻¹, 4 h after p.o. and i.v. phosphate-buffered saline administration, respectively). In the p.o. group creatinine was elevated until 4 h after ALA administration (P<0.007), urea was not changed. In the i.v. group creatinine was transiently elevated 1 h after ALA administration (P<0.001); urea was not changed.

ALA distribution

ALA peak concentration was higher after p.o. than after i.v. ALA administration in parts of the gastrointestinal tract (stomach (P=0.004), duodenal aspirate (P=0.007), jejunum (P<0.001) and kidney (P=0.02) and less pronounced (not significantly) in liver and plasma (Figure 7.1).

In the p.o. group, ALA concentration in oesophagus, colon, spleen, bladder, heart, lung, muscle, fat, skin, brain and nerve were similar to the i.v. group. A rapid rate of reduction led to undetectable concentration of ALA in the i.v. group.
already at 3 h after administration in oesophagus, stomach, colon, liver, heart, lung, muscle, fat, skin and nerve. In duodenal aspirate, jejunum, spleen, kidney and bladder, ALA concentration was no longer detectable at 6 h after ALA administration. In brain ALA concentration only rose marginally. ALA concentration reached undetectable levels somewhat later in the p.o. group compared to the i.v. group in stomach, duodenal aspirate, jejunum, liver, kidney and bladder. In plasma, ALA concentration was higher in the p.o. than in the i.v. group already at 1 h after administration \((P<0.001)\). Plasma ALA levels in both groups declined sharply with a half-life of approximately 50 min. In urine ALA was found already 1 h after administration, both in the p.o. and i.v. group. Peak concentration was found at 2 h and only small amounts of ALA were found in urine after 4 h.

![Figure 7.1 Tissue distribution of 5-aminolevulinic acid (ALA) in various tissues measured at 0, 1, 2, 3, 4, 6, 12 and 24 h (tick marks on x-axis) after administration of 200 mg kg\(^{-1}\) ALA p.o. (dashed line) or i.v. (solid line). Each point represents ALA concentration obtained from three different rats. ALA concentrations are plotted on a logarithmic y-axis.](image)

**Porphyrin distribution**

Concentrations are given in nmol per gram wet tissue because for PDT absolute porphyrin concentrations are important (Figure 7.2). A representation of values per mg protein obscured the results as there is a large variance in protein content between different tissues. Peak concentration of porphyrins in all tissues was...
similar after p.o. or i.v. ALA administration ($P > 0.05$). The amount of porphyrins was highest in duodenal aspirate, followed by jejunum, liver and kidney (> 10 nmol per gram tissue). Porphyrins accumulated less in oesophagus, stomach, colon, spleen, bladder, heart, lung and nerve (2-10 nmol g$^{-1}$), and only slightly in plasma, muscle, fat, skin and brain (< 2 nmol g$^{-1}$). The patterns of all tissues except kidney show a rapid rise following ALA administration. Peak levels are found between 2 and 4 h after ALA administration in the p.o. group and between 1 and 3 h in the i.v. group. Twelve hours after administration of ALA, porphyrin concentration in all tissues except kidney returned to background levels. In the kidney a relatively high basal concentration of porphyrins was found (5.2±0.4 nmol g$^{-1}$) and the concentration climbed throughout the observed period, being approximately 2.5 times as high at 24 h compared to the basal concentration. The highest concentration of porphyrins in plasma was found at 2 h after the i.v. ALA dose and at 3 h after the p.o. ALA dose. The plasma concentration declined to levels close to baseline after 12 h. In urine the first porphyrins above baseline level could be detected after 4 h. At 24 h after ALA administration porphyrin concentrations in urine returned to normal.

Figure 7.2 Time-dependent accumulation of porphyrins in various tissues following p.o. (dashed line) and i.v. (solid line) administration of 200 mg kg$^{-1}$ 5-aminolevulinic acid (ALA). Porphyrin concentrations were measured at 8 time-points: 0, 1, 2, 3, 4, 6, 12 and 24 h (tick marks on x-axis) after ALA administration. Each point represents mean values of three rats. Porphyrin concentrations are plotted on a logarithmic scale.

111
Fluorescence microscopy

In laser scanning images of various tissues, 3 h after oral ALA administration, fluorescence due to porphyrin accumulation was most pronounced in epithelial lining of the tongue, oesophagus, jejunum, colon, bladder, prostatic glands and pancreatic duct, the ocular conjunctiva and ciliary body of the eye (Figure 7.3). In the submucosa and muscular layers fluorescence was at background levels. In liver diffuse fluorescence was located in the lobules, while the central veins showed no fluorescence. In kidney, fluorescence was located in the tubules and was at background levels in the glomeruli. Fluorescence images of control rats showed no fluorescence, besides autofluorescence of the luminal surface of the keratin layer of the tongue and oesophagus and autofluorescence in the colonic lumen.

Total ALA biodistribution

To study ALA biodistribution after intravenous and oral administration, we combined ALA, the intermediate porphobilinogen, and porphyrin concentrations for each tissue at each time point to get the total amount of ALA. The formation of porphobilinogen requires two molecules of ALA and that of porphyrins eight molecules, so that the porphobilinogen and porphyrin concentrations were multiplied by two and eight respectively. Thereafter, the amount of total ALA per organ or per total volume (blood, duodenal aspirate and urine) was determined. Because ALA in the urine is not used for porphyrin synthesis, the cumulative amount of ALA in urine was subtracted from the amount of all tissues together at each time point. After that, the amount of total ALA in each tissue was divided by the total amount of ALA in the body, giving a stable pattern during the first 4 h (Table 7.2).

Predictive value of ALA concentration

After determining the area under the curve of ALA and porphyrin concentrations in each tissue, no correlation (correlation coefficient < 0.5) was found between maximum porphyrin concentration and maximum or total ALA concentration. A correlation coefficient of 0.87 was found between total amounts of accumulated porphyrins (area under the curve) and maximum or total ALA concentration respectively.
Figure 7.3 Laser scanning microscopic images of frozen sections (10 μm) of various tissues, 3 h after oral administration of 200 mg kg\(^{-1}\) 5-aminolevulinic acid, together with the corresponding haematoxylin & eosin sections. Fluorescence in this set-up is produced by porphyrins. Only autofluorescence of the luminal surface of the keratin layer of the tongue and oesophagus and autofluorescence of the colonic lumen were also present in images of control rats. Fluorescence intensity must not be extrapolated to porphyrin concentration as the optical absorption properties of tissues vary widely.

Left in descending order: bladder, jejunum, prostate, pancreas, colon.
Right in descending order: eye, liver, kidney, oesophagus, tongue.
Table 7.2 5-aminolevulinic acid (ALA) biodistribution. Total ALA (ALA or converted into porphobilinogen or porphyrins) in each tissue as percentage of total ALA in the body.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>percentage of total ALA after p.o. ALA administration</th>
<th>percentage of total ALA after i.v. ALA administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>oesophagus</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>stomach</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>duodenal aspirate</td>
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<td>5</td>
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<tr>
<td>small bowel</td>
<td>17</td>
<td>7</td>
</tr>
<tr>
<td>colon</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>liver</td>
<td>45</td>
<td>55</td>
</tr>
<tr>
<td>spleen</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>blood</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>kidney</td>
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<td>13</td>
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<tr>
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<td>brain</td>
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</tr>
<tr>
<td>nerve</td>
<td>&lt;0.1</td>
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</tr>
</tbody>
</table>

Discussion

The interest for ALA-PDT as a cancer treatment has increased rapidly during the past few years due to comparatively high tumour selectivity and short-lasting skin photosensitivity. Until now, ALA has been administered without any detailed information about its pharmacokinetics. This animal study was designed to determine systematically the time course of ALA biodistribution and subsequent accumulation of porphyrins after oral and intravenous ALA administration in the normal body. A tumour model was not used, as ALA-PDT is most suitable for carcinoma in situ or premalignant lesions. Many premalignant lesions, such as Barrett's oesophagus, are composed of metaplastic epithelium, which, besides their metaplastic appearance, are normal rather than cancer-like tissues. Besides that, the literature shows that different tumours behave quite differently with regard to porphyrin accumulation after administration of ALA. For optimal PDT, data from each specific tumour or premalignant tissue are needed.
ALA concentration was highest in kidney, bladder and urine and, after p.o. ALA administration, also in jejunum. Porphyrins accumulated mainly in duodenal aspirate, jejunum, liver and kidney and were an order of magnitude lower in plasma, muscle, fat, skin and brain.

The elevated ALAT and ASAT levels of the i.v. control rats can be explained by the use of ether anaesthesia in this group. Thus, in the i.v. group, the effect of ALA administration on liver function was mixed with the effect of ether anaesthesia. Twelve hours after ALA (and ether) administration liver functions returned to normal. If i.v. ALA administration had an effect on liver function, it was only short lasting. In the p.o. group no anaesthesia was used and ALA did not cause elevation of ALAT or ASAT. Renal function tests were unchanged by ether, and ALA only caused mild transient elevation of creatinine after both p.o. and i.v. administration.

Figure 7.1 shows that the concentration of ALA in some tissues was higher after p.o. than after i.v. ALA administration; in stomach, duodenal aspirate and jejunum this pattern exceeded 6 h. This suggests that after oral ALA administration, some ALA is absorbed in the stomach (probably passively due to its neutral charge at low pH), but most ALA is absorbed in the small intestine. The lower ALA peak concentrations in liver, plasma and kidney in the i.v. group compared the p.o. group are probably due to delayed absorption from the gastrointestinal tract and the timing of the sampling. It is still not clear whether ALA is able to pass the blood-brain barrier. In our study, ALA concentration in brain only rose slightly; porphyrin concentrations however, were indeed elevated after ALA administration. Because porphyrins cannot pass an intact blood-brain barrier, the porphyrin accumulation in this study suggests that small amounts of ALA do pass the barrier. An enterohepatic circulation of ALA possibly occurs, because ALA was found in duodenal aspirate after i.v. ALA administration and the peak ALA concentration in jejunum was reached later than in other tissues. Most tissues converted all ALA into porphyrins. In kidney, only a small part of ALA was converted into porphyrins; a large proportion was excreted unchanged in urine. In liver, besides conversion into haem, porphyrins (and a small amount of ALA) were excreted via the bile.

Despite differences in ALA concentration in some tissues after oral and intravenous ALA administration, there was no difference in the porphyrin concentration in the organs we studied (Figure 7.2). Both peak porphyrin concentrations and total accumulation were similar between the two routes of administration, suggesting that the amount of ALA given was above that needed to give a maximum rate of porphyrin synthesis.

The time course of ALA and porphyrin concentration changes in tissues provides evidence for in situ synthesis of porphyrins rather than uptake of PpIX.
from the systemic circulation. Furthermore, it seems unlikely that the liver is the main supplier of PpIX, because peak porphyrin concentration in the liver did not appear before that in other tissues and PpIX concentration in jejunum showed the same pattern as was found in other tissues (Figure 7.2).

For PDT it is important to know which tissues are photosensitized at what time. No studies comparing porphyrin concentration and photosensitization after ALA are available. Most studies have used fluorescence techniques to determine porphyrin concentration in arbitrary gray units. The optical absorption properties of tissues vary widely so that the fluorescence levels between different tissues cannot be compared. Other studies have evaluated the PDT effects after various doses or routes of ALA administration but did not determine porphyrin concentration at a tissue level. Comparison of two reports from a single group suggests that 2.2 μg PpIX per gram tissue (3.6 nmol g⁻¹) can already cause necrosis in normal rat colonic mucosa on exposure to light. Another study has reported delayed tumour growth after irradiation of a tumour in rats with an amount of 4 μg porphyrins per gram tumour (6.6 nmol g⁻¹). In our study these "photosensitive" concentrations were reached in all tissues except in muscle, fat, skin and brain.

Quantitative data of PpIX concentrations in different tissues after ALA administration are only available from three other studies. The first found that rat liver and intestine contained the highest levels of PpIX (approximately 6.3 nmol per gram tissue), followed by aorta (4.3 nmol g⁻¹) and oesophagus (2.1 nmol g⁻¹), 3 h after 300 mg kg⁻¹ ALA i.v. Serum, muscle and skin hardly contained any porphyrins (approximately 0.5 nmol per gram tissue). Although we found higher concentrations of porphyrins, the order of ranking is comparable. In humans, after oral administration of 60 mg kg⁻¹ ALA, peak PpIX concentration in liver (10.1 nmol per gram tissue) was also found to be higher than in skin, muscle or omentum (<1 nmol g⁻¹). In the third study in two rabbits, surprisingly, the highest concentration of PpIX was found in plasma (454.20 μg g⁻¹ = 748.76 nmol ml⁻¹) compared to only 11.4 μg g⁻¹ in liver (18.8 nmol g⁻¹ liver), 3 h after 100 mg kg⁻¹ i.v. ALA administration. In our study PpIX concentration in plasma reached a maximum of only 1.35 nmol ml⁻¹, compared to 16.5 nmol g⁻¹ in liver. This is consistent with the results reported in a human study showing a maximum PpIX concentration in plasma of 1.22 nmol ml⁻¹, 7 h after oral administration of 40 mg kg⁻¹ ALA. In red blood cells we found a maximum PpIX concentration of 11 nmol ml⁻¹ red blood cells, 1 h after ALA administration (data not shown).

Besides quantification, porphyrin localization is also important. Fluorescence microscopy confirmed that fat and muscle contained low concentrations of porphyrins, while epithelial linings showed strong fluorescence. This combination makes ALA-PDT highly suitable for treatment of epithelial lesions such as premalignant and malignant skin and bladder lesions and Barrett's oesophagus.
Maximal porphyrin concentration was found in most tissues after 2 to 3 h (Figure 7.2), with porphyrin concentrations close to baseline levels at 12 h after ALA administration. The only exception is the kidney. There is supply of ALA to the kidney from 1 to 24 h, as ALA is also excreted in the urine during that time. Probably porphyrin synthesis in the kidney is at maximum rate at least during the first 24 h after ALA administration.

With regard to tumours, literature on porphyrin concentrations in rat tumours is sparse. Hua et al. found a maximum porphyrin concentration of 6.9 nmol per gram R3230AC mammary adenocarcinoma (3 h after 300 mg kg\(^{-1}\) ALA i.v.) and 8.2 nmol per gram NMU mammary adenocarcinoma (4 h after 300 mg kg\(^{-1}\) ALA i.v.).\(^{14}\) In a previous study of our group a maximum ALA-induced porphyrin concentration in mammary tumour BN472 of only 2.0 nmol g\(^{-1}\) after 2 mg ml\(^{-1}\) ALA in drinking water for 9 days was reported.\(^{31}\) Loh et al. described a maximum porphyrin concentration of 11.7 nmol g\(^{-1}\) in chemically induced duodenal tumours, 4.5 h after 200 mg kg\(^{-1}\) ALA i.v.\(^{27}\) These data show that there is a wide variance in tumour porphyrin concentration after administration of ALA.

Further studies of ALA-induced porphyrin production in different tumours \textit{in situ} are needed before the dosing scheme which provides an optimal ratio between tumour and surrounding tissue can be determined. On the basis of the described data in this study, various tissues can now be excluded from contributing to photosensitization.

After photobleaching, re-appearance of fluorescence has been reported, what can be used to induce more pronounced PDT effect by fractionating the illumination.\(^{32,33}\) Three hours after ALA administration, some ALA was still present in duodenal aspirate, jejunum, spleen, kidney and bladder and after p.o. ALA administration additionally in the stomach. Duodenal contents, small intestine and kidney could play a role as ALA depot (Figure 7.1), or small amounts of ALA present in each tissue could be the source of new porphyrins after an initial light dose.

It seems not unlikely that various tissues have a different demand for haem synthesis related to their function and therefore a different quantity of haem-synthesizing enzymes. Tissues with a short cell doubling time or high rate of metabolism have been suggested to be more likely to accumulate porphyrins after ALA; however, \textit{in vitro} studies do not fully support this.\(^{31}\) Due to the bypass of the feedback regulation, cells in most tissues are not capable of converting all the ALA entering the cell into haem. Iron stores probably will be insufficient, resulting in accumulation of PpIX. Another explanation for selective porphyrin accumulation is a difference in activity of specific enzymes of haem biosynthesis. Porphobilinogen deaminase has been reported to be higher and ferrochelatase lower in tissues that accumulate more PpIX.\(^{35,36}\) However, a simple relationship between the
activity of these enzymes and porphyrin concentration in different tissues seems unlikely. Another explanation is that the uptake of ALA is greater or its efflux lower in tissues that accumulate high concentrations of porphyrins. To approach this, we evaluated the correlation between maximum or total ALA concentration and maximum or total porphyrin concentration in each tissue. In contrast to peak porphyrin concentration, total porphyrin accumulation (area under the curve) correlated well with maximum and total ALA concentration in the tissues. A difference in rate of porphyrin synthesis rather than a difference in ALA uptake between tissues might therefore be responsible for selective ALA-induced porphyrin accumulation. In time most tissues are capable of converting porphyrins into haem as might be concluded from our results.

In conclusion: this study describes the biodistribution of ALA and subsequent porphyrin accumulation in different tissues. Administration of 200 mg kg\(^{-1}\) ALA resulted in accumulation of photosensitive concentrations of porphyrins, 1 to 6 h after ALA administration, in all tissues except muscle, fat, skin and brain. No difference in porphyrin concentrations was found between systemic and oral ALA administration. Knowledge of the time-concentration relationship should be helpful in selecting dosages, routes of administration and timing of ALA-PDT.
REFERENCES

19. Mauzerall D, Granick S. The occurrence and determination of 5-aminolevulinic acid and
CHAPTER 8

KINETICS, LOCALIZATION AND MECHANISM OF 5-AMINOLEVULINIC ACID-INDUCED ENDOGENOUS PHOTOSENSITIZATION OF RAT NORMAL AND BARRETT'S OESOPHAGUS

This chapter has been adapted from Van den Boogert et al. Lasers in Surgery and Medicine 1999;24:3-13.
**INTRODUCTION**

Since 1970 the incidence of oesophageal adenocarcinoma is rising both in Europe and in the United States, at a rate greater than that of any other malignancy.\(^1\) Barrett’s epithelial metaplasia is a premalignant condition, probably arising from (duodeno)gastro-oesophageal reflux, causing a 30- to 50-fold greater risk of developing oesophageal adenocarcinoma (Chapter 1).\(^2\) Antireflux medication such as proton-pump inhibitors, H-2 receptor blockers, or possibly antireflux surgery are the current treatment options. Although reflux complaints disappear in almost 100% of treated cases, no effect on Barrett’s epithelium itself has been shown, and malignant degeneration towards adenocarcinoma can still proceed.\(^3\) Thus, most studies have advised using endoscopic surveillance for patients with Barrett’s oesophagus. New forms of therapy are under investigation to completely eliminate Barrett’s epithelium. Laser therapy is one of these new modalities. Local intraluminal laser coagulation, which causes thermal destruction and ablation of the lesion in combination with antireflux therapy, may lead to regeneration with squamous epithelium (Chapter 2).\(^4\) A disadvantage is the relative uncontrolled damage through the entire wall, which often leads to strictures and perforations. Regeneration with both squamous and columnar epithelium has also been described.\(^5\)

In photodynamic therapy (PDT), laser light is used to activate a photosensitizing drug previously administered to accumulate in (pre)malignant tissues. This results in a photochemical reaction, with the generation of singlet oxygen causing cell death (Chapter 3). The best known clinical photosensitizers are haematoporphyrin derivate (HpD) and its more purified form, Photofrin®. Although clinical studies have shown some effectiveness in the treatment of Barrett’s oesophagus and superficial oesophageal cancer, they have three main drawbacks.\(^6,7\) They cause prolonged skin sensitivity to light, thus requiring avoidance of sunlight, and offer limited tumour selectivity.\(^8,10\) Moreover, strictures requiring dilatations are a frequent complication when using HpD or Photofrin®.\(^6,7\) A new method of photosensitization is the administration of the natural porphyrin precursor 5-aminolevulinic acid (ALA).\(^11\) The synthesis of ALA is rate limiting in the haem-synthetic pathway.\(^12\) The administration of exogenous ALA bypasses both the rate-limiting step and the feedback control in the haem biosynthesis, and leads to accumulation of protoporphyrin IX (PpIX) in certain types of cells and tissues (Chapter 7).\(^13\) ALA-PDT could therefore be a suitable therapy for Barrett’s oesophagus. Although some clinical case reports have described promising results, remaining islands of columnar epithelium after therapy have been found in several studies.\(^14\) These therapy-resistant cells may be eliminated by adjusting the ALA-PDT treatment.
The present study was carried out to determine the optimal timing of ALA administration with respect to subsequent therapeutic illumination. In a first experiment, the kinetics and localization of ALA-induced porphyrin accumulation in the rat normal oesophagus were studied. To understand the mechanism of porphyrin accumulation, ALA and iron concentrations and activity of porphobilinogen deaminase and ferrochelatase were determined in the different layers of the oesophagus. In the second experiment, with laser scanning microscopy, localization and relative concentration of ALA-induced porphyrin accumulation in Barrett's and adjacent normal oesophagus was studied in an animal model (Chapters 4 and 5).

MATERIALS AND METHODS

Animals and materials

The experimental protocol was approved by "The Committee on Animal Research" of the Erasmus University Rotterdam. Ninety-two male adult Wag/Rij rats (Harlan CPB, Austerlitz, The Netherlands), weighing 180-200 g were used for the experiments. They had free access to rat chow and tap water. ALA was obtained from Sigma Chemical Company (Zwijndrecht, The Netherlands) with a purity of 98%. The following reagents were purchased from Porphyrin Products (Logan, Utah, USA): PpIX, disodium salt, and zinc PpIX. Tris-HCl was purchased from Boehringer (Mannheim, Germany), and other chemicals were purchased from Merck (Darmstadt, Germany).

Experimental design

Forty-eight animals were randomly assigned to two study groups of 21 animals each and one control group of six animals. In the study groups, the animals were administered 200 mg kg\(^{-1}\) ALA dissolved in phosphate-buffered saline either by intravenous injection (i.v.) into the penile vein under ether anaesthesia (n=21), or by oral gavage (p.o., n=21). Control animals received phosphate-buffered saline either i.v. (n=3) or p.o. (n=3). In the study groups, after administration of ALA, the animals were placed in subdued light. They were sacrificed at 1, 2, 3, 4, 6, 12 or 24 h after administration in groups of three. Control animals were sacrificed at 4 h. In subdued light, a small piece of the distal oesophagus was excised and immediately snap frozen and stored at -80°C for fluorescence microscopic study. The rest of the oesophagus was then resected and opened longitudinally. The mucosa was surgically separated from the underlying muscularis. The separated tissues were frozen immediately and stored at -80°C until chemical extraction.
Twenty additional untreated rats were used to determine ferrochelatase and porphobilinogen deaminase activity (n = 10) and iron concentration (n = 10) in both oesophageal mucosa and underlying muscular layer.

**Barrett's oesophagus**

Twenty-four animals underwent an oesophagojejunostomy with total gastrectomy according to the model of Levrat *et al.* to induce duodeno-oesophageal reflux and subsequent Barrett's columnar epithelium (Chapters 4 and 5). A median laparotomy was performed, and the oesophagus was dissected 2 mm above its entry in the stomach. The stomach was resected and the duodenum closed. Thereafter, the oesophagus was anastomosed end-to-side to the jejunum, approximately 5 cm distal to the Treitz ligament. After 18 weeks of induced duodenal reflux, 21 rats received 200 mg kg⁻¹ ALA intravenously. Rats were sacrificed at 1, 2, 3, 4, 6, 8 or 12 h after ALA administration (n = 3/time point). Three controls received phosphate-buffered saline i.v. and were sacrificed 3 h later. The oesophagus was dissected and cut into seven 5-mm pieces; the most distal piece was approximately 2 mm proximal to the anastomosis. The samples were immediately snap frozen and stored at -80°C for fluorescence microscopic study.

**Determination of ALA concentration**

ALA was extracted from tissue samples with a mixture of dimethylsulfoxide (DMSO) and methanol (MeOH; 30/70 vol./vol.) and quantified by using a fluorimetric enzyme assay, as described in the previous chapter. In brief, tissue homogenates were mixed with 480 μl DMSO/MeOH and centrifugated; 200 μl of ALA standard solution (0.1-2 nmol ml⁻¹) or diluted tissue homogenates were mixed with 50 μl of an enzyme mixture of porphobilinogen deaminase and ALA deaminase in Tris-HCl buffer or with 50 μl of Tris-HCl buffer for blank detection. The samples were incubated overnight, and the reaction was stopped by addition of 750 μl stop buffer (one part Tris-HCl and two parts trichloroacetic acid). The samples were then placed on a ultraviolet light source (350 nm Woods light) to convert porphyrinogens into porphyrins and centrifugated. The porphyrin concentration in the supernatant was determined in a Perkin Elmer fluorimeter (LS 5B, Nieuwerkerk aan den IJssel, The Netherlands) by using a red-sensitive photomultiplier. The ALA standard line was used to calculate the original ALA concentration in tissue concentration (nmol per mg protein).

**Porphyrin analysis**

The analysis was carried out according to Chisolm and Brown with some modifications (Chapter 7). To tissue homogenates, HCL and ethyl-acetate/glacial acid were added. After centrifugation, the ethyl-acetate phase with
some protein was removed, and after a second centrifugation the HCL phase was measured in a fluorescence spectrometer LS 5B using a red-sensitive photomultiplier (Perkin Elmer), at an excitation wavelength of 410 nm and an emission wavelength of 650 nm. Emission at 650 nm in a direct extraction assay as described above was found to be proportional (≥90%) to PpIX quantification as determined by a limited number of high performance liquid chromatography porphyrin separations. Porphyrin standards were analysed separately for their actual concentration using ultraviolet spectroscopy and the molar extinction coefficient ($\varepsilon_{407} = 0.275 \text{mol}^{-1} \text{cm}^{-1}$). Recovery of porphyrins was checked by adding standard PpIX to the samples. A recovery between 90 and 100% was achieved.

Protein was measured using the method of Lowry et al.23

**Fluorescence microscopy**

The method has been discussed in detail in the previous chapter.20 In brief, in subdued light, tissue blocks were cut into sections of 10 μm thick (four sections per sample) and stored at -80°C. Just prior to fluorescence microscopy, the sections were thawed. Preparations were imaged by using a helium/neon laser (633 nm) for fluorescence excitation. After passing a 665 nm longpass filter, the emitted light was detected by a photomultiplier. All images were recorded in one session to avoid errors due to daily differences in microscope settings. The digital images were analysed with the Kontron KS-400 image analysis program (Kontron Electronik, München, Germany). The respective areas of interest were outlined manually or determined by interactive thresholding. After that, the mean fluorescence (integrated fluorescence/surface area) in each area was automatically calculated. After fluorescence microscopy, sections were fixed in formalin and stained with haematoxylin and eosin (H&E) for comparative histological analysis.

**Determination of porphobilinogen deaminase and ferrochelatase activity**

The samples were homogenized in sterile water (1:10 wt./wt.) using a Potter Elvehjem homogenizer. Porphobilinogen deaminase was measured as described previously by Wilson et al. after an initial incubation at 55°C for 60 min and cooling to room temperature to destroy the activity of uroporphyrinogen decarboxylase and to prevent further metabolism of uroporphyrinogen.24 Ferrochelatase was measured by a modification of the method of Li et al.25 50 μl of the homogenate was added to 100 μl 0.25 mol l⁻¹ TrisHCl buffer, pH 8.2, containing 8 g l⁻¹ Triton X-100 and 1.75 mmol l⁻¹ palmitic acid. 50 μl of a 250-μmol l⁻¹ solution of PpIX in KOH 0.01 mol l⁻¹ were added, and the reaction was started by the addition of 50 μl of a 200-μmol l⁻¹ solution of zinc acetate in water. The mixture was incubated at 37°C for 60 min, and the reaction was stopped by the addition of 1 ml of DMSO/MeOH (30:70). After subsequent centrifugation in an Eppendorf
centrifuge for 5 min at 1800g, 100 μl of supernatant was injected on a Perkin Elmer high performance liquid chromatography with a reversed-phase Chrompack RP18 column, with acetone/methanol/water/formic acid (560:240: 200:2) 1 ml min⁻¹ as mobile phase. Zinc PpIX was detected by a Perkin Elmer LS40 fluorometer, with an excitation wavelength of 415 nm and an emission wavelength of 580 nm. Results were expressed as nmol per mg protein.

**Determination of iron concentration**

The concentration of iron was measured spectrophotometrically by using a modification of the method described by Lauber, with a wavelength of 562 nm.²⁶

**Statistical analysis**

The values are expressed as means ± standard error of the mean (SEM). Comparisons were made using the Student's t-test. Total accumulation of porphyrins after oral and intravenous ALA administration was calculated using an area under the curve analysis. A value of \( P<0.05 \) was considered significant.

**RESULTS**

**ALA distribution**

Maximum ALA concentration in mucosa and muscularis was reached at 1 h after p.o. and i.v. ALA administration. In the p.o. group, peak ALA concentration was higher in mucosal tissue than in muscularis (2.08±0.24 versus 1.11±0.18 nmol per mg protein, \( P=0.03 \)); in the i.v. group this was not statistically significantly different (1.46±0.22 versus 1.26±0.30 nmol per mg protein, \( P=0.06; \) Figure 8.1). There was no difference in maximum ALA concentration in both mucosa and muscularis after p.o. or i.v. administration. After 1 h, in both groups and tissue layers, ALA concentration declined sharply, with a half-life of approximately 1 h, and was at background levels at 4 h after ALA administration.

**Porphyrin distribution**

Basal porphyrin concentration was higher in the mucosa, compared to the muscularis of the squamous-lined oesophagus (\( P=0.049; \) Figure 8.1). Peak porphyrin levels, reached at 3 h after ALA administration, were higher in mucosa (90.1±10.0 pmol per mg protein in the p.o. group and 86.8±12.8 pmol per mg protein in the i.v. group) than in muscularis (26.7±3.1 and 32.6±3.4 pmol per mg protein; \( P=0.003 \) in the p.o. group and \( P=0.015 \) in the i.v. group). A maximal mucosa:muscularis ratio of approximately 3.5 was found at 2 h after ALA administration in both groups. Total accumulation of porphyrins (area under the
ALA-kinetics in oesophagus

curve) was also higher in the mucosa than in the muscularis and was equal in the p.o. and i.v. groups. At 6, 12 and 24 h after receiving ALA, porphyrin levels had returned to those levels found in control animals.

Figure 8.1 Extracted porphyrin levels (solid lines, left y-axis) and tissue 5-aminolevulinic acid (ALA) concentration (dashed lines, right y-axis) after administration of 200 mg kg\(^{-1}\) ALA. Each point indicates the mean level of 3 rats ± standard error of the mean. The ALA and porphyrin levels of control rats are given at 0 h. O, mucosa, intravenous ALA; ●, mucosa, oral gavage ALA; □, muscularis, intravenous ALA; ■, muscularis, oral gavage ALA; Δ, ALA concentration in oesophageal mucosa; ▲, ALA concentration in oesophageal muscularis.

Fluorescence imaging

Areas with strong autofluorescence were found in control animals (Figure 8.3). Comparison with the H&E stainings showed that this fluorescence was restricted to the luminal surface of the oesophageal mucosa with nests of food (e.g., chlorophyll and bacteria). In all sections, these autofluorescent areas were present.

After administration of ALA, a strong, broad, homogeneous fluorescence of the basal cell layer of the squamous epithelium was found (Figure 8.4). Weak fluorescence was apparent in the muscularis mucosae. The keratin layer, lamina propria of the mucosa, the submucosa and the muscularis showed fluorescence near background level. The fluorescence intensity of the various oesophageal layers after analysis of the digital images is shown in Figure 8.2. Fluorescence level of the basal cell layer increased rapidly, reaching a maximum at 3 h after ALA administration. Thereafter, fluorescence decreased to background levels at 24 h.
Fluorescence of the muscularis mucosae showed the same pattern at lower intensity levels. The fluorescence level of the other oesophageal layers was barely above background levels. The maximal fluorescence ratio between the squamous basal cell layer and muscularis mucosae was 3 (3 h after ALA administration). This ratio was 7 between the squamous basal cell layer and submucosa or muscularis (also 3 h after ALA). The results of the p.o. and i.v. groups did not differ significantly.

Figure 8.2 Grey-scale values of fluorescence ± standard error of the mean (SEM) in tissue layers of the normal oesophagus as a function of time after 5-aminolevulillic acid (ALA) administration. Each point indicates the mean level of three rats ± SEM. The levels of control rats are given at 0 h. ○, mucosal basal cell layer, intravenous ALA; ●, mucosal basal cell layer, oral gavage ALA; △, muscularis mucosae, intravenous ALA; ▲, muscularis mucosae, oral gavage ALA; □, rest of the oesophageal wall (lamina propria, submucosa, muscularis), intravenous ALA; ■, rest of the oesophageal wall (lamina propria, submucosa, muscularis), oral gavage ALA.

Porphobilinogen deaminase and ferrochelatase activity

Porphobilinogen deaminase activity in the mucosa was almost threefold higher than in the muscularis (78.29 ± 10.8 versus 30.72 ± 7.5 pmol h⁻¹ of uroporphyrin per mg protein, \( P < 0.001; \) Table 8.1). The ferrochelatase activity in oesophageal mucosa and muscularis did not differ significantly (0.68 ± 0.08 versus 0.63 ± 0.09 nmol h⁻¹ of zinc PpIX per mg protein, \( P = 0.16 \)).

Iron concentration

The amount of iron in mucosa (1.40 ± 0.13 \( \mu \)mol per g dry weight) was lower than in muscularis (2.15 ± 0.12 \( \mu \)mol per g dry weight, \( P < 0.001; \) Table 8.1).
Figure 8.3 A: Laser scanning microscopic image of a frozen section (10 μm) of a normal rat oesophagus 4 h after receiving intravenous phosphate-buffered saline. B: Light microscopic image of the same section after staining with haematoxylin and eosin. B, mucosal basal cell layer; K, keratin layer; L, lumen; LP, lamina propria; M, muscularis; MM, muscularis mucosae; SM, submucosa.

Figure 8.4 Fluorescence image (A) of a frozen section (10 μm) of a normal rat oesophagus, 3 h after intravenous administration of 5-aminolevulinic acid (200 mg kg⁻¹), together with corresponding section stained with haematoxylin and eosin (B). Fluorescence image (C) and corresponding haematoxylin and eosin section (D) of the same section at higher magnification. B, mucosal basal cell layer; K, keratin layer; L, lumen; LP, lamina propria; M, muscularis; MM, muscularis mucosae; SM, submucosa.
Table 8.1 Porphobilinogen deaminase (PBGD), ferrochelatase activity, and iron concentration in oesophageal mucosa and muscularis*.

<table>
<thead>
<tr>
<th></th>
<th>Mucosa</th>
<th>Muscularis</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBGD (pmol h$^{-1}$)</td>
<td>78.29±10.77</td>
<td>30.72±7.47</td>
<td>$P&lt;0.001$</td>
</tr>
<tr>
<td>of uroporphyrin per mg protein</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Ferrochelatase (nmol h$^{-1}$)</td>
<td>0.68±0.08</td>
<td>0.63±0.09</td>
<td>$P=0.16$</td>
</tr>
<tr>
<td>of zinc PpIX per mg protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iron (µmol per g dry tissue)</td>
<td>1.40±0.13</td>
<td>2.15±0.12</td>
<td>$P&lt;0.001$</td>
</tr>
</tbody>
</table>

*Values are expressed as mean±standard error of the mean; n=10 for all groups.

PpIX=protoporphyrin IX.

Barrett’s oesophagus

Eighteen weeks after oesophagojejunostomy, several histological abnormalities were observed: regenerative thickening, basal cell hyperplasia, keratin cysts, and Barrett’s columnar metaplasia (Chapters 4 and 5). The Barrett’s mucosa in the rat had the same histological aspect as found in man. It consisted of gastric epithelium with glands and foveolae rather than crypts and villi characteristic of jejunal mucosa. In most animals, the columnar oesophageal epithelium was not in continuum with the jejunal epithelium, but it was separated at the site of the anastomosis (marked by the sutures) by a small area lined by squamous epithelium.

Fluorescence imaging in Barrett’s oesophagus

Eighteen of the 21 animals showed Barrett’s oesophagus in the distal segment. In these sections, fluorescence was also restricted to the epithelium of the mucosa but had a heterogeneous aspect (Figures 8.5 and 8.6). Some areas showed strong fluorescence, others, especially some deep glands, showed considerably less, and some even no detectable fluorescence. The stromal tissue between the glands showed fluorescence near background level.

Fluorescence intensity did not depend only on the time of illumination after ALA administration but also on the histologic appearance of the columnar mucosa and the quantity of glands in particular. Highest mean fluorescence was found at 3 h after ALA administration, but the values within each animal and between animals sacrificed at the same time after ALA administration, differed considerably. Within each animal, we compared the grey values of the columnar mucosa with that of the adjacent squamous epithelium (with regenerative thickening and basal cell hyperplasia, up to 20 cell layers thick, also showing homogeneous fluorescence restricted to the epithelial layer) and the squamous epithelium in the proximal sections (3 to 5 rows of cells lining the surface).
For determination of the grey value of the columnar mucosa, all mucosal glands were included, at some places extending up to the muscularis mucosae and up to 50 rows thick with, besides epithelial cells, stromal cells. At none of the studied time points after ALA administration was the fluorescence of the Barrett’s epithelium higher than that of the normal mucosa.

**DISCUSSION**

This study shows that, in the rat oesophagus, ALA-induced porphyrin accumulation occurs selectively in mucosa, whether squamous or Barrett’s, compared to the muscularis. No selectivity of PpIX accumulation in Barrett’s epithelium in favour of adjacent squamous epithelium was detected. Maximal porphyrin concentration was found at 3 h after ALA administration. A possible explanation for the selective accumulation of porphyrins is a higher ratio of porphobilinogen deaminase:ferrochelatase in the mucosa compared to the muscularis and a relatively low iron concentration.

ALA-PDT treatment for Barrett’s oesophagus is, in theory, based on selective ALA-induced porphyrin production in Barrett’s mucosa and a much lower production in surrounding normal mucosa. Within each animal, we determined fluorescence in both Barrett’s and adjacent squamous epithelium at several hours after ALA administration. With respect to its premalignancy, because treatment of Barrett’s oesophagus requires elimination of all the columnar mucosa, we determined the grey value of the Barrett’s mucosa including the deepest glands and not just of the columnar cells at the surface. No selectivity in porphyrin fluorescence of Barrett’s versus adjacent normal squamous mucosa was found. Furthermore, the heterogeneity of fluorescence in Barrett’s epithelium may explain why ALA-PDT for Barrett’s oesophagus in clinical case reports is not always successful and may lead to pseudoregression.

In the normal oesophageal mucosa and muscularis, we quantified porphyrin concentration with the extraction method. Although surgical excision of the mucosa is not at cellular accuracy, the standard errors of the mean were relatively small. The results obtained from the quantitative chemical extraction method and laser scanning microscopy were supplementary and show that, in normal oesophageal mucosa, a considerable amount of porphyrins accumulates. Besides the precise localizing properties, the laser scanning microscopy also provided information on (relative) porphyrin concentrations. Pilot studies (unpublished data) and results of others have indicated that the porphyrin concentration in the normal rat oesophagus after administration of 200 mg kg⁻¹ ALA is high enough to induce excessive photodynamic damage.
Figure 8.5 Fluorescence image (A) and a subsequent light microscopic image after staining with haematoxylin and eosin (B), of a rat oesophagus 3 h after intravenous administration of 5-aminolevulinic acid and 18 weeks after an oesophagojejunostomy, showing columnar epithelium. C, D: The same section at higher magnification. B, Barrett’s epithelium; L, lumen.

Figure 8.6 Fluorescence image (A) and a subsequent light microscopic image after staining with haematoxylin and eosin (B), of a rat oesophagus 3 h after intravenous administration of 5-aminolevulinic acid and 18 weeks after an oesophagojejunostomy. Both squamous epithelium (S) and Barrett’s mucosa (B) are shown. Fluorescence of Barrett’s mucosa was more heterogeneous, but fluorescence intensity did not differ between squamous and Barrett’s epithelium. Fluorescence in the underlaying submucosa and muscularis was lower compared to the mucosa.
A possible mechanism for selective ALA-induced porphyrin production is that ALA uptake is higher in tissues that accumulate porphyrins.\textsuperscript{28,29} Indeed, ALA concentration in mucosa was higher than in muscularis (significance was reached only in the p.o. group, $P=0.03$). No difference in ALA concentration in either mucosa or muscularis was seen between p.o. and i.v. routes of ALA administration. Until detailed information of cellular mechanisms of ALA uptake and efflux is obtained, the reason for ALA to accumulate selectively in specific tissue layers remains unknown.

Another reason for selective porphyrin accumulation in mucosa might be a difference in activity of the porphyrin-forming enzyme porphobilinogen deaminase and the porphyrin-converting enzyme ferrochelatase between mucosa and muscularis.\textsuperscript{11,30,31} We found no difference in ferrochelatase activity in the mucosa versus the muscularis. However, the porphobilinogen deaminase activity was almost threefold higher in the mucosa than in the muscularis. The ratio porphobilinogen deaminase:ferrochelatase was higher and thus more advantageous for porphyrin accumulation in mucosa (1.15) than in muscularis (0.49, $P<0.001$).

Previous investigations have shown that a difference in iron concentration could be an explanation for selective porphyrin accumulation in certain cells and that iron chelators could enhance ALA-induced porphyrin accumulation.\textsuperscript{32,33} Iron is needed for formation of haem from PpIX. A relatively low iron concentration could be a limiting factor for the formation of haem when high levels of ALA are present, which may result in accumulation of PpIX. Iron concentration in the mucosa was significantly lower than in muscularis ($P<0.001$). Whether this relatively low concentration is indeed restricting for haem formation is not clear. Low iron concentration is essential for bacteriostatic and bactericidal properties in blood, lymph, and exudates.\textsuperscript{34} For the same reason, iron concentration in epithelial lining, being the first barrier to micro-organisms, may be low. However, no other information about iron concentration in epithelial surfaces is available. Iron determination in more tissues and comparison with the level of porphyrin accumulation in these tissues is needed to further understand the role of iron.

Finally, a reason for selectivity of ALA-induced porphyrin accumulation may be a shorter cell-doubling time of tissues that accumulate high porphyrin concentrations.\textsuperscript{32} A higher cell turnover implies a higher metabolic activity, especially a higher requirement for haem, an important component in vital respiratory pigment, and therefore a higher production of porphyrins. Although the high fluorescence of the epithelial basal cell layer could be explained by the high turnover of these cells, the fluorescence of the muscularis mucosae cannot. However, a marked similarity between the fluorescence images and corresponding haematoxylin and eosin-stained sections was noticed; strong porphyrin fluorescence was associated with intense haematoxylin staining, not only of nuclei, but also of
the cytoplasm. Further research is needed to determine the relevance of this observation.

In conclusion, ALA-induced porphyrin accumulation occurs selectively in the normal and Barrett's epithelial mucosa compared to the muscularis. Therefore, ALA-induced endogenous photosensitization seems highly suitable for PDT treatment of Barrett’s oesophagus. Further clinical studies must be done to determine whether ALA-PDT radically eliminates Barrett’s epithelium or whether some columnar cells remain because not all cells show porphyrin fluorescence. Probably destruction of all columnar cells requires a second course of ALA-PDT. Moreover, the normal squamous epithelium adjacent to the Barrett’s epithelium accumulates porphyrins in a concentration comparable to Barrett’s oesophagus. Therefore, the place of the illumination during treatment is crucial and will determine the semi-selectivity of ALA-PDT for Barrett’s oesophagus. Furthermore, oral ALA administration results in the same kinetic pattern of porphyrins as intravenous administration in the rat oesophagus. Maximal porphyrin concentration in the mucosa was found at 3 h; thus, the most efficient PDT effect will be achieved with illumination at 3 h after ALA administration. However, most selective epithelial damage probably will be attained with treatment at 2 h after administration because then the ratio of porphyrin concentration between mucosa and muscularis is maximal.
REFERENCES

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PART IV

OPTIMIZATION OF PHOTODYNAMIC THERAPY
CHAPTER 9

TIMING OF ILLUMINATION IS ESSENTIAL FOR SAFE AND EFFICIENT PHOTODYNAMIC THERAPY

This chapter has been adapted from Van den Boogert et al. *British Journal of Cancer* 1999;79:825-830.
INTRODUCTION

The incidence of oesophageal adenocarcinoma has been rising more rapidly over the past decade than that of any other solid tumour (Chapter 1). The majority of these carcinomas arises in a Barrett’s oesophagus, which is characterized by metaplastic specialized columnar epithelium. The development of cancer is thought to progress morphologically through low- and high-grade dysplasia, to invasive carcinoma. Oesophagus resection has been advocated for patients with high-grade dysplasia. However, this is controversial because its associated morbidity and mortality have been judged too high in context of a pre-neoplastic disease (Chapter 2). A less mutilating treatment is required that would be acceptable to more patients and applicable in patients at high risk for surgery and general anaesthesia. Photodynamic therapy (PDT) could be such a treatment as it ideally causes only necrosis of the mucosa and submucosa, leaving the muscularis propria intact. Large areas of tissue can be treated and general anaesthesia is not required. PDT is a technique combining photosensitizing agents and illumination in the presence of tissue oxygen to produce photochemical tissue destruction (Chapter 3). In combination with acid suppression, PDT may lead to re-epithelialization with normal-appearing squamous epithelium. In some cases however, islands of residual columnar epithelium and thus of potentially pre-malignant cells have been described underneath regenerated squamous mucosa, so-called pseudoregression. These therapy-resistant cells may be eliminated by improving the PDT treatment.

In this study the optimal time of illumination after administration of 5-aminolevulinic acid (ALA) was determined in a rat model. The effectiveness of destroying epithelial cells as well as the possible impact on oesophageal function in the healing phase, were evaluated.

MATERIALS AND METHODS

Animals

The experimental protocol was approved by "The Committee on Animal Research" of the Erasmus University Rotterdam. Sixty-two male Wistar rats (Harlan CPB, Zeist, The Netherlands), weighing 275-325 grams were used. They had free access to tap water and rat chow (AM II, Hope Farms, Woerden, The Netherlands). To avoid skin lesions, animals were put in a cage in subdued light.

Experimental design

Fifty-six animals were randomly allocated to seven groups of eight animals each. In six treatment groups the animals received 200 mg kg\(^{-1}\) ALA (Sigma...
Chemical Company, St Louis, MO, USA) dissolved in phosphate-buffered saline, by single oral gavage. The animals in the seventh group served as controls and received phosphate-buffered saline only. PDT was carried out under intramuscular ketamine and xylazine anaesthesia. In the six treatment groups, illumination was performed at 1, 2, 3, 4, 6 or 12 h after ALA administration. In control animals, illumination was performed at 3 h. Four rats in each group were sacrificed at 48 h after illumination to study the acute effects, as a pilot study had shown a maximum PDT effect at 48 h. The other four rats in each group were sacrificed on day 28 after illumination to study healing because, by then, a pilot study had shown complete re-epithelialization. Furthermore, that time interval was found to show full expression of stenosis after oesophageal corrosive burns.  

Additionally, six animals were used for separate temperature and pressure measurements and were sacrificed 48 h after the procedure.

Light delivery

A balloon catheter (PTA Balloon Catheter Opta 5, Cordis, Roden, The Netherlands) was used to deliver homogeneous and circumferential light to the oesophagus. The device consisted of a semiflexible catheter and an inflatable cylindric optically clear balloon (length 20 mm; outside diameter 3.5 mm inflated with 0.4 ml air) attached to the distal end of the catheter (Figure 9.1). A 400-μm fiber with a 10-mm length cylindrical diffusing tip of 760 μm diameter (Lightstic 360, Rare Earth Medical Inc, West Yarmouth, MA, USA) was placed exactly in the centre of the balloon and thus centrally in the oesophagus.

Light of 633 nm (600 Series Dye Module pumped by KTP/532 laser, Laserscope, San Jose, CA, USA) was transmitted through the fiber. A spectroscope (WaveMate, Coherent, Auburn, CA, USA) was used to verify the accuracy of the laser wavelength. The output power emitted by the fiber tip, 100 mW, was calibrated and measured before and after treatment by the built-in power meter of the dye laser.

Illumination was performed with a radiant energy of 25 J, giving an incident light dose of approximately 22.7 J per cm² tissue surface (area of a 1-cm long, 3.5-mm diameter cylinder).

Fluorescence and dosimetry

True light fluence (J cm⁻²) and protoporphyrin IX (PpIX) fluorescence spectra were monitored during illumination at 15 s interval with a spherical isotropic probe of 800 μm diameter (Rare Earth Medical inc., West Yarmouth, MA, USA), which was positioned halfway along the cylindrical diffuser and pressed against the oesophageal wall by the balloon catheter (Figure 9.1). The distal fibre end was led to a 50/50% beam splitter (Rare Earth Medical inc., West Yarmouth, MA, USA).
to transport part of the light to a light dose meter and the other part to an optical multichannel analyser system (Multispec 77400 spectrometer + Instaspec-IV 77131 CCD-camera, Oriel Instruments, CT, USA) with a built-in combined 630 nm notch plus 665 nm long wavelength pass filter to block the laser light (Figure 9.1). The response of the isotropic probe was calibrated for true fluence rate measurements using an integrating sphere with a homogeneous diffuse light field.\textsuperscript{8}

![Figure 9.1 Experimental set-up for oesophagus photodynamic therapy facilitating a homogeneous illumination and real-time measurements of the true light fluence rate and protoporphyrin IX fluorescence spectra. Illumination parameters: 1 cm cylindrical diffuser, 25 J radiant energy, power output 100 mW.](image)

Recorded \textit{in vivo} fluorescence spectra were normalized by dividing them by the fluence rate, which was simultaneously recorded by the light dose meter. This yields, in first order approximation, spectra that are corrected for different tissue optical properties, geometrical aberrations and different output power of the cylindrical diffuser. Next, the mean normal tissue autofluorescence spectrum measured in the control group was subtracted from the fluorescence spectra measured in the animals with oral ALA administration. The remaining PpIX fluorescence peak was integrated over a 15-nm range resulting in a number that is a measure for the average local PpIX concentration.

**Measurement of temperature and pressure effect**

To monitor the thermal effects of the procedure, temperature was measured
every 10 s in three rats during illumination at 3 h after administration of ALA. Three thermal probes (SMM probe for model 790 fluoroptic thermometer, Luxtron, Santa Clara, CA, USA) were placed, either in the suprahepatic space or supragastric space next to the oesophagus, or in the oesophagus between the inflated balloon and the oesophageal wall.

The effect of pressure from the inflated balloon was studied by histological examination of the oesophagus of three rats, 48 h after a sham procedure in which the balloon was inflated during 250 s without illumination and without previous ALA administration.

Analysis of tissue damage

In rats sacrificed 48 h after PDT, the oesophagus with a small piece of stomach was removed. The oesophagus was opened longitudinally, the circumference at the laser site was measured and the macroscopic and microscopic appearance were noted. Thereafter the oesophagus was curled up from distal to proximal, fixed in formalin, sectioned and stained with haematoxylin and eosin for conventional light microscopy. Damage was scored quantitatively on a scale from 0 to 3 for each separate oesophageal layer by a pathologist who was not aware of the performed treatment. Oedema of the submucosa and serosa was scored based on the thickness of the submucosa (-, normal; +, 2 times thicker than normal; + +, 3-5 times thicker; + + +, ≥5 times thicker). Inflammation of the submucosa, muscularis propria and serosa was scored on the basis of the amount of inflammatory cells (mostly lymphocytes) using a grid (-, none; +, <1 per grid; + +, 1-2 per grid; + + +, ≥3 per grid). The severity of the necrosis of the muscularis propria was scored on basis of the presence of vital muscle cells (-, 100%; +, >75%; + +, >25%; + + +, ≤25%). In rats sacrificed at day 28 after PDT, a barium oesophagogram under intramuscular ketamine (60 mg kg⁻¹) and xylazine (2.5 mg kg⁻¹) anaesthesia was performed before sacrifice to diagnose stenotic changes and dilatations. Thereafter, rats were sacrificed and the oesophagus was taken out, opened longitudinally and the circumference was measured at 0.5-cm intervals. Apart from the haematoxylin and eosin staining, the sections were stained with anti-S-100 (labels Schwann cells of the peripheral nervous system) to investigate possible changes in nerve tissue. The number of anti-S-100 positive areas along the oesophagus was scored using a grid. Thereafter the average number of anti-S-100 positive areas at the laser site and at the non-laser site was compared with oesophageal diameter on the oesophagogram.

Statistical analysis

The values are expressed as mean ± standard error of the mean (SEM). Comparisons of oesophageal circumference, diameter on oesophagogram, weight
and fluorescence intensity were made using Student's \( t \)-test. Comparison of weight was controlled by the Mann-Whitney \( U \)-test. Spearman's rank order correlation for anti-S-100 staining against oesophageal diameter was used to analyse the relation between nerve damage and oesophageal diameter. A difference was considered to be significant at \( P \) values of \(<0.05\).

**RESULTS**

One animal (illuminated at 3 h after ALA administration) died of anaesthesia during illumination. One animal (illuminated at 6 h after ALA administration) died at day 7 after PDT of oesophageal necrosis and perforation.

**Temperature and pressure effects**

Temperature of the suprahepatic and supragastric space maximally rose 0.6\(^\circ\)C to a maximum of 36.7\(^\circ\)C. The temperature in the oesophagus maximally rose 1.2\(^\circ\)C to a maximum of 37.1\(^\circ\)C. Histological analysis of the oesophagi of the rats after sham balloon insufflation procedure showed no abnormalities.

**Tissue fluorescence**

The *in vivo* fluorescence spectrum recorded in rats after administration of ALA showed the PpIX-specific fluorescence peak at 705 nm, and was virtually the same as in the *in vitro* fluorescence of PpIX dissolved in Triton (2 nmol ml\(^{-1}\)) measured under the same conditions (Figure 9.2).

The PpIX peak at 635 nm interferes with the illumination light (633 nm) and is blocked by the long wavelength pass filter. Besides the typical 705 nm PpIX fluorescence peak, an additional 680 nm peak was observed in five animals (one control, one at 1 h, one at 3 h, and two at 4 h after ALA administration), probably due to mucus, food components or bacteria (Figure 9.2). Largest initial PpIX fluorescence was observed in the group illuminated at 3 h after ALA administration (Figure 9.3).

Rats illuminated at 1, 6 or 12 h after ALA administration showed a significantly smaller initial fluorescence peak (\( P<0.01, P<0.02, \) and \( P<0.02, \) respectively) compared to values at 3 h, whereas animals illuminated at 2 or 4 h after ALA administration did not differ significantly (\( P=0.07 \) and \( P=0.2, \) respectively). Fluorescence intensity declined with illumination time to intensities of around background level after 150 s in the groups illuminated at 1, 6 or 12 h after ALA administration. In the other treatment groups, fluorescence was still declining at the end of illumination.
Figure 9.2 Typical protoporphyrin IX fluorescence (solid line) with a peak at 705 nm and anomalous fluorescence with a peak at 680 nm observed in five animals (dashed line). Spectra are corrected for the normal tissue auto-fluorescence.

Figure 9.3 Mean integrated protoporphyrin IX fluorescence at 705 nm during optical irradiation. The error bars are standard errors of the mean and are indicative of the inter-animal variation (n=8 for each group). Time of illumination after 5-aminolevulinic acid administration: 1 h (---), 2 h (---), 3 h (---), 4 h (--), 5 h (- - -) and 12 h (---).

Dosimetry
During optical irradiation, the true fluence rate was on average 3.0±0.1 (mean±SEM, n=56) times higher than the incident non-scattered irradiation, with a minimum of 1.4 and a maximum of 4.1.

Histopathological changes 48 h after PDT
On microscopic examination, no abnormalities were seen in control animals (Table 9.1). Diffuse loss of epithelium in all animals was only seen in the group illuminated at 2 h after ALA administration. In this group, full thickness necrosis with acute inflammatory cell infiltration and diffuse swelling of the submucosa was observed. The epithelial damage was almost complete; however, some patches with one layer of epithelial cells were found, showing mitotic activity (Figure 9.4).

In the groups illuminated at 3, 4 or 6 h after ALA administration, loss of epithelium was only seen in one of four animals. In the other three, the epithelial layer was normal, with different degrees of submucosal oedema and inflammation and necrosis of the muscularis (Figure 9.4). Illumination at 1 or 12 h after ALA administration did not cause any epithelial lesions.
Table 9.1 Histopathological changes of the oesophageal wall at 48 h after 5-aminolevulinic acid (ALA)-mediated photodynamic therapy in different treatment groups (n=4 for all groups).

<table>
<thead>
<tr>
<th>Illumination time after ALA (h)</th>
<th>loss of epithelium (n)</th>
<th>submucosa</th>
<th>muscularis propria</th>
<th>serosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>++</td>
<td>+++</td>
<td>+ +</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>+</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

Treatment parameters: 200 mg kg⁻¹ ALA, 1 cm cylindrical diffuser, 25 J radiant energy, 100 mW power output. Inflam = inflammation. Changes within layers: -, none; +, mild; ++, moderate; ++++, severe (see materials and methods section).

Figure 9.4 Histopathological section of the rat oesophagus at the laser site 48 h after photodynamic therapy with illumination at 2 h (A) and at 3 h (B) after administration of 5-aminolevulinic acid. E, epithelium; LP, lamina propria; M, muscularis propria; MM, muscularis mucosae; SM, submucosa (x 40, haematoxylin and eosin stain).

Histological and radiological changes 28 days after PDT

Twenty-eight days after illumination, four of 11 rats treated at 4, 6 or 12 h after ALA administration had lost more than 30% of their original weight (Table 9.2). However, no statistically significant difference in the groups illuminated at 4 or 12 h was reached because of large variations in gaining weight. In both groups, one animal lost and one animal gained considerable weight (4 h, loss 154 g and gain 59 g; 12 h, loss 102 g and gain 77 g). Weight in these four animals correlated negatively with the oesophageal circumference (4 h, -154 g/12 mm oesophageal circumference and +59 g/9 mm; 12 h, -102 g/11 mm and +77 g/7 mm).

None of the animals showed any strictures on the oesophagogram. The oesophagus was significantly dilated at the laser site in all animals (P < 0.02) except
Timing of Illumination

in the group treated at 1 h after ALA administration (Figure 9.5). Almost a
doubling of the circumference at the laser site was found in the groups treated at
4, 6 or 12 h ($P < 0.02$) (Table 9.2).

Table 9.2 Circumference and diameter of the oesophagus at the laser site and weight gain of the
rats at 28 days after photodynamic therapy ($n=3$ for groups illuminated at 3 and 6 h, $n=4$ for the
other groups).

<table>
<thead>
<tr>
<th>Illumination time after ALA (h)</th>
<th>Circumference in mm ± SEM</th>
<th>Diameter on radiograph in mm ± SEM</th>
<th>Weight gain in g ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>4.3 ± 0.3</td>
<td>3.3 ± 0.2</td>
<td>80 ± 5</td>
</tr>
<tr>
<td>1</td>
<td>4.5 ± 0.3</td>
<td>3.5 ± 0.2</td>
<td>74 ± 5</td>
</tr>
<tr>
<td>2</td>
<td>5 ± 0.3</td>
<td>4.3 ± 0.2*</td>
<td>50 ± 4*</td>
</tr>
<tr>
<td>3</td>
<td>5.7 ± 0.3*</td>
<td>5.1 ± 0.2*</td>
<td>65 ± 2*</td>
</tr>
<tr>
<td>4</td>
<td>10.3 ± 0.8*</td>
<td>6.4 ± 0.3*</td>
<td>-2 ± 51</td>
</tr>
<tr>
<td>6</td>
<td>10 ± 1.7*</td>
<td>6.0 ± 0.8*</td>
<td>-32 ± 38*</td>
</tr>
<tr>
<td>12</td>
<td>10 ± 1.1*</td>
<td>6.3 ± 0.4*</td>
<td>5 ± 39</td>
</tr>
</tbody>
</table>

* $P$ value $< 0.05$ compared to the control group, SEM = standard error of the mean.

Treatment parameters: 200 mg kg$^{-1}$ 5-aminolevulinic acid (ALA), 1 cm cylindrical diffuser, 25
J radiant energy, 100 mW power output.

Figure 9.5 Oesophagogram at 28 days after 5-aminolevulinic acid (ALA) mediated
photodynamic therapy: (A) illumination at 1 h after ALA, (B) illumination at 12 h after ALA. In
rat A, no dilatations nor strictures were seen. In rat B, the oesophagus was dilated at the laser site
with a doubling of the diameter. Arrows indicate the site of laser treatment.

Microscopic examination showed complete re-epithelialization and a normal
appearing submucosa, muscularis and serosa in all groups. Oesophageal diameter

147
on the oesophagogram was significantly negatively correlated with the intensity of Schwann cell staining (anti-S-100) at the laser site \( (P=0.02, r=-0.56) \) (Figures 9.6 and 9.7). No correlation was found between the intensity of Schwann cell staining and diameter at the non-laser site \( (P=0.39, r=-0.17) \).

**Figure 9.6** Nerve tissue staining at the non-laser site (A) and at the laser site (B), 28 days after photodynamic therapy with illumination at 12 h after administration of 5-aminolevulnic acid. Arrows indicate the myenteric plexus, E, epithelium; M, muscularis propria; SM, submucosa \( (x \times 40, \text{anti-S-100 stain}) \).

**Figure 9.7** Anti-S-100 staining at the laser site (■) and non-laser site (○) versus oesophageal diameter at the laser site on oesophagogram, together with the corresponding trendlines at the laser site (solid line) \( (P=0.02, r=-0.56) \), and at the non-laser site (dashed line) \( (P=0.39, r=-0.17) \).

**DISCUSSION**

This study considers the efficacy and safety of ALA-PDT of the normal rat oesophagus. Maximal efficacy was found when rats were treated 2 h after oral administration of 200 mg kg\(^{-1}\) ALA. Illumination at 4, 6 or 12 h after ALA administration caused oesophageal dilatation, functional impairment and weight loss.
Timing of Illumination

In a previous study, we found that, after oral administration of 200 mg kg\(^{-1}\) ALA, ALA-induced fluorescence of PpIX in the normal rat oesophagus was located almost exclusively in the basal cell layer of the oesophageal epithelium (Chapter 8).\(^{10}\) Fluorescence of the submucosa and muscularis propria was near background level. We therefore expected to find selective damage to the epithelial layer only. Diffuse destruction of the epithelium in all animals was found only in the group treated 2 h after ALA administration. Even in this group, small areas of epithelial cells showing mitotic activity remained after treatment. It is not clear whether the epithelium in these areas indicates re-epithelialization or incomplete epithelial damage, which may lead to pseudoregression as observed in clinical situations.\(^{4,5}\)

The present illumination schemes were chosen, based on results from pilot studies, with the aim of achieving a range of biological responses (mild to severe damage). Further alterations in the illumination schedule may possibly eliminate these remaining areas of viable epithelium. For example, a longer illumination time in the groups treated at 2 to 4 h could still increase the PDT effect because significant PpIX fluorescence was present at the end of illumination (Figure 9.3). Furthermore, adjustment of the power output, or fractionated illumination, may improve the efficacy of PDT.\(^{11,12}\)

In the groups illuminated at 1, 3, 4, 6 or 12 h after ALA, the oesophageal epithelium was mostly completely intact. However, in different degrees, damage to the submucosa, muscularis and serosa was always present (Table 9.1). One explanation for this damage pattern is that the photodynamic threshold (tissue photosensitizer concentration \(\times\) true light dose) to induce necrosis is lower for muscle than for epithelium.\(^{13}\) One reason for that could be a difference in sensitivity to oxygen-radicals (the damaging agents formed during PDT) between epithelium and muscularis. Several studies have suggested that muscle tissue is particularly prone to oxygen radical-mediated cell membrane damage.\(^{14}\) Non-protein sulphydryls in the epithelium are capable of binding free radicals, and rapid cell turn over and the process of restitution contribute to an intact epithelial lining.\(^{15}\) Another explanation for the observed muscle damage could be a high production rate of haemoglobin and myoglobin in muscle. As part of this fast dynamic process, only few of the intermediates between ALA and haem will accumulate. This may lead to a low level of the intermediate PpIX and hence of a low fluorescence intensity on point fluorescence measurement. However, this may be translated into a high cumulative level of PpIX during PDT, resulting in a high production rate of singlet oxygen.

Although we found extensive damage to the submucosa and muscle, at long-term we did not find any strictures. However, severe dilatations occurred in all rats treated 4, 6 or 12 h after ALA administration. This phenomenon could be explained by muscle fibre necrosis, a peripheral neuropathy or a combination of both. Muscle
fiber necrosis seems unlikely as at 48 h after PDT muscle damage was most pronounced in the groups illuminated at 2 and 3 h after ALA administration. In this study, a significant relation between the absence of anti-S-100 staining and oesophageal dilatation at the treatment site was found, indicating nerve tissue damage in the myenteric plexus at the laser site. This may be caused by a relative late production of PpIX in nerve tissue, predominantly in Schwann cells rather than in nerve cells. Furthermore, the brain and nervous system are considered to be more susceptible to oxidative damage than other tissues due to their high content of polyunsaturated lipid-rich neural parenchyma, high oxygen consumption, and low content of scavengers.

From an electron microscopic study of the normal mouse skin, it appeared that, besides endothelial cells and fibroblasts, nerve fibers are most sensitive to photodynamic therapy (using haematoporphyrin derivative). This study confirms earlier studies by Murrer et al. and Van Staveren et al., i.e. that the true fluence rate in hollow organs is larger than the calculated incident fluence rate due to the strong light scattering nature of tissue. In a clinical setting, real-time light dosimetry and PpIX fluorescence measurements may be used to refine the PDT treatment by delivering equal light doses to different areas and monitoring PpIX levels to determine the duration of the illumination.

In conclusion, our results show that both the effectiveness and safety of ALA-PDT treatment for oesophageal lesions largely depend on the time between ALA administration and illumination. Further improvement of the illumination parameters leading to complete epithelial destruction is needed before it can be used as therapy for Barrett's oesophagus in clinical practice.
REFERENCES


CHAPTER 10

PHOTODYNAMIC THERAPY FOR OESOPHAGEAL LESIONS: SELECTIVITY DEPENDS ON WAVELENGTH, POWER AND LIGHT DOSE

This chapter has been adapted from Van den Boogert et al. Annals of Thoracic Surgery 1999;68:1764-1770.
INTRODUCTION

Since 1970 the incidence of oesophageal adenocarcinoma has increased, both in Europe and in the United States, at a rate greater than that of any other malignancy.\textsuperscript{1} Barrett's epithelial metaplasia is a premalignant condition that causes a 30- to 50-fold greater risk of oesophageal adenocarcinoma (Chapter 1).\textsuperscript{2} The current treatment option for patients with Barrett's oesophagus with high-grade dysplasia or oesophageal adenocarcinoma is oesophageal resection (Chapter 2). For Barrett's oesophagus with high-grade dysplasia, however, this treatment is controversial because the mortality and morbidity associated with oesophageal resection is judged to be too high for premalignant lesions.\textsuperscript{3,5} 5-Aminolevulinic acid (ALA)-induced protoporphyrin IX (PpIX)-mediated photodynamic therapy (PDT) is an experimental therapy for Barrett's oesophagus. Pharmacokinetic studies showed selective PpIX fluorescence in oesophageal mucosa (squamous or Barrett's) compared with submucosa and muscularis after ALA administration (Chapter 8).\textsuperscript{4,5} Wavelength-dependent activation of accumulated PpIX ideally selectively destroys the Barrett's epithelium. In clinical studies, ALA-PDT has been shown to eliminate Barrett's epithelium followed by re-epithelialization with squamous epithelium.\textsuperscript{6,7} However, incomplete ablation leading to remaining islands of residual Barrett's epithelium underneath regenerated squamous epithelium has been described.\textsuperscript{6,7}

The present study aimed to find illumination parameters for selective and complete epithelial damage by varying the power output and total light dose applied. A pharmacokinetic study (Chapter 8) showed similar ALA-induced photosensitization in squamous and Barrett's epithelium, so the normal rat oesophagus was chosen to compare lesions in a standardized and reproducible model.\textsuperscript{4} Because PDT damage to the underlying muscle layer or nerve tissue has been found to result in oesophageal dilatation (previous chapter), we compared 633 nm light with 532 nm light, which penetrates tissue less deeply.\textsuperscript{8,9} In addition, fluorescence was measured during illumination and light dosimetry was done.

MATERIALS AND METHODS

Animals

The experimental protocol was approved by "The Committee on Animal Research" of the Erasmus University Rotterdam. Seventy male Wistar rats (Harlan CPB, Zeist, The Netherlands), weighing 250-300 grams, were used. They had free access to tap water and rat chow (AM II, Hope Farms, Woerden, The Netherlands). Photodynamic therapy was done using intramuscular ketamine and xylazine anaesthesia.
Experimental design

The animals were randomly allocated to two groups of 35 animals each. In each group illumination was done with either 633 nm or 532 nm light. In both groups, 30 animals received 200 mg kg\(^{-1}\) ALA (Sigma Chemical Company, St. Louis, MO, USA) dissolved in phosphate-buffered saline to a volume of 1 ml in one oral gavage directly into the stomach. In both groups, animals were allocated to six groups of five animals each. Illumination was done at 3 h after ALA administration, with either 8.3 or 25 J per cm diffuser length applied with 33, 100 or 300 mW per cm diffuser length. Control animals (n=5 in both 633 nm and 532 nm light group) received phosphate-buffered saline orally and were illuminated 3 h thereafter with 300 mW and 25 J. Animals were sacrificed at 48 h after PDT.

Photodynamic therapy treatment conditions

To deliver homogeneous and circumferential light to the oesophagus, a custom made optically clear double-lumen balloon catheter (PTA Balloon Catheter Opta 5, Cordis, Roden, The Netherlands) was used (length 20 mm, diameter 3.5 mm when inflated with 0.4 ml air), with the lumen for the laser fibre exactly in the centre. Light was transmitted through a 400 \(\mu\)m fibre with a 10 mm long cylindrical diffusing tip of 760 \(\mu\)m in diameter (Lightstic 360, Rare Earth Medical Inc, West Yarmouth, MA, USA). When the balloon catheter and diffuser were used as described, the illuminated area was 1.1 cm\(^2\). Fluence and fluence rates in the present study are expressed per cm diffuser length (and thus per 1.1 cm\(^2\) surface area). Illumination with 633 nm was done with a dye laser, Model 630 (600 Series Dye Module, Laserscope, San Jose, CA, USA), pumped by a KTP/532 surgical laser (Laserscope, San Jose, CA, USA). The power emitted was calibrated with a built-in power meter and could not be tuned below 100 mW. Adjustment of 33 mW was achieved by placing neutral density filters in the light path between the output port of the laser and the fibre of the linear diffuser.

The application of 532 nm was done with the KTP/532 laser. With a linear diffuser, the built-in power meter of the KTP laser could not be used and so the power emitted was measured with an integrating sphere (Optometer Model 370, Graseby Optronics, Orlando, FL, USA). Each desired output power could be achieved by using neutral density filters. A spectroscope (WaveMate, Coherent, Auburn, CA, USA) was used to verify the accuracy of the wavelength in both groups.

Fluorescence and dosimetry

True light fluence and PpIX spectra were monitored during PDT at 15 s intervals. A spherical isotropic probe of 800 \(\mu\)m in diameter (Rare Earth Medical Inc., West Yarmouth, MA, USA) was positioned halfway between the balloon and
the oesophageal surface. Its distal fibre end led to a 50/50% beam splitter (Rare Earth Medical Inc., West Yarmouth, MA, USA), transporting part of the light to a light dose meter and the other part to an optical multi-channel analyzer system (Multispec 77400 spectrometer + Intaspec-IV 77131 CCD-camera, Oriel Instruments, CT, USA) with built-in a combined 630 nm notch plus 665 nm long wavelength pass filter during 633 nm illumination, and a 570 nm long wavelength pass filter during 532 nm irradiation. The response of the isotropic probe was calibrated for true fluence rate measurements using an integrating sphere with a homogeneous diffuse light field.10

The mean autofluorescence measured in control animals was subtracted from the recorded spectra and the remaining PpIX fluorescence peak (at 705 nm using 633 nm light and at 635 nm using 532 nm light) was integrated over a 30 nm range. Values are given relative to the start value, which was set to 1.

**Histology**

Animals were sacrificed at 48 h after PDT. After inspection of the thoracic and abdominal cavity, the oesophagus with a small piece of stomach was dissected and opened longitudinally. The width at the laser-site was measured and the oesophageal appearance noted. The oesophagus was curved up from distal to proximal, fixed in formalin, sectioned and stained with haematoxylin and eosin for conventional light microscopy. Damage was scored semi-quantitatively on a scale from 0 to 3 for each separate oesophageal layer by two investigators, masked to the treatment (Table 10.1).

**Table 10.1** Histological scoring system for oesophageal damage at the laser site.

<table>
<thead>
<tr>
<th>oesophageal layer</th>
<th>damage score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>epithelium</td>
<td>normal</td>
</tr>
<tr>
<td>submucosa</td>
<td>no</td>
</tr>
<tr>
<td>oedema</td>
<td>0 inflam cell/grid</td>
</tr>
<tr>
<td>inflammation</td>
<td>0 inflam cell/grid</td>
</tr>
<tr>
<td>muscularis propria</td>
<td>100% vital muscle</td>
</tr>
</tbody>
</table>

Inflam cells = inflammatory cells (mostly leukocytes).

Additionally, a selectivity factor (SF) for epithelial damage was introduced. The SF is defined as the epithelial damage score divided by the muscular damage score (muscular damage score = (muscular necrosis + muscular inflammation)/2).
Statistical analysis

Fluorescence intensity, true fluence rates and damage scores are expressed as mean ± standard error of the mean (SEM). Comparisons were made using Student’s t-test. A difference was considered to be significant at \( P \) values less than <0.05.

RESULTS

All animals survived until sacrifice. No abnormalities were seen in the thoracic cavity at autopsy. In the 633 nm light group at all doses with 100 and 300 mW, a white spot (10 to 25 mm²) was apparent on the cranial surface of the liver next to the oesophagus. In animals treated with 33 mW 633 nm light, a smaller (1 to 5 mm²) white spot was apparent on the liver in seven of 10 animals (two in the 8.3 J and five in the 25 J group). In controls and animals treated with 532 nm light, no macroscopic liver abnormalities were observed.

Fluorescence

When PpIX fluorescence was plotted versus illumination time, fluorescence in the 633 nm group declined faster when a higher power output was used (Figure 10.1A). When fluorescence was plotted versus the applied energy, fluorescence was increasingly bleached with decreasing output power (Figure 10.1B). When 33, 100 or 300 mW 633 nm light was used, PpIX fluorescence after 8.3 J was not fully bleached because fluorescence after 25 J was significantly lower than after 8.3 J (\( P=0.01 \), \( P=0.04 \) and \( P=0.002 \), respectively). The inversed fluorescence versus the applied energy shows a linear relationship (Figure 10.1C) as was found previously by Robinson et al.\textsuperscript{11} The slope of the straight lines is a measure for the bleaching rate (with respect to the applied energy). The slopes of the 33, 100 and 300 mW lines differed significantly (\( P<0.02 \)) and were 0.106±0.005, 0.083±0.005, and 0.046±0.005 J\textsuperscript{-1}, respectively.

In the 532 nm groups, PpIX fluorescence versus illumination time declined fastest with 300 mW output (data not shown). The fluorescence after 25 J was significantly lower than after 8.3 J for the 100 and 300 mW groups (\( P=0.03 \) and \( P=0.04 \) respectively). Fluorescence intensity after long illumination (25 J) with 33 mW did not differ from the intensity after short illumination (8.3 J) (\( P=0.11 \)). Again, for a certain energy applied, the fluorescence was bleached most for the lowest output power.
Figure 10.1 Mean integrated protoporphyrin IX (PpIX) fluorescence at 705 nm during 633 nm illumination plotted against illumination time (A) and applied energy (B). The error bars are standard errors of the mean. In Figure C the reciprocal of the normalized PpIX fluorescence is plotted as a function of light dose, the lines are least-square best fits of the data. Energy is applied with 33 (- - - -), 100 (--), or 300 mW (—-).  

**Dosimetry**  
In the 633 nm light group, during optical irradiation the measured true fluence rate (scattered plus non-scattered light) at the surface was $2.4 \pm 0.05$ times higher than the calculated irradiance (non-scattered light), with a minimum of 1.9 and a maximum of 3.0. In the 532 nm light group the true fluence rate was $1.3 \pm 0.05$ times higher (less than using 633 nm, $P<0.001$) than the irradiance, with a minimum of 1.0 and a maximum of 2.0.
Histology after 633 nm light illumination

No histological abnormalities were found in the control group. Most damage to the epithelium was observed in animals treated with 33 mW, although the difference was not significant compared with animals treated with 100 mW, 25J (Table 10.2).

Table 10.2. Histopathological changes of the oesophageal wall at 48 h after 5-aminolevulinic acid-mediated photodynamic therapy in different groups, treated with 633 nm light.1

<table>
<thead>
<tr>
<th>lightdose (J)</th>
<th>power (mW)</th>
<th>epithelium</th>
<th>submucosa</th>
<th>muscularis propria</th>
<th>SFb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>oedema</td>
<td>inflam</td>
<td>inflam</td>
<td>necrosis</td>
</tr>
<tr>
<td>8.3</td>
<td>33</td>
<td>2.0±0.6</td>
<td>2.0±0.5</td>
<td>1.4±0.5</td>
<td>0.8±0.4</td>
</tr>
<tr>
<td>100</td>
<td>0.8±0.6</td>
<td>2.2±0.2</td>
<td>1.4±0.2</td>
<td>2.0±0.3</td>
<td>1.2±0.2</td>
</tr>
<tr>
<td>300</td>
<td>0</td>
<td>0.8±0.4</td>
<td>0.8±0.2</td>
<td>0.6±0.2</td>
<td>0.6±0.2</td>
</tr>
<tr>
<td>25</td>
<td>33</td>
<td>2.8±0.2</td>
<td>2.6±0.2</td>
<td>2.2±0.4</td>
<td>3.0±0</td>
</tr>
<tr>
<td>100</td>
<td>1.6±0.6</td>
<td>2.8±0.2</td>
<td>1.2±0.2</td>
<td>2.6±0.4</td>
<td>2.4±0.6</td>
</tr>
<tr>
<td>300</td>
<td>0.8±0.6</td>
<td>2.0±0.4</td>
<td>1.2±0.2</td>
<td>2.0±0.3</td>
<td>2.2±0.3</td>
</tr>
</tbody>
</table>

a Mean damage score of the layer ± standard error of the mean on a scale from 0 to 3. Control animals showed no damage (n=5 for all groups).

b SF = selectivity factor of epithelial damage = epithelial damage score : muscular damage score, expressed as mean selectivity factor ± standard error of the mean.

c Selectivity factor is significantly higher (P<0.04) compared with all other 633 nm groups.

Using 33 mW and a total light dose of 25 J, complete epithelial ablation was achieved in four out of five animals. In one animal, one epithelial cell layer remained. With 33 mW and a total light dose of 8.3 J complete epithelial ablation was achieved in three out of five animals. Damage to the submucosa was comparable in all groups, only illumination with 300 mW and 8.3 J caused less damage. Damage to the muscularis propria was more severe in the 25 J than in the 8.3 J groups (P<0.01). Given a fixed total light dose, lowering the power from 300 mW to 33 mW resulted in more severe epithelial than muscular damage and thus in a higher selectivity factor (SF) (for 8.3 J: 100 mW versus 33 mW, P=0.02; 300 mW versus 33 mW, P=0.04, for 25 J: P=0.049 and P=0.04, respectively). Most selective epithelial damage was found after applying 8.3 J with 33 mW (SF=3.7±0.7, P<0.04 versus all other 633 nm illumination schemes), and most complete epithelial damage after applying 25 J with 33 mW (SF=1.1±0.1) (Figure 10.2). Other treatment regimes showed less damage to the epithelium than to the muscularis propria (SF<1).
Figure 10.2 Histologic characteristics of a rat oesophagus at the photodynamic therapy site after 633 nm illumination with 33 mW and 8.3 J (A) and 25 J (B). The arrow in panel A indicates the beginning of the illuminated area (from arrow to the right), showing very selective and complete loss of epithelium, whereas the muscle layer was not damaged. Long illumination (B) caused, besides loss of epithelium, severe damage to the muscularis propria. E=epithelium, K=keratin layer, LP=lamina propria, M= muscularis propria, MM= muscularis mucosae, SM= submucosa (x 40, haematoxylin and eosin stain).

**Histology after 532 nm light illumination**

No histological abnormalities were found in the control group. A light dose of 8.3 J did not cause any epithelial or muscular damage and caused only slight oedema and inflammation of the submucosa (Table 10.3). Only in the groups treated with 25 J applied with 33 or 100 mW, was complete epithelial ablation found in one and two animals, respectively. Most selective epithelial damage was achieved with 33 mW (selectivity factor=2.0±0.7).

**Table 10.3** Histopathologic changes of the oesophageal wall at 48 h after 5-aminolevulinic acid-mediated photodynamic therapy in different groups treated with 532 nm light.\(^a\)

<table>
<thead>
<tr>
<th>light dose (J)</th>
<th>power (mW)</th>
<th>epithelium</th>
<th>submucosa</th>
<th>muscularis propria</th>
<th>SF(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.3</td>
<td>33</td>
<td>0</td>
<td>0.2±0.2</td>
<td>0.2±0.2</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>0.4±0.2</td>
<td>0.2±0.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>300</td>
<td>0</td>
<td>0.2±0.2</td>
<td>0.4±0.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>33</td>
<td>0.8±0.6</td>
<td>1.2±0.4</td>
<td>1.2±0.5</td>
<td>0.8±0.2</td>
</tr>
<tr>
<td>100</td>
<td>1.2±0.7</td>
<td>2.2±0.5</td>
<td>1.6±0.4</td>
<td>1.8±0.5</td>
<td>1.2±0.6</td>
</tr>
<tr>
<td>300</td>
<td>1.8±0.6</td>
<td>1.0±0.0</td>
<td>1.4±0.4</td>
<td>0.8±0.2</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) Mean damage score of the layer ± standard error of the mean on a scale from 0 to 3. Control animals showed no damage (n=5 for all groups).

\(^b\) SF = selectivity factor of epithelial damage = epithelial damage score : muscular damage score, expressed as mean selectivity factor ± standard error of the mean. Inflam = inflammation.
Histology after 633 nm versus 532 nm light illumination

Comparing 633 with 532 nm treatment regimens with the same output power and energy, 532 nm light caused less damage to the epithelium and muscularis propria than 633 nm light (Figure 10.3). Analysis of the different 532 nm and 633 nm groups showed that illumination with 33 mW (8.3 J in the 633 nm group and 25 J in the 532 nm group) caused the most selective epithelial damage.

Figure 10.3 Histopathological section of a rat oesophagus at the laser site after 633 nm (A) and 532 nm (B) light irradiation with 100 mW and 8.3 J. 633 nm illumination caused considerably more damage than 532 nm illumination. E=epithelium, K=keratin layer, LP=lamina propria, M=muscularis propria, MM= muscularis mucosae, SM=submucosa (x 40, haematoxylin and eosin stain).

DISCUSSION

Our results indicate that the selectivity of ALA-PDT for inducing oesophageal epithelial damage depends largely on the combination of wavelength, output power and light dose. Most selective epithelial damage was achieved with 33 mW illumination, whereas illumination with 300 mW caused damage to the underlying muscularis propria with less damage to the epithelium. Very selective damage to the epithelium was found after 633 nm light illumination with 33 mW and 8.3 J.

For improving the results of PDT, most attention has been paid to the development of a photosensitizer which accumulates selectively in (pre)malignant lesions. ALA has been a great step forward for PDT of superficial (epithelial) lesions. Until recently, less attention has been given to the influence of illumination parameters. Incomplete destruction of Barrett's epithelium after ALA-PDT in patients and the proved importance of the time interval between ALA administration and illumination (Chapters 8 and 9) suggests that adjusting the
illumination protocol can further improve the results. 6-8

Because green light (532 nm) penetrates tissue less deep than red light (633 nm), we expected to find more superficial and thus more selective damage to the epithelium using 532 nm light. 12 Indeed, we found no macroscopic liver damage in the green light groups. In general however, comparison of the damage between the 633 and 532 nm groups with the same power and energy delivered showed that 532 nm light caused less epithelial damage than 633 nm light. We choose 532 nm light because PpIX extinction coefficients and the ratio of photon energies are approximately equal at 532 nm and 633 nm. Equivalent rates of photon absorption are therefore achieved with approximately equal energy fluence rates at 532 nm and 633 nm. 12 However, the optical absorption and scattering of tissues also vary at different wavelengths. The in vivo dosimetric results showed that for a given output power the in situ measured fluence rate for 532 nm light is lower than that for 633 nm light (fluence rate multiplication factor \( \beta = \frac{\text{measured fluence rate}}{\text{calculated irradiance}} \), \( \beta_{532} = 1.3 \) and \( \beta_{633} = 2.4 \)). Dosimetry in this study suggests a matching "true light dose" for 532 and 633 nm illumination with a 2 times higher output power or a 2 times longer illumination for 532 nm compared with 633 nm light. Based on the results of a pilot study, we had chosen a factor 3 in varying the incident light dose. When the damage in the 633 nm light group was compared with the damage in the 532 nm light group with a comparable true light dose (3 times longer illumination or a 3 times higher power output), there were no differences in epithelial or muscular damage or in SF.

No other studies comparing wavelengths in ALA-PDT have been described. Studies using hematoporphyrin derivatives as a photosensitizer and 514 nm versus 630 nm light drew different conclusions because some used equivalent rates of photon absorption, whereas others did not account for the differences in optical tissue properties at these wavelengths. 9,12-15 The former studies concluded that 514 nm light has equal or more effect and toxicity than 630 nm light. 9,13-15 The latter studies concluded that 514 nm light has less effect than 630 nm light. 12

In the present study, equal doses of incident light led to the finding that 532 nm light is less effective than 633 nm light, because of the lower true in vivo light doses. In clinical ALA-PDT practice, the assumption that the illumination time can be shortened by using 514 nm in stead of 633 nm light to achieve equal damage is based on the higher PpIX excitation coefficient at 514 nm but does not take into account the lower true in vivo light dose at 514 nm. The presented results can be extrapolated to indicate that the incident light doses must be approximately equal for 514 nm and 633 nm ALA-PDT to achieve equal damage at the surface. Our results further show the importance of monitoring both the true light dose and the fluorescence in a clinical setting, because both the fluence rate (due to the different light scattering properties of tissue) and concentration of the photosensitizer vary
Results of PDT could be improved by varying the power output for a given total light dose. In both the 532 and 633 nm groups, most selective epithelial damage was achieved using low output power. This finding is consistent with two other studies of ALA-PDT on normal mouse skin and transplanted rat tumours.\textsuperscript{11,16} Similar results were found in tumours in mice using haematoporphyrin derivative.\textsuperscript{17-19} This is probably due to the fact that for achieving an equal light dose, the duration of the illumination increases with decreasing power. Rapid \textit{PpIX} photobleaching and photochemical oxygen consumption during high irradiances can lead to relative hypoxia and reduced PDT effect. Long illumination implies high oxygen supply (oxygen saturation of blood $\times$ vascular flow $\times$ time). Thus, there seems to be a critical combination of total light dose (low enough to cause minor muscular damage) and illumination time (long enough to supply oxygen needed for epithelial damage) to achieve selective epithelial ablation.

Although ALA-induced photosensitization occurs almost exclusively in the oesophageal mucosa, almost all animals in the present study showed damage to the submucosa or muscularis, which suggests that the photodynamic threshold to induce damage to the submucosa and muscle is lower than that to the epithelium.\textsuperscript{4,5,8} As described in the previous chapter, a difference in vascular supply, sensitivity to singlet oxygen or \textit{PpIX} metabolism might explain this observation.\textsuperscript{8}

Besides the selectivity of the epithelial damage, the completeness of the epithelial ablation is important. In the present study the optimal illumination scheme was between 8.3 and 25 J applied with 33 mW and 633 nm light. One animal in the 25 J group did not have complete epithelial ablation. A second treatment therefore could ensure complete elimination of the Barrett’s epithelium in patients.

In summary, we demonstrated the importance of light dosimetry, fluorescence measurements, and, moreover, the choice of the illumination protocol for ALA-PDT of oesophageal lesions. Using equal doses of incident light, illumination with 532 nm light was less effective than 633 nm light, because of the lower true light dose. The application of low power 633 nm light caused selective epithelial damage when a low light dose was given and caused complete epithelial damage when a high light dose was given. With these optimized illumination parameters, efficient and safe oesophageal ALA-PDT can be performed.
REFERENCES


CHAPTER 11

FRACTIONATED ILLUMINATION: EFFECT ON FERROCHELATASE ACTIVITY

This chapter has been adapted from Van den Boogert et al. Journal of Photochemistry and Photobiology B Biology (submitted).
INTRODUCTION

Photodynamic therapy (PDT) is an experimental treatment modality for (pre)malignant oesophageal lesions. Apart from exogenously administered porphyrins, endogenously formed porphyrins are used as photosensitizers. Although in the clinical set-up, promising results have been described with both ways of photosensitization, each has its specific drawbacks. PDT with exogenous porphyrins (haematoporphyrin derivative and Photofrin®) causes deeper lesions but may result in strictures or even oesophageal perforations. On the other hand, PDT with endogenous photosensitization (5-aminolevulinic acid, ALA) causes more superficial lesions resulting in less complications but incomplete ablation in various cases (Chapter 2).

One possibility to optimize the treatment with endogenous photosensitization might be a fractionated delivery of light. Depending on the fractionation scheme, various theories are cited to explain the effect. The first reason for the enhanced effect of fractionated light doses as compared to continuous illumination is a delayed vascular shutdown or relaxation of the vasoconstriction, observed as part of PDT-damage. This results in improved tissue oxygenation, essential for the photochemical reaction. Secondly, longer fractionation intervals (1-3 h) can produce a renewed PDT effect, probably because new porphyrins are formed from the still apparent ALA. Re-appearance of porphyrin fluorescence has been demonstrated several hours after the first illumination. Chapter 12 focusses on these two theories regarding fractionated illumination.

Results from others and our personal unpublished experience from in vitro work have lead to another, biochemical explanation for the enhanced effect of fractionated light doses. ALA is a natural haem precursor and formation of ALA is rate limiting in the haem-synthetic pathway (Chapter 3, Figure 11.1). Furthermore, the synthesis of ALA is regulated via a negative feedback mechanism governed by the concentration of free haem. Exogenous ALA bypasses both the rate limiting enzyme ALA synthase and the feedback mechanism. This results in the accumulation of the photosensitive intermediate protoporphyrin IX (PpIX) in certain types of cells and tissues. The reason for PpIX accumulation in (premalignant) epithelium might be a difference in the activity of certain enzymes of the haem biosynthetic pathway. Ferrochelatase activity might be lower and porphobilinogen deaminase (PBGD) activity higher in these tissues (Chapter 8). After ALA synthase, PBGD is rate limiting in PpIX formation whereas ferrochelatase converts photochemically active PpIX into photochemically inactive haem by incorporating ferrous iron.

We hypothesize that a short period of illumination, relative early after ALA administration, may selectively damage ferrochelatase because this enzyme is
Fractionated illumination temporarily bound to PpIX. Selective destruction of ferrochelatase will further enhance the accumulation of PpIX as it can no longer be transformed into haem. This possibility has been studied in vitro by Lim and coworkers with variable results.\textsuperscript{10,11} The purpose of the study presented in this chapter was to test this hypothesis in an in vivo situation in the normal rat oesophagus using various ALA doses and illumination schemes.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure11.1.png}
\caption{Simplified mechanism of 5-aminolevulnic acid (ALA)-induced protoporphyrin IX (PpIX) accumulation. PBG = porphobilinogen.}
\end{figure}

**MATERIALS AND METHODS**

**Animals and photosensitization**

The experimental protocol was approved by "The Committee on Animal Research" of the Erasmus University Rotterdam. Sixty male Wistar rats (Harlan CPB, Zeist, The Netherlands), weighing 325-375 grams, were used. They had free access to tap water and rat chow (AM II, Hope Farms, Woerden, The Netherlands). Photosensitization was achieved by gastric gavage of ALA (Sigma Chemical Company, St. Louis, MO, USA) dissolved in phosphate-buffered saline. The solution was freshly prepared for each animal and immediately given.

**Design of the study**

Animals were randomly allocated to 2 groups of 30 animals each. In the first group, ferrochelatase, porphobilinogen deaminase (PBGD), and PpIX concentration were measured. Animals received 200 mg kg\textsuperscript{-1} ALA and were divided into six groups of five animals each. Three groups served as controls and did not
receive illumination. Animals in the three control groups were sacrificed at 3 h (n=5) or 4 h (n=5) after ALA administration, or received a second dose of 200 mg kg\(^{-1}\) ALA, 4 h after the first dose and were sacrificed 3 h thereafter. The distal 4 cm of the oesophagus was taken out for enzyme and PpIX measurements. Specimens were immediately kept on ice and stored at -80\(^\circ\)C. After enzyme determinations, additionally the ratio between PBGD and ferrochelatase activity was determined in every animal. In the experimental three groups the animals received ALA and were sacrificed according to the three former groups but, additionally, the distal oesophagus was illuminated at 1 h after the first dose of ALA with 12.5 J per cm diffuser length.

The second group of 30 animals were used for histological assessment of PDT treatment and was also divided into six groups of five animals each, receiving ALA as outlined above. In three groups, the oesophagus was illuminated continuously with 20 J cm\(^{-1}\) (n=5) or 32.5 J cm\(^{-1}\) (n=5) at 3 h after ALA administration, or at 3 h after a second dose of ALA with 20 J cm\(^{-1}\) (n=5). In the other three experimental groups, the distal oesophagus was illuminated with 12.5 J cm\(^{-1}\) at 1 h after ALA and additionally with 20 J cm\(^{-1}\) at 3 h (n=5) or 4 h (n=5) after ALA administration, or animals received a second dose of ALA at 4 h after the first dose and were illuminated at 1 h (12.5 J cm\(^{-1}\)) after the first and 3 h (20 J cm\(^{-1}\)) after the second dose of ALA. Animals were sacrificed at 48 h after PDT.

**PDT treatment**

PDT was performed with 633 nm light from a dye laser (600 Series Dye Module, Laserscope, San Jose, CA, USA), pumped by a KTP/532 surgical laser (Laserscope, San Jose, CA, USA). Light was transmitted through a 400 \(\mu\)m fiber with a 10 mm length cylindrical diffusing tip of 760 \(\mu\)m diameter (Lightstic 360, Rare Earth Medical Inc, West Yarmouth, MA, USA). To deliver homogeneous and circumferential light to the oesophagus, an optically clear double-lumen balloon catheter (PTA Balloon Catheter Opta 5, Cordis, Roden, The Netherlands) was used (length 20 mm, diameter 3.5 mm when inflated with 0.4 ml air), with the lumen for the laser fiber exactly in the centre (Figure 11.2).\(^{17}\) This resulted in an illuminated area of 1.1 cm\(^2\). Fluence and fluence rates in the present study are given per cm diffuser length. An output power of 100 mW per cm diffuser length was used in all experiments. The power emitted was calibrated with a built-in power meter. The accuracy of the wavelength was verified with a spectroscope (WaveMate, Coherent, Auburn, CA, USA). During PDT at 15 s intervals, PpIX fluorescence spectra were measured and light dosimetry was performed with a spherical isotropic probe of 800 \(\mu\)m diameter (Rare Earth Medical inc., West Yarmouth, MA, USA), positioned halfway along the balloon. Its distal fiber end led to a 50/50% beam splitter (Rare Earth Medical inc., West Yarmouth, MA,
Fractionated illumination

USA). Part of the light was conveyed to a light dose meter and the other part to an optical multi-channel analyzer system (Multispec 77400 spectrometer + Intstaspec-IV 77131 CCD-camera, Oriel Instruments, CT, USA) with built-in a combined 630 nm notch plus 665 nm long wavelength pass filter. The response of the isotropic probe was calibrated for true fluence rate measurements using an integrating sphere with a homogeneous diffuse light field. The mean autofluorescence measured in control animals was subtracted from the recorded spectra and the remaining PpIX fluorescence peak at 705 nm was integrated over a 15 nm range. Values are given relative to the start value, which was set to 1.

Illumination at 1 h after ALA in the groups for enzyme measurements was performed with a 2 cm cylindrical fiber, also using a balloon catheter. After 12.5 J per cm diffuser illumination the fiber was retracted 2 cm and again 12.5 J per cm diffuser length was given. This was necessary because an oesophageal length of 4 cm was needed for the determinations. Neither dosimetry nor fluorescence spectra were recorded during illumination in these groups.

![Figure 11.1](image)

**Figure 11.1** The optically clear double-lumen balloon catheter with the lumen for the laser fibre exactly in the centre that was used to deliver homogeneous and circumferential light to the oesophagus. Halfway along the balloon a spherical isotropic probe (arrow) was positioned to measure fluorescence spectra and perform light dosimetry during photodynamic therapy.

**Determination of ferrochelatase and porphobilinogen deaminase (PBGD)**

Determinations were performed as described in detail in Chapter 8. First, samples were homogenized in sterile water (1:10 w/w). PBGD was measured as previously described by Wilson *et al.* and performed according to the adapted procedure described by Hinnen *et al.* Due to storage of tissue samples at -80°C, uroporphyrin decarboxylase activity is destroyed. Ferrochelatase activity was determined as described previously by Van Hillegersberg *et al.*

**Determination of PpIX concentration**

PpIX was analysed spectrophotometrically according to Chisolm and Brown with some modifications using a red-sensitive photomultiplier (Perkin Elmer,
Nieuwerkerk a/d IJssel, The Netherlands) at an excitation wavelength of 410 nm and an emission wavelength of 650 nm.\textsuperscript{12,20} Porphyrin standards were analysed separately for their actual concentration using ultraviolet-spectroscopy and the molar extinction coefficient ($\epsilon_{407} = 0.275$ l mol$^{-1}$ cm$^{-1}$).

Protein content was measured using the method of Lowry et al.\textsuperscript{21}

**Histology**

The animals were sacrificed at 48 h after PDT. The oesophagus was taken out in toto, opened longitudinally and curled up from distal to proximal. Specimens were fixed in formalin, sectioned and stained with haematoxylin and eosin for conventional light microscopy. Damage was scored semi-quantitatively for epithelium (0 = normal, 1 = more than 1 cell layer left, 2 = 1 cell layer left, 3 = complete ablation) and muscularis propria (0 = 100% vital muscle, 1 = more than 75% vital muscle, 2 = 25-75% vital muscle, 3 = less than 25% vital muscle) by two investigators, masked to the treatment. Additionally, the selectivity factor (SF) for epithelial damage, defined as the epithelial damage score : muscular damage score, was determined in every animal.

**Statistical analysis**

All values are expressed as mean ± standard error of the mean (SEM). Comparisons were made using (paired) Student's $t$-test. A difference was considered to be significant at $P$ values of $<0.05$.

**RESULTS**

**Enzyme determinations and porphyrin concentration**

Enzyme determinations showed no difference in PBGD activity at 4 or 7 h after ALA between animals who were not illuminated and those who were illuminated at 1 h after ALA administration (Table 11.1). In animals sacrificed at 3 h, illumination at 1 h caused a decrease in PBGD activity ($37.8 ± 1.9$ versus $28.7 ± 0.8$ pmol h$^{-1}$ of uroporphyrin per mg protein, $P=0.02$). Ferrochelatase activity had significantly declined in animals illuminated at 1 h and sacrificed at 3 or 4 h after ALA ($0.56 ± 0.06$ versus $0.86 ± 0.04$ nmol h$^{-1}$ of zinc PpIX per mg protein, $P=0.002$ and $0.26 ± 0.03$ versus $0.80 ± 0.03$ nmol h$^{-1}$ of zinc PpIX per mg protein, $P<0.001$ respectively). However, in animals receiving two times 200 mg kg$^{-1}$ ALA ferrochelatase did not change by illumination at 1 h after the first ALA dose ($0.96 ± 0.06$ versus $1.04 ± 0.02$ nmol h$^{-1}$ of zinc PpIX per mg protein, $P=0.3$). Illumination at 1 h caused a significant increase in the ratio PBGD:ferrochelatase in animals sacrificed at 4 h ($0.12 ± 0.01$ with illumination at 1 h versus
0.042±0.004 without illumination, \( P < 0.001 \). In animals sacrificed at 3 h after the first or second dose of ALA the ratio did not differ between illuminated and non-illuminated animals.

Porphyrin accumulation did not differ between animals illuminated at 1 h after ALA and non-illuminated animals (\( P = 0.4 \), \( P = 0.8 \) and \( P = 0.5 \) at 3, 4 and 7 h, respectively).

Table 11.1 Porphobilinogen deaminase (PBGD), ferrochelatase activity, and porphyrin concentration in the rat oesophagus after oral administration of 200 mg kg\(^{-1}\) 5-aminolevulinic acid (ALA). Data are expressed as mean±standard error of the mean (\( n = 5 \) for each group).

<table>
<thead>
<tr>
<th>Illumination</th>
<th>sacrifice time after ALA (h)</th>
<th>PBGD pmol h(^{-1}) of URO per mg protein</th>
<th>ferrochelatase ratio PBGD: pmol ferrochelatase per mg protein</th>
<th>porphyrin pmol per mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>no</td>
<td>3</td>
<td>37.8±1.9</td>
<td>0.86±0.04</td>
<td>0.044±0.0003</td>
</tr>
<tr>
<td>no</td>
<td>4</td>
<td>33.3±2.4</td>
<td>0.80±0.03</td>
<td>0.042±0.004</td>
</tr>
<tr>
<td>no</td>
<td>7*</td>
<td>27.4±0.9</td>
<td>1.04±0.02</td>
<td>0.026±0.005</td>
</tr>
<tr>
<td>1h (12.5 J cm(^{-1}))</td>
<td>3</td>
<td>28.7±0.8*</td>
<td>0.56±0.06**</td>
<td>0.053±0.005</td>
</tr>
<tr>
<td>1h (12.5 J cm(^{-1}))</td>
<td>4</td>
<td>29.9±0.6**</td>
<td>0.26±0.03**</td>
<td>0.12±0.01**</td>
</tr>
<tr>
<td>1h (12.5 J cm(^{-1}))</td>
<td>7*</td>
<td>30.8±0.9</td>
<td>0.96±0.06</td>
<td>0.33±0.005</td>
</tr>
</tbody>
</table>

\* animals received 200 mg kg\(^{-1}\) ALA at 0 h and at 4 h and were sacrificed at 7 h after the first ALA dose. \*\* \( P = 0.02 \) (with versus without illumination at 1 h), \*\*\* \( P < 0.002 \) (with versus without illumination at 1 h). PpIX = protoporphyrin IX, URO = uroporphyrin.

Dosimetry

During optical irradiation the true fluence rate (scattered plus non-scattered light) at the oesophageal surface in the groups illuminated at 3 or 4 h with or without illumination at 1 h was 3 to 3.5 times higher than the calculated irradiance, with a minimum of 2.75±0.12 and a maximum of 3.59±0.28 (Table 11.2).

Table 11.2 The fluence rate multiplication factor \( \beta \) (true fluence rate:calculated irradiance) during illumination (light dose expressed per cm diffuser) at various time-points after 5-aminolevulinic acid (ALA) administration.

<table>
<thead>
<tr>
<th>Light dose</th>
<th>1h</th>
<th>3h</th>
<th>4h</th>
<th>7h</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 J cm(^{-1}) or 32.5 J cm(^{-1}) (3h)</td>
<td>3.41±0.38</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.5 J cm(^{-1}) (1h)+20 J cm(^{-1}) (3h)</td>
<td>2.75±0.12</td>
<td>3.52±0.25*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.5 J cm(^{-1}) (1h)+20 J cm(^{-1}) (4h)</td>
<td>3.59±0.28</td>
<td>3.29±0.19*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 J cm(^{-1}) (7h)*</td>
<td></td>
<td>1.53±0.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.5 J cm(^{-1}) (1h)+20 J cm(^{-1}) (7h)*</td>
<td>1.52±0.14</td>
<td>1.48±0.05*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\* \( P = 0.055 \) (\( \beta_{1h} \) versus \( \beta_{7h} \)), \* \( P = 0.84 \) (\( \beta_{1h} \) versus \( \beta_{4h} \)), \* \( P = 0.78 \) (\( \beta_{1h} \) versus \( \beta_{7h} \)).

\* animals received 200 mg kg\(^{-1}\) ALA at 0 h and additionally at 4 h.
In animals illuminated at 7 h with or without illumination at 1 h true fluence rate was 1.5 times higher than the incident (calculated) irradiance. In the fractionated groups, illumination at 1 h after ALA caused a fluence rate multiplication factor \( P \) (true fluence rate:calculated irradiance) similar to the \( P \) during the second illumination at 3, 4 or 7 h \((P=0.055, P=0.84 \) and \( P=0.78 \), respectively). Furthermore, \( P \) did not differ between animals illuminated only at 3, or 7 h or animals illuminated additionally at 1 h (\( 3.41 \pm 0.38 \) versus \( 3.52 \pm 0.25 \), \( P=0.83 \) and \( 1.33 \pm 0.09 \) versus \( 1.48 \pm 0.05 \), \( P=0.68 \), respectively).

**In vivo porphyrin fluorescence**

*In vivo* porphyrin fluorescence declined in all groups during optical irradiance (Figure 11.3). Initial porphyrin fluorescence in animals treated only at 3 or 7 h after ALA was higher compared to illumination at 1 h \((P<0.001)\). In the fractionated groups, 705 nm fluorescence intensity was not higher at second illumination at 3 or 4 h than at illumination at 1 h \((P>0.1)\). Fluorescence intensity at second illumination at 7 h was higher than at initial illumination at 1 h \((P=0.02)\).

**Figure 11.3** Mean integrated *in vivo* apparent porphyrin fluorescence of the oesophageal surface at 705 nm during optical irradiation at 3 h with or without illumination at 1 h (A), 4 h with illumination at 1 h (B) and 7 h with or without illumination at 1 h (C). The error bars are standard errors of the mean \((n=5 \) for each group). As the illumination time is very short in comparison with the time of illumination after 5-aminolevulinic acid (ALA) administration, two time scales have been used. Fluorescence during illumination is given on a linear time scale (15 s between tick marks) and only the onset of the illumination corresponds with the time after ALA administration as given on the X-axis. (Lightdose:A: —■—=20 J cm\(^{-1}\) at 3 h, —△—=32.5 J cm\(^{-1}\) at 3 h, —Δ—=12.5+20 J cm\(^{-1}\) at 1 and 3 h, respectively; B: —□—=12.5+20 J cm\(^{-1}\) at 1 and 4 h, respectively; C: —●—=20 J cm\(^{-1}\) at 7 h, —○—=12.5+20 J cm\(^{-1}\) at 1 and 7 h, respectively).
Histology

No difference in epithelial or muscular damage or SF was found between the groups receiving one ALA dose ($P>0.13$) (Table 11.3). Illumination at 3 h caused complete epithelial ablation in 7 out of 10 animals, extra illumination at 1 h resulted in complete ablation in 4 out of 10 animals (2 in both groups) (Figure 11.4).

Table 11.3 Damage of the epithelium and muscularis propria of the normal rat oesophagus produced with 5-aminolevulinic (ALA)-photodynamic therapy ($n=5$ for each group). Three groups were illuminated at 3 or 7 h after ALA (200 mg kg$^{-1}$ orally). Three groups were illuminated at 1 h after ALA to induce selective damage to ferrochelatase and additionally at 3, 4 or 7 h.

<table>
<thead>
<tr>
<th>illumination time after ALA</th>
<th>light dose</th>
<th>epithelium$^a$</th>
<th>muscularis$^a$</th>
<th>SF$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 h</td>
<td>20 J cm$^{-1}$</td>
<td>2.8±0.2</td>
<td>3.0±0</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td>3 h</td>
<td>32.5 J cm$^{-1}$</td>
<td>2.0±0.6</td>
<td>2.6±0.4</td>
<td>0.7±0.2</td>
</tr>
<tr>
<td>7 h</td>
<td>20 J cm$^{-1}$</td>
<td>1.4±0.6</td>
<td>1.6±0.4</td>
<td>1.0±0.4</td>
</tr>
<tr>
<td>1 h + 3 h</td>
<td>12.5 J cm$^{-1}$ + 20 J cm$^{-1}$</td>
<td>2.0±0.5</td>
<td>2.6±0.2</td>
<td>0.7±0.2</td>
</tr>
<tr>
<td>1 h + 4 h</td>
<td>12.5 J cm$^{-1}$ + 20 J cm$^{-1}$</td>
<td>1.6±0.7</td>
<td>2.2±0.5</td>
<td>0.5±0.2</td>
</tr>
<tr>
<td>1 h + 7 h</td>
<td>12.5 J cm$^{-1}$ + 20 J cm$^{-1}$</td>
<td>1.8±0.6</td>
<td>1.6±0.4</td>
<td>1.0±0.3</td>
</tr>
</tbody>
</table>

$^a$ Selectivity factor for epithelial damage (epithelial damage score : muscular damage score). $^b P>0.1$ between the four groups.

Figure 11.4 Histopathological section of the rat oesophagus after 5-aminolevulinic acid (ALA)-mediated photodynamic therapy (PDT) with 633 nm light irradiation. The animal was illuminated at 1 h after ALA (12.5 J cm$^{-1}$) and received PDT at 3 h after ALA (20 J cm$^{-1}$). The arrow indicates the beginning of the illuminated area (from arrow to the left), showing complete epithelial ablation. The submucosa shows oedema and the muscle layer necrosis and cellular infiltration. C= cellular infiltration, E=epithelium, M= muscularis propria, N=necrosis, SM= submucosa.
Chapter 11

DISCUSSION

When used in treatment of (pre)malignant lesions, ALA-PDT must ensure complete ablation of abnormal tissue. At present however, this is not always achieved. One possibility to improve the results is to attain a higher photosensitizer concentration in the tissue to be treated. As ALA by itself is not a photosensitizer but results in endogenous formation of the photosensitizing agent PpIX via the haem biosynthetic pathway (Figure 11.1), several biochemical mechanisms could be used to optimize PpIX accumulation. This study focussed on selective inhibition of the enzyme ferrochelatase.

Under normal (no exogenous ALA) circumstances, the concentration of substrates and intermediates is usually far below the Michaelis constant of the haem synthetic enzymes. For example, carriers of erythropoietic protoporphyria have a 50% or more reduction of the normal ferrochelatase activity but this does not result in clinical symptoms. After administration of ALA, the normal amount of ferrochelatase might be rate limiting in the synthesis of haem. Inhibition of ferrochelatase could then significantly increase the accumulation of the intermediate PpIX. The ratio between PBGD and ferrochelatase is of particular interest as this ratio, at least in part, seems to determine the level of photosensitization of a specific tissue.

The results of enzyme measurements in animals receiving a single ALA dose show that ferrochelatase activity can be significantly reduced by illumination with 12.5 J per cm diffuser length at 1 h after ALA administration. The ratio PBGD:ferrochelatase has significantly increased in animals illuminated at 1 h and sacrificed at 4 h. Under the present experimental conditions this did result in neither more porphyrin accumulation nor more damage to the oesophageal epithelium or muscle, probably because part of the PpIX present at 1 h after ALA was bleached during illumination at 1 h. Furthermore, from previous studies it appeared that the amount of ALA present at 1 h decreases rapidly (Chapters 7 and 8). It might therefore be beneficial to give a second dose of ALA after the first illumination to ensure that the relative ferrochelatase deficit becomes relevant due to a high intracellular ALA concentration. Furthermore, the selectivity of the ALA-PDT treatment can be further enhanced by such a procedure as only the area to be treated is enclosed in the first illumination.

Based on these considerations and the first results, a second study was performed in which animals received a second dose of ALA at 4 h after the first dose, as suggested above. However, a second dose of ALA did result in neither more PpIX accumulation, nor more PDT-induced damage. Probably, maximal PpIX formation has already been achieved with an ALA dose lower than 200 mg kg⁻¹ and this can not be further increased by more ALA. In addition, it is possible
that the viability of cells is decreased when the activity of ferrochelatase is decreased by illumination at 1 h.

Proper comparison of the damage induced by PDT with and without illumination at 1 h was possible because illumination at 1 h after ALA can be performed without inducing damage to the normal rat oesophagus as a former study has shown (Chapter 9). Furthermore, the dosimetric results presented here show no difference between the fluence rate multiplication factor $\beta$ during illumination at 1 h compared to illumination in the same animals at 3, 4 or 7 h after ALA administration. Thus, illumination at 1 h does not cause changes in tissue optical properties and therefore does not cause major changes in tissue histology (e.g. oedema). All illuminations in animals at 3 and 4 h with or without illumination at 1 h were performed on one day. The study performing illuminations at 7 h with or without illumination at 1 h was performed on a different day. The difference between the dosimetric results between animals in the 3 and 4 h groups and animals in the 7 h groups may be due to daily differences in laser and isotropic probe settings. However, recorded in vivo porphyrin fluorescence spectra were normalized by dividing them by the fluence rate. This yields, in first order approximation, spectra that are corrected for different tissue optical properties, geometrical aberrations and different output power of the cylindrical diffuser. From the results within the various groups it can be concluded that illumination at 1 h does not result in higher porphyrin fluorescence during the second illumination than non-fractionated illumination alone.

In conclusion, although illumination at 1 h after ALA administration can cause selective destruction of ferrochelatase, resulting in a higher ratio of PBGD:ferrochelatase, this does not result in accumulation of more porphyrins, even when a second dose of ALA is given. Therefore, it appears that fractionated illumination in a scheme studied in this chapter does not enhance ALA-PDT-induced epithelial ablation of the rat oesophagus.
REFERENCES


19. Wilson JHP, De Rooij FWM, Te Velde K. Acute intermittent porphyria in the Netherlands:


CHAPTER 12

FRACTIONATED ILLUMINATION: EFFECT ON BLOOD FLOW AND PROTOPORPHYRIN IX FORMATION

This chapter has been adapted from Van den Boogert et al., *Lasers in Medical Science* (submitted).
INTRODUCTION

Photodynamic therapy (PDT) is a new treatment modality for (pre)malignant oesophageal lesions like Barrett's oesophagus (Chapter 1). In PDT, laser light is used to activate a photosensitive drug previously administered to accumulate in (pre)malignant tissue (Chapter 3). This activation causes a photochemical reaction, in which singlet oxygen is formed, resulting in tissue damage.\(^1\) In Barrett's oesophagus, endoscopic PDT can eliminate Barrett's epithelium after which normal squamous epithelium regenerates (Chapter 2).\(^2-9\) However, the use of exogenous photosensitizers has been found to cause side effects, such as strictures and perforation.\(^2-6\) With the introduction of the endogenous photosensitizer 5-aminolevulinic acid (ALA), these side effects may be reduced as endogenous photosensitization selectively occurs in the oesophageal mucosa. This results in selective PDT damage to the epithelial layer without the occurrence of strictures.\(^7-9\)

In contrast, complete ablation often could not be achieved and Barrett's cells remained at the surface or beneath normal appearing squamous epithelium (pseudoregression).\(^7-9\) Optimization of ALA-PDT should lead to a more complete ablation of Barrett's epithelium.

One option for improving the results is fractionating the illumination.\(^10-16\) Depending on the length of the time interval between the light fractions, different theories are cited to explain the effect. In the former chapter one theory has been studied. The results did not support the hypothesis that the supposed enhanced PDT-effect by fractionating the illumination can be explained by selective damage of ferrochelatase during the first illumination.

Another reason for the enhanced effect of fractionated light doses as compared to continuous illumination is a delayed vascular shutdown or relaxation of the vasoconstriction, observed as part of PDT-damage.\(^17-21\) This results in improved tissue oxygenation, essential for the photochemical reaction. Probably a single interval between the illuminations is enough to achieve relieve of vasoconstriction.\(^13\) Using ALA, intervals varying between 30 s and 180 s are reported to be beneficial by reducing vasoconstriction.\(^12,13\) However, the effect of ALA on vessels is not well defined. Both vasoconstriction and vasodilatation have been found.\(^21-23\)

Furthermore, relocation of the photosensitizer during the dark interval is a theory behind light fractionation.\(^24\) With use of ALA this seems unlikely as not ALA itself but endogenously produced protoporphyrin IX (PpIX) is the photosensitive compound. However, due to the histological heterogeneity of Barrett's oesophagus, maximal endogenous formation of PpIX following ALA is probably not achieved in all cells of the Barrett's oesophagus at the same time, which results in heterogeneous porphyrin fluorescence.\(^25\) Fractionation therefore could be used to completely ablate the heterogeneous Barrett's epithelium.
Longer fractionation intervals (1 to 3 h) can produce renewed PDT effect, probably because new porphyrins are formed from the still apparent ALA.\textsuperscript{11} Re-appearance of PpIX fluorescence has been demonstrated several hours after the first illumination.\textsuperscript{11,26}

The present study was carried out to investigate both hypothesis on enhanced PDT effect with fractionated illumination using short and long time intervals in the normal rat oesophagus.

**Materials and Methods**

**Animals and photosensitization**

The experimental protocol was approved by "The Committee on Animal Research" of the Erasmus University Rotterdam. Forty male Wistar rats (Harlan CPB, Zeist, The Netherlands), weighing 325-375 grams, were used. They had free access to tap water and rat chow (AM II, Hope Farms, Woerden, The Netherlands). Photosensitization was achieved by oral administration of 200 mg kg\(^{-1}\) ALA (Sigma Chemical Company, St. Louis, MO, USA) dissolved in phosphate-buffered saline. The solution was freshly prepared for each animal and immediately given. All study animals were sacrificed at 48 hours after PDT.

**Design of the study**

Animals were randomly allocated to two groups of 20 animals each.

In the first group the vascular effects of fractionation were studied by laser Doppler flowmetry (see below). Animals were randomly allocated to four groups of five animals. Two groups served as control: five animals received ALA but no illumination and five animals were illuminated in two equal light doses of 10 J per cm diffuser length with a 150 s interval in between without prior ALA administration. In the study groups five animals received endo-oesophageal ALA-PDT in one continuous light dose of 20 J cm\(^{-1}\) at 3 h after ALA administration. The other five animals were illuminated at 3 h after ALA in two equal light doses of 10 J cm\(^{-1}\) with a 150 s interval in between.\textsuperscript{13}

In the second group new porphyrin forming was studied by PpIX fluorescence measurements. Animals were randomly allocated to four groups of five animals. One group served as control: animals were illuminated (40 J cm\(^{-1}\)) without ALA administration (n=5). Two study groups were illuminated at 3 h after ALA, with either 20 J cm\(^{-1}\) (n=5) or 40 J cm\(^{-1}\) (n=5). Animals in the third study group were illuminated at 3 h (20 J cm\(^{-1}\)) plus 6 h (20 J cm\(^{-1}\)) after ALA. In all animals PpIX fluorescence was measured at 3, 4, 5 and 6 h after ALA.
PDT treatment

PDT was performed with 633 nm light from a dye laser, (600 Series Dye Module, Laserscope, San Jose, CA, USA), pumped by a KTP/532 surgical laser (Laserscope, San Jose, CA, USA). Light was transmitted through a 400 µm fiber with a 10 mm length cylindrical diffusing tip of 760 µm diameter (Lightstic 360, Rare Earth Medical Inc, West Yarmouth, MA, USA). To deliver homogeneous and circumferential light to the oesophagus, an optically clear double-lumen balloon catheter (PTA Balloon Catheter Opta 5, Cordis, Roden, The Netherlands) was used (length 20 mm, diameter 3.5 mm when inflated with 0.4 ml air), with the lumen for the laser fibre exactly in the centre. This resulted in an illuminated area of 1.1 cm². An output power of 100 mW per cm diffuser length was used in all experiments, which corresponds with an irradiance of 91 mW cm². The power emitted was calibrated with a built-in power meter. The accuracy of the wavelength was verified with a spectroscope (WaveMate, Coherent, Auburn, CA, USA). During PDT at 15 s intervals, PpIX fluorescence spectra were measured and light dosimetry was performed with a spherical isotropic probe of 800 µm diameter (Rare Earth Medical Inc., West Yarmouth, MA, USA), positioned halfway along the balloon (Figure 12.1).

![Figure 12.1](image)

**Figure 12.1** Experimental set-up for photodynamic therapy of the rat oesophagus. A cylindrical light diffuser in the centre of a balloon catheter was used to deliver homogeneous and circumferential light to the oesophagus. With the isotropic probe, the true fluence rate and protoporphyrin IX fluorescence spectra were simultaneously recorded.

Its distal fiber end led to a 50/50% beam splitter (Rare Earth Medical Inc., West Yarmouth, MA, USA). Part of the light was conveyed to a light dose meter
and the other part to an optical multi-channel analyzer system (Multispec 77400 spectrometer + Intastpec-IV 77131 CCD-camera, Oriel Instruments, CT, USA) with built-in a combined 630 nm notch plus 665 nm long wavelength pass filter. The response of the isotropic probe was calibrated for true fluence rate measurements using an integrating sphere with a homogeneous diffuse light field. The mean autofluorescence measured in control animals was subtracted from the recorded spectra and the remaining PpIX fluorescence peak at 705 nm was integrated over a 15 nm range. Values are given relative to the start value, which was set to 1. In order to minimize additional PDT effect of the fluorescence measurements performed every hour in the "3 h fractionation" group, a helium/neon laser (633 nm) with an output power of 4.5 mW per cm diffuser length was used. Dosimetry was performed and fluorescence spectra were recorded as described above but were integrated during 60 seconds. This resulted in an additional light dose of approximately 0.3 J cm$^2$ per measurement.

**Oesophageal blood flow**

A commercially available laser Doppler flowmeter (LDF) (Periflux PF 2B, Perimed, Järfalla, Sweden) equipped with a special angled endoscopic probe (PF 306S, Perimed, Järfalla, Sweden) was used (helium/neon laser, 633 nm, 2 mW). Hereby a flow-dependent output is extracted from the power and frequency distribution of Doppler-shifted signals generated by laser light scattered from perfused tissue. The LDF output should maintain a linear relationship between output and capillary blood cell flux (mean blood cell speed x red blood cell concentration). The sensitivity of the LDF was checked using a calibration standard consisting of 2-μm latex spheres (PF 1001, Perimed, Järfalla, Sweden). From a pilot study it appeared that the flow through the *ex vivo* kidney was grossly linearly related to the LDF signal. Flowmetry during PDT was not possible as the laser light interfered with the Doppler signal. Therefore, blood flow measurements were performed just before and immediately after illumination. To prevent differences in the groups due to laser Doppler flowmetrically induced damage, blood flow was measured in all animals during 240 s. In the continuously illuminated animals, blood flow was measured during 120 s before and after PDT. In the fractionated group flow was measured during 45 s before the first light dose, during the 150 s dark interval and during 45 after the second light dose. Additionally, in all animals oesophageal blood flow was measured during 30 s at 30, 60 and 90 min after PDT.

**Histology**

Animals were sacrificed at 48 h after PDT and the oesophagus was dissected and opened longitudinally. The oesophagus was curled up from distal to proximal, fixed in formalin, sectioned and stained with haematoxylin and eosin for
conventional light microscopy. Damage was scored semi-quantitatively for epithelium (0 = normal, 1 = more than 1 cell layer left, 2 = 1 cell layer left, 3 = complete ablation) and muscularis propria (0 = 100% vital muscle, 1 = more than 75% vital muscle, 2 = 25-75% vital muscle, 3 = less than 25% vital muscle) by two investigators, masked to the treatment. Additionally, the selectivity factor (SF) for epithelial damage, defined as the epithelial damage score: muscular damage score was determined. 30

Statistical analysis
Fluorescence intensity, fluence rate multiplication factors and damage scores are expressed as mean ± standard error of the mean (SEM). Comparisons were made using (paired) Student's t-test. A difference was considered to be significant at \( P \) values of <0.05.

RESULTS

One animal (illuminated at 3 h after ALA with 20 J cm\(^{-2}\)) in the "3 h fractionation" group died of anaesthesia during illumination.

Dosimetry
During optical irradiation at 3 h after ALA, the true fluence rate (scattered plus non-scattered light) at the oesophageal surface was approximately 3 times higher than the calculated irradiance (fluence rate multiplication factor \( \beta = \frac{\text{true fluence rate}}{\text{calculated irradiance}} \)), with a minimum of 2.60±0.35 and a maximum of 3.61±0.33 (Table 12.1). At 3 h after ALA \( \beta \) did not differ between the various study groups (\( P \geq 0.13 \)) or between the study and control groups (\( P \geq 0.08 \)). \( \beta \) was not changed by a single dark interval of 150 s (3.35±0.29 versus 2.97±0.18, \( P = 0.20 \)).

At 4, 5 and 6 h after ALA in animals illuminated at 3 h after ALA, \( \beta \) was lower than during the initial illumination at 3 h (\( P \leq 0.03 \), \( P \leq 0.01 \) and \( P \leq 0.001 \) respectively). In control animals without ALA, illumination did not cause a change in \( \beta \).

Blood flow
The pattern of the oesophageal blood flow did not differ between animals illuminated continuously or with a 150 s interval between the light fractions. In general, oesophageal blood flow had not declined directly after PDT treatment, whether performed with continuous or fractionated illumination. Moreover, no difference in blood flow pattern was observed between animals illuminated with and
without prior ALA administration. Administration of ALA itself did not lead to a change in oesophageal blood flow.

Table 12.1 The fluence rate multiplication factor $\beta$ (true fluence rate:calculated irradiance) in the various treatment groups.

<table>
<thead>
<tr>
<th>hours after ALA</th>
<th>0h</th>
<th>3h</th>
<th>4h*</th>
<th>5h*</th>
<th>6h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150 s light fractionation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control - after 10 J cm$^{-1}$</td>
<td>3.61±0.33</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>continuous 20 J cm$^{-1}$</td>
<td>2.62±0.19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fractionated - after 10 J cm$^{-1}$</td>
<td>2.60±0.35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- after 20 J cm$^{-1}$</td>
<td>3.35±0.29</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 h light fractionation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control (40 J cm$^{-1}$)</td>
<td>2.89±0.12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>continuous 20 J cm$^{-1}$</td>
<td>2.97±0.06</td>
<td>1.96±0.13*</td>
<td>1.62±0.06a</td>
<td>1.47±0.05**</td>
<td></td>
</tr>
<tr>
<td>continuous 40 J cm$^{-1}$</td>
<td>3.09±0.10</td>
<td>2.08±0.08b</td>
<td>1.76±0.06a</td>
<td>1.39±0.0724*</td>
<td></td>
</tr>
<tr>
<td>fractionated 20 J cm$^{-1}$ + 20 J cm$^{-1}$</td>
<td>2.70±0.11</td>
<td>2.17±0.11b</td>
<td>2.10±0.05c</td>
<td>1.83±0.08a</td>
<td></td>
</tr>
</tbody>
</table>

Light dose expressed as J per cm diffuser length (cm$^{-1}$), ALA =5-aminolevulinic acid, * dosimetry performed during low power fluorescence measurements (additional light dose 0.3 J cm$^{-1}$), $^a$ P≤0.001 (versus 3h), $^b$ P=0.03 (versus 3h), $^c$ P=0.01 (versus 3h).

Tissue fluorescence

After administration of ALA the in vivo fluorescence spectrum showed the PpIX specific fluorescence peak at 705 nm. In some animals, during PDT a new fluorescence peak appeared at approximately 675 nm (Figure 12.2). This is probably due to the formation of photoproducts and especially of the hydroxyaldehyde-chlorin photoproducts of PpIX with an emission band at 674 nm.$^{31-33}$

In animals illuminated at 3 h continuously or with a 150 s interval between the two light doses, porphyrin fluorescence equally declined during continuous and fractionated illumination (Figure 12.3). In animals in the "3 h interval group" PpIX fluorescence intensity after 20 J cm$^{-1}$ illumination at 3 h after ALA has equally declined in all groups ($P<0.001$) (Figure 12.4). In the fractionated and 40 J cm$^{-1}$ group, fluorescence at 705 nm has significantly increased at 4 h after ALA compared to fluorescence at the end of the illumination at 3 h ($P=0.03$ and $P<0.01$, respectively). In these groups PpIX fluorescence at 5 h was higher than at 4 h after ALA ($P<0.01$). In the 20 J cm$^{-1}$ group significance was not reached. At 6 h after ALA, relative 705 nm fluorescence intensity in the fractionated group was lower than in the continuous groups ($P<0.001$) and, in all groups, did not differ

185
from PpIX fluorescence at 5 h after ALA. During the second illumination at 6 h, 705 nm fluorescence intensity declined to background levels.

**Figure 12.2** Typical protoporphyrin IX (PpIX) fluorescence with a peak at 705 nm seen early during 633 nm illumination with 100 mW cm\(^{-1}\) at 3 h after 5-aminolevulinic acid. During illumination, after several minutes, in some animals a new fluorescence peak appeared at 674 nm, probably due to the formation of (hydroxyaldehyde-chlorin) photoproducts of PpIX. (– at start of the illumination, – after 1 min, – = after 3 min, – = after 6 min, – = after 7 min).

**Figure 12.3** Mean integrated protoporphyrin IX (PpIX) fluorescence at 705 nm during 633 nm photodynamic therapy (20 J per cm diffuser length, 100 mW cm\(^{-1}\)) at 3 h after 5-aminolevulinic acid (ALA) administration. The irregularity of the curves of the fractionated group near 100 s illumination time, is due to repositioning of the isotropic probe after the dark interval. The error bars are standard errors of the mean (n=5 for each group). – – – = continuous illumination, – – – = fractionated illumination with 150 s dark interval between 2 x 10 J cm\(^{-1}\).
Figure 12.4 Mean integrated porphyrin fluorescence at 705 nm during and after 633 nm photodynamic therapy. Illumination was performed with 100 mW per cm diffuser length at 3 h after 5-aminolevulinic acid (ALA) (---0--- = 20 J per cm diffuser length, n=4; ...,•••••• = 40 J per cm diffuser length, n=5) or with 20 J per cm diffuser length at 3 and 6 h after ALA, n=5 (---■---). The error bars are standard errors of the mean.

Histology

None of the control animals showed any damage to the epithelium or muscle layer. A single interval of 150 s halfway the illumination did neither increase epithelial or muscular damage nor the selectivity factor compared to continuous illumination (P=0.9, P=0.3 and P=0.3, respectively) (Table 12.2). Three animals in the fractionated and two in the continuous group showed complete epithelial ablation.

Table 12.2 Histological damage of the "150 s interval" groups, 48 h after illumination (n=5 for each group). Damage is expressed as mean±standard error of the mean. None of the control animals showed any damage to the epithelium or muscle layer.

<table>
<thead>
<tr>
<th>illumination</th>
<th>epitheliuma</th>
<th>damage muscularisa</th>
<th>SFa</th>
</tr>
</thead>
<tbody>
<tr>
<td>continuous (20 J cm⁻¹)</td>
<td>1.9±0.6</td>
<td>2.4±0.4</td>
<td>0.7±0.2</td>
</tr>
<tr>
<td>fractionated</td>
<td>1.8±0.7</td>
<td>1.6±0.5</td>
<td>1.0±0.3</td>
</tr>
<tr>
<td>(1 stop of 150 s between 2x10 J cm⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Light dose expressed as J per cm diffuser length, SF=selectivity factor for epithelial damage (epithelial damage score: muscular damage score). a P≥0.3 between the two groups. Score (see materials and methods section): 0= no damage, 1= minor damage, 2= major damage, 3= complete epithelial ablation or <25% vital muscle.
The selectivity factor and the damage to the epithelial and muscular layer did not differ significantly between animals illuminated at 3 h only or at 3 h plus 6 h (P≥0.2, P≥0.2 and P≥0.1, respectively) (Table 12.3).

Table 12.3 Histological damage of the "3 h interval" groups, 48 h after illumination. Damage is expressed as mean ± standard error of the mean. Animals in the control group did not show any damage to the epithelium or muscle layer.

<table>
<thead>
<tr>
<th>illumination time after ALA</th>
<th>light dose</th>
<th>epithelium</th>
<th>muscularis</th>
<th>SF</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 h&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20 J cm&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>2.8±0.3</td>
<td>2.4±0.4</td>
<td>1.3±0.2</td>
</tr>
<tr>
<td>3 h&lt;sup&gt;c&lt;/sup&gt;</td>
<td>40 J cm&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>3.0±0</td>
<td>2.6±0.2</td>
<td>1.2±0.1</td>
</tr>
<tr>
<td>3 h + 6 h&lt;sup&gt;c&lt;/sup&gt;</td>
<td>20 J cm&lt;sup&gt;-1&lt;/sup&gt; + 20 J cm&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>3.0±0</td>
<td>3.0±0</td>
<td>1.0±0</td>
</tr>
</tbody>
</table>

Light dose expressed as J per cm diffuser length (cm<sup>-1</sup>), SF = selectivity factor for epithelial damage (epithelial damage score:muscular damage score). <sup>a</sup> P≥0.1 between the three groups, <sup>b</sup> n=4, <sup>c</sup> n=5. Score (see materials and methods section): 0 = no damage, 1 = minor damage, 2 = major damage, 3 = complete epithelial ablation or <25% vital muscle.

All animals in the groups receiving 40 J cm<sup>-1</sup> in one single dose at 3 h or in two equal fractions at 3 and 6 h, showed complete epithelial ablation (Figure 12.5). One animal that had received 20 J cm<sup>-1</sup> had a lower epithelial damage score of 2.

Figure 12.5 Histopathological section of the rat oesophagus after 5-aminolevulinic acid-mediated photodynamic therapy with 633 nm light irradiation of 100 mW per cm diffuser length and 40 J per cm diffuser length. The arrow indicates the beginning of the illuminated area (from arrow to the right), showing complete epithelial ablation. The submucosa shows oedema and the muscle layer necrosis and cellular infiltration. C=cellular infiltration, E=epithelium, M=muscularis propria, N=necrosis, SM=submucosa.
DISCUSSION

The study described in this chapter was carried out to reveal whether a fractionated light delivery could improve the results of ALA-PDT for superficial oesophageal lesions. In the normal rat oesophagus, ALA-PDT did not cause a reduction in microvascular blood flow as measured by Doppler flowmetry. During an interval of 3 h new PpIX is formed. In the present study, fractionated illumination using short or long time intervals did not result in more damage.

To study the effect of fractionated illumination on oesophageal blood flow, a single dark interval of 150 s was chosen on the basis of results from previous studies in which it appeared that a single short dark interval results in a five times larger area of necrosis after ALA-PDT. In the studies performing fractionated ALA-PDT however, blood flow was not measured. Other studies, in which blood flow during and after ALA-PDT was measured show conflicting results and were not combined with fractionation of the light dose. The time needed for relieve of vasoconstriction ranges between 0.05 s (pulsed laser light) and several minutes. Pulsed laser light, investigated in an in vitro study using meta-tetrahydroxy-phenylchlorin (mTHPC) as photosensitizer, may act via short phases of recovery during the dark intervals, probably resulting in more singlet oxygen. However, using Photofrin®, pulsed and continuous wave laser light induced comparable injury in the dog oesophagus. Furthermore, the rate of photobleaching of PpIX, an oxygen dependent process, did not differ between continuous or fractionated (150 s stop) light delivery, indicating similar oxygen and thus vascular circumstances. The histological sections at 48 h after PDT, show severe inflammation of the treated area with infiltration of inflammatory cells and hyperaemia and show no signs of vasoconstriction.

The observed hyperaemia could explain the difference in tissue optical properties between the first illumination at 3 h after ALA and measurements at 4, 5 and 6 h after ALA. The resulting decrease in the fluence rate multiplication factor is probably caused by an increase of the absorption of light by blood due to hyperaemia.

The observation that a single 150 s dark interval did not result in more complete epithelial ablation is clearly in contradiction with the observed five to eight times larger area of necrosis mentioned by Messman et al. They performed ALA-PDT in the normal rat colon, and so the differences may be explained by the organ investigated.

The second part of this study dealt with fractionating the light to make ongoing PpIX formation possible. In accordance with two other studies, we observed new PpIX fluorescence in the hours following the initial illumination. At 6 h after ALA, PpIX fluorescence in animals illuminated at 3 h plus 6 h after ALA was
lower than in animals illuminated at 3 h after ALA only. This can be explained by the fact that in the former group of animals PpIX fluorescence has more bleached as it was recorded during PDT treatment with the Dye laser (fluorescence measurement after 20 s, 2 J cm\(^{-2}\)) whereas PpIX fluorescence in the latter group of animals was recorded with the helium/neon laser (60 s, 0.3 J cm\(^{-2}\)).

An ALA-kinetic study we performed earlier showed that maximal PpIX concentration in the rat oesophagus is found at 3 h after ALA administration (Chapter 8).\(^{25,27}\) Furthermore, at 3 h after ALA administration, ALA can still be detected in oesophageal mucosa and muscle layer.\(^{25}\) This provides possibilities for ongoing PpIX formation but to ensure this, cells must at least have the haem synthetic pathway enzymes up to PpIX formation working. \textit{In vitro} experiments will be needed to determine cell viability shortly after ALA-PDT. Furthermore, from the results of studies by He and coworkers and the study described in Chapter 11 it appears that during PDT the enzyme ferrochelatase can be selectively destructed as PpIX is temporarily bound to this enzyme.\(^{36,37}\) Ferrochelatase converts PpIX into photochemically inactive haem and a lower activity of this enzyme therefore theoretically leads to accumulation of more PpIX. However, although the study in Chapter 11 shows damage to ferrochelatase by fractionating the illumination, no enhancement of the PDT-induced epithelial ablation was achieved.

Other possible producers of PpIX after initial illumination are attracted inflammatory cells. After the first illumination inflammatory cells migrate quickly to the damaged area. Neutrophils and mast cells are found already at 5 minutes after PDT.\(^{38}\) Being not activated by the laser light, these cells carry their un-bleached ALA-induced PpIX and may additionally use the ALA that leaks from the damaged oesophageal cells for PpIX formation. Whether oesophageal cells formed new PpIX out of still apparent ALA or that PpIX was carried to the treated area by inflammatory cells attracted by cell damage induced during the initial illumination, needs to be established.

In conclusion, ALA-PDT of the normal rat oesophagus did not cause a decrease in oesophageal microvascular blood flow. During an interval of 3 h after initial illumination at 3 h after ALA administration, new PpIX fluorescence was observed, which was bleached during a second treatment at 6 h. Whether oesophageal cells formed new PpIX out of still apparent ALA or that PpIX was carried to the treated area by inflammatory cells attracted by cell damage induced during the initial illumination, needs to be established. Under the present treatment conditions, fractionated illumination using short (150 s) or long (3 h) time intervals did not result in more damage. Thus, this study shows no evidence for improved PDT effect with fractionated light delivery.
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PART V

GENERAL DISCUSSION
CHAPTER 13

GENERAL DISCUSSION: CONSIDERATIONS FOR CLINICAL APPLICATION
Introduction

The studies described in this thesis were prompted by the realization that although Barrett's oesophagus is the known precursor of oesophageal adenocarcinoma, there is no curative therapy for Barrett's oesophagus. At present, patients with Barrett's oesophagus and reflux complaints are treated with antireflux medication or possibly antireflux surgery. However, these therapies have no effect on the Barrett's epithelium itself nor on the development of adenocarcinoma. It would be a great step forward if Barrett's epithelium could be ablated before adenocarcinoma has developed. Furthermore, a minimally invasive treatment as an alternative to oesophagectomy as treatment for Barrett's oesophagus with high-grade dysplasia or superficial cancer is needed because of the high morbidity and mortality associated with this operation. This alternative treatment should also be applicable in patients at high risk for surgery and general anaesthesia.

Photodynamic therapy (PDT) with 5-aminolevulinic acid (ALA)-induced photosensitization is one of the new modalities for the endoscopic treatment of Barrett's oesophagus. Some promising case-reports have been described. However, until now, very few clinical studies on ALA-based PDT for Barrett's oesophagus have been performed (Chapter 2). Complete reversal of the Barrett's epithelium has been achieved in 10% of patients. Therefore, the results have to be improved before ALA-PDT can routinely be used for the treatment of patients with Barrett's oesophagus.

At present, PDT has not yet been approved for the treatment of Barrett's oesophagus. Approval for PDT has been given for palliation in obstructive oesophageal cancer in The Netherlands, USA and Canada. The U.S. Food & Drug Administration approved only Photofrin® as photosensitizer and no second- or third-generation photosensitizers. However, approvals for ALA-PDT for the treatment of patients with Barrett's oesophagus are currently being sought. Then, the treatment is no longer confined to use in clinical trials or inoperable patients. This implies that patients fit for surgery will be treated by a still experimental treatment modality with a change of incomplete ablation of the premalignant epithelium and the occurrence of side-effects. This incomplete ablation is extra dangerous because the phenomenon of pseudoregression (regenerated squamous mucosa covering remnant Barrett's cells) makes the remnant premalignant cells invisible at endoscopic surveillance.

Our research was therefore directed towards improvement of ALA-PDT in inducing selective and complete epithelial ablation of the oesophagus with a low occurrence of side-effects. Furthermore, the experiments described in the present thesis provide insight in the working mechanism of the therapy resulting in a well-founded treatment proposal for further clinical studies on ALA-PDT for Barrett's oesophagus.
ANIMAL MODEL

Present

The first step necessary to investigate ALA-PDT for Barrett’s oesophagus in animals was the development of an animal model for Barrett’s oesophagus. This proved to be difficult. Especially because studies in human suggest that Barrett’s oesophagus is a lesion that takes years to develop. We are the first who managed to develop an animal model for macroscopic Barrett’s oesophagus in the rat (Chapter 4). Besides rats we used opossums because the upper gastrointestinal tract of this species resembles the human situation. Although not actually the scope of this thesis, several important conclusions concerning the pathogenesis and etiology of Barrett’s oesophagus can be drawn from our studies.

First, Barrett’s oesophagus does not originate from submucosal gland ducts as rats lack these structures. Furthermore, longstanding duodeno-oesophageal reflux, and bile reflux in particular, induces Barrett’s oesophagus in rats and it provides an imperative environment for the development of carcinoma when combined with a carcinogen. Opossums only developed microscopic foci of specialized metaplasia in a minority of cases probably because they have less severe bile reflux after oesophagojejunostomy and they have a different composition of bile.

Future

In the rat model for Barrett’s oesophagus several questions concerning the pathogenesis and treatment of Barrett’s oesophagus can be investigated. Surgical modifications (for example, Roux-en-Y procedure) inducing selective reflux of bile acids or pancreatic enzymes or adding possible promoting or protecting factors (for example, pepsine, HCl, H-2 blockers, proton pump inhibitors, taurinedeoxycholaat, glycinedeoxycholaat) should lead to further insight in the pathogenesis of Barrett’s oesophagus. On the other hand, it is possible that not one particular component in the refluxate induces intestinal metaplasia but that reflux in general leads to chronic irritation and a higher cell turn-over. As the various bile acids differ in their irritating action (depending in part on the pH of the refluxate) the composition of the refluxate remains important. In addition, studies influencing the inflammatory reaction (for example, non-steroidal anti-inflammatory drugs, corticosteroids) must be performed.

Pathogenetic studies on Barrett’s oesophagus can be combined with studies on the prevention and treatment of Barrett’s oesophagus and malignant degeneration of the entity. As mentioned before, the most widely used form of management of patients with Barrett’s oesophagus is acid suppression, although this does not address the issue of bile reflux. Moreover, the observation that the recent increase in the incidence of oesophageal adenocarcinoma parallels the introduction of H-2
Blockers and proton pump inhibitors might indicate that bile reflux is also (or even more) damaging in an alkaline environment. Besides that, antireflux medication induces bacterial overgrowth in the stomach (and these bacteria are able to deconjugate bile acids). Antireflux surgery is practised on the basis that it abolishes both acid and alkaline reflux. It has been shown to arrest or even regress the progression of columnarisation but occasionally the development of adenocarcinoma has been reported following successful antireflux surgery. Our findings support the need for a multi-centre, randomised, controlled clinical trial in which acid suppression by proton pump inhibition will be compared to antireflux surgery on the risk of development of adenocarcinoma.

It is theoretically possible that surgery may prevent adenocarcinoma if performed sufficiently early in the sequence of genomic instability, but that the point may reached beyond which no form of treatment can prevent the development of adenocarcinoma. The only way to reduce the predetermined cancer risk would be to remove the abnormal epithelium and encourage a normal lining to regenerate. This could be achieved by endoscopic mucosal ablation, which must be followed or even preceded by antireflux surgery. At present, most patients are maintained on a proton pump inhibitor after endoscopic ablation therapies (including PDT) for Barrett's oesophagus. Some failures of the treatment are attributed to "inadequate antireflux therapy" or "intractable reflux". Considering the pathogenesis of Barrett's oesophagus this seems unlikely. Despite the debate, it is believed that suppressive acid therapy must be maintained after PDT for Barrett's oesophagus. The optimal management of Barrett's oesophagus should be identified by comparing the long-term efficacy and influence on the risk of developing adenocarcinoma of PDT alone, PDT together with antireflux surgery and PDT with pharmacological acid suppression. To investigate this, studies in the animal model and prospective clinical trials are supplementary.

**Pharmacokinetics**

**General**

In chapter 7 it is shown that a relative high dose of 200 mg kg$^{-1}$ ALA is not toxic in rats, neither after oral nor after intravenous administration. A considerable amount of ALA was lost via the urine. Therefore, a dose of 40 or 60 mg kg$^{-1}$ ALA as is used in patients seems to be safe. In contrast to what is found in approximately one third of patients, we did not find a transient increase in serum aminotransaminase level after oral ALA administration. Furthermore, an important outcome of the study was that oral administration induces similar porphyrin accumulation in almost every organ compared to intravenous administration, making it more easy and likely safer to use in patients.
Oesophageal

We were especially interested in the pharmacokinetics of ALA and subsequent porphyrin accumulation in the normal rat oesophagus and in rats with Barrett’s oesophagus. The study in the normal rat oesophagus was more easy to perform. The entire oesophagus provided enough tissue to perform ALA and porphyrin measurements and porphyrin fluorescence was homogeneous resulting in an intensity value with a rather small standard deviation. In animals with (microscopic) Barrett’s oesophagus chemical extraction was not possible as, after 18 weeks of duodeno-oesophageal reflux, animals only had small areas of microscopic Barrett’s epithelium. Furthermore, porphyrin fluorescence in these animals was more heterogeneous due to the histological heterogeneity of Barrett’s oesophagus.

From the fluorescence study it appears that, in contrast to what is often cited and what many involved in ALA-PDT believe, the selectivity of ALA-induced porphyrin accumulation lies in the difference between epithelium, whether squamous or Barrett’s, and the muscularis and not between Barrett’s and squamous epithelium. However, although porphyrin accumulation did not selectively occur in Barrett’s epithelium, ALA-induced endogenous photosensibilization seems highly suitable for PDT treatment of Barrett’s oesophagus, since, for the treatment of oesophageal lesions it is more important to leave the muscle layer intact than the fast-regenerating adjacent normal squamous epithelium.

Whether a difference in ALA-induced porphyrin accumulation does appear in case of Barrett’s oesophagus with high grade dysplasia or adenocarcinoma needs to be established. Some studies show a higher intracellular accumulation of endogenous protoporphyrin IX in areas of high-grade dysplasia and malignant lesions than in the surrounding mucosa in oesophagus, duodenum and colon. This preferential accumulation in dysplastic cells can be used for diagnosis. Initial experience with ALA-induced photodynamic diagnosis seems to indicate reliable tumour photosensitization. However, the diagnostic predictive value depends on the ALA dose (local application of ALA with a spray catheter is also under investigation) and the time interval between administration and endoscopy. Further investigations are needed to evaluate the sensitivity and specificity of photodynamic diagnosis for different tumour entities.

From the present study it is clear that photodynamic diagnosis based on ALA-induced photosensitization will not be able to differentiate between normal squamous epithelium and Barrett’s epithelium with no dysplasia. In addition, one needs to keep in mind that performing ALA-PDT for Barrett’s oesophagus will always also induce damage to the adjacent normal squamous epithelium. The position of the laser fibre finally determines the semi-selectivity of the treatment.
OPTIMIZATION OF ALA-PDT

The major limiting factor in using PDT is the restricted depth of ablation. Furthermore, from both clinical and preclinical studies it appears that part of the treated tissues and tumours respond with unpredictable depths of necrosis. This is complicated by the dependence of PDT on numerous complex variables: photosensitizer localization, tissue concentration, oxygenation and blood flow, target tissue optical properties, activation wavelength, power density, light source and treatment regimen.24

To optimize the results of ALA-PDT for oesophageal lesions we performed four studies (chapter 9 to 12).25-28 The most important outcome of these studies is that the way in which illumination is performed determines to a great extent the results of ALA-PDT. The sole combination of photosensitizer and laser light is no guarantee for success. Although there has been far less attention to the physical performance of illumination than to the selectivity of the photosensitizer, our studies proof that the boundary between success and failure depends on the accuracy of the illumination. There is no reason to assume that herein Barrett's oesophagus differs from the normal oesophagus, nor that the human oesophagus differs from the rat one.

First, the time of illumination after administration of ALA is important and there is a rather narrow time span in which illumination has to be performed, in our study between 2 and 3 h after ALA administration (Chapter 9).25 Otherwise only minor damage to the epithelium can be achieved, although ALA induces "selective" PpIX accumulation in the oesophageal epithelium. Furthermore, not only for the completeness of the epithelial ablation but also for the occurrence of side-effects it is important to choose the right time interval between ALA administration and illumination. Fortunately, we saw the lowest occurrence of side effects at the time points where maximal epithelial damage was induced (2 and 3 h after ALA administration). Oesophageal dilatation and weight loss, probably caused by nerve damage, occurred in animals illuminated relatively late after ALA administration.

Second, the choice of the laser parameters is important. Wavelength, total light dose and power output need to be chosen accurately. In our study, 633 nm light was more effective in inducing epithelial damage than 532 nm light, probably because fluence rate at the oesophageal surface was highest for 633 nm light.26 Furthermore, delivering 8.3 J cm⁻¹ did not cause any damage to the epithelium when it was applied with 300 mW cm⁻¹, some damage when it was applied with 100 mW cm⁻¹ and very selective and complete epithelial ablation using 33 mW cm⁻¹. The importance of this observation is not in the exact figures but in the realization that choosing a relatively low power output dramatically increases the induced epithelial damage. Increasing the total light dose will further increase the damage, both to the
epithelial and muscle layer. We found no difference in (epithelial) damage between fractionated (using short or long time intervals) and continuous illumination.\textsuperscript{27,28}

In patient studies performing PDT it is important that the way in which PDT is performed is mentioned in detail, including the time of illumination after administration of the photosensitizer and all laser parameters. In general, most reports of PDT are anecdotal or preliminary and include patients with different types of tumours and different stages. Further, many treatment parameters are not comparable. These variables make it difficult to interpret the various data.

In addition, our studies demonstrate the importance of light dosimetry and fluorescence measurements during illumination. Only when studies comply with these requirements, the various results can be properly compared, leading to a better insight in ALA-PDT-induced damage and a well-considered performance of the treatment in the future.

Further optimization of ALA-PDT should include use of ALA esters, addition of iron chelators, raising the concentration and activity of neutrophils, enhancement of the apoptotic response and the combination with hyperthermia to PDT.\textsuperscript{29-34}

\textbf{CONSIDERATIONS FOR CLINICAL APPLICATION}

In the studies described in this thesis, ALA-PDT has been found to produce selective and complete epithelial ablation of the normal rat oesophagus. The induced damage was found to heal fast and complete.

Besides the possible difference in reaction on ALA-PDT between normal squamous and Barrett's epithelium in rats, there might also be a difference between the rat and human oesophagus. The studies in the present thesis did not intend to provide a "ready-made" PDT scheme for use in clinical practice and the results cannot be translated literally to the human situation. However, important results on the pharmacokinetics, mechanism, optimization and dangers of ALA-PDT treatment in general and ALA-PDT for oesophageal lesions in particular described in the present thesis provide insight that is of use in both clinical and preclinical studies. For further "fine-tuning" patient studies, parallel to further preclinical studies as outlined above, are indispensable.

We believe that the experimental studies presented in this thesis have justified phase I clinical studies to investigate the feasibility of ALA-PDT for Barrett's oesophagus. Hereby it is most important that the results of the treatment are accurately histologically controlled. Taking random biopsies is not enough as small islands of Barrett's mucosa and especially the presence of Barrett's cells beneath normal appearing squamous mucosa (pseudoregression) can be overlooked. A study in which additional ALA-PDT is performed in patients, who will have to undergo
oesophagectomy for high-grade dysplasia or adenocarcinoma at various time intervals after PDT, is preferable. Like we performed in our animal studies, thorough transmural histological examination of the resection specimen provides essential information on the completeness of PDT-induced epithelial ablation. In our opinion this is the only way in which a "promising new treatment modality" like ALA-PDT, can become an "approved treatment modality" for patients with Barrett's oesophagus. It is such an elegant therapy that we should "treat" it with caution in midst of more aggressive ablation and surgical therapies.

The studies in this thesis have provided a strong fundament for the further development of ALA-PDT as clinical therapy for patients with Barrett's oesophagus. However, randomized trials are certainly necessary before ALA-PDT can be recommended as primary treatment for Barrett's oesophagus and early oesophageal cancer. Until then, given the good long-term results after surgery for Barrett's oesophagus and high-grade dysplasia or superficial adenocarcinoma, along with the fact that it eliminates the need for repeated endoscopies and acid suppression, we believe surgery should remain the preferred treatment for these entities. On the other hand, the fact that PDT can be performed on an out-patient base, the minimally invasive character of the therapy and the low morbidity rate make ALA-PDT well suited for non-operative candidates and for continued clinical trials aimed at improving its efficacy.
REFERENCES


Since 1970 the incidence of oesophageal adenocarcinoma has increased in many Western countries, at a rate that exceeds that of any other malignancy. One assumes that the majority of these adenocarcinoma arise in a Barrett’s oesophagus. A generally accepted definition of Barrett’s oesophagus is the presence of columnar mucosa with intestinal metaplasia, characterized by goblet cells, in the oesophagus. Photodynamic therapy with use of 5-aminolevulinic acid is a new treatment option ideally leading to selective endoscopic ablation of the Barrett’s epithelium. Before photodynamic therapy can routinely be used for the treatment of patients with Barrett’s oesophagus in clinical practice the technique must be optimized to ensure complete ablation of all (pre)malignant cells and minimize the occurrence of unwanted side effects. The preclinical studies described in this thesis intend to provide insight in the therapy and to investigate the modalities for effective results. As such, this thesis forms the fundamentals for future clinical patient studies on photodynamic therapy with use of 5-aminolevulinic acid for oesophageal lesions.

Chapter 1 presents the state of the art of the pathophysiology, diagnosis and management of Barrett’s oesophagus. Barrett’s oesophagus is probably caused by longstanding gastro(duoden)-oesophageal reflux. Its diagnosis requires endoscopic examination with biopsy and histology from the lower oesophagus. Carcinogenesis of Barrett’s oesophagus is thought to follow a sequence from intestinal metaplasia, to low-grade dysplasia, through high-grade dysplasia, to adenocarcinoma. Research on objective markers to determine the risk of malignant degeneration must lead to efficient surveillance of a selected group of patients in the future. At present, cost and time consuming surveillance programs -with still doubtful efficacy- are carried out in a few clinics only. Antireflux medication or eventually antireflux surgery are the current treatment options. Although reflux complaints disappear in almost 100%, no effect on the Barrett’s epithelium itself has been shown and malignant
Summary

degeneration can still proceed.

Experimental endoscopic therapies, therefore, focus on the ablation of the columnar epithelium with restoration of the squamous epithelium. Endoscopic ablation can be performed by either a thermal, chemical or mechanical method. The characteristics and results of these therapies are outlined in the next chapter.

Chapter 2 reviews the current management and results of experimental endoscopic ablation therapies for patients with Barrett’s oesophagus and high-grade dysplasia. The various methods of endoscopic ablation, including multipolar electrocoagulation, argon plasma beam coagulation, contact laser photoablation and photodynamic therapy and their advantages and disadvantages are considered. Furthermore, this chapter presents an overview of the results of 37 published patient studies, case reports, and abstracts on experimental endoscopic therapies for Barrett’s oesophagus. It seems that all endoscopic ablation therapies, in combination with antireflux treatment, can result in reversal of both high-grade dysplasia and Barrett’s oesophagus in a variable percentage of patients. However, the treatment parameters of the various ablation therapies should be optimized and the long-term benefits should be carefully evaluated in prospective randomised trials. The present chapter therefore concludes that, until then, the use of endoscopic ablation therapy for Barrett’s oesophagus with high-grade dysplasia should remain investigational.

In Chapter 3 the fundamentals of photodynamic therapy (PDT) and especially of 5-aminolevulinic acid (ALA)-based PDT are outlined. PDT is concerned with the use of laser light to activate a photosensitive drug previously administered to accumulate selectively in (pre)malignant tissues such as Barrett’s epithelium. Subsequent illumination with laser light of an appropriate wavelength results in a photochemical reaction with the generation of singlet oxygen what causes cell death and thus destruction of the Barrett’s epithelium. Porphyrins are most widely used as photosensitizers. The photosensitive porphyrins can be administered exogenously or produced endogenously. ALA, the photosensitizer that is used in the present thesis, induces endogenous production of porphyrins and especially of protoporphyrin IX (PpIX). The photophysics, photochemistry and photobiology of ALA-based PDT are discussed.

Chapter 4 describes the development of an animal model for Barrett’s oesophagus. Assuming that reflux of duodenal contents is a causal factor in the pathogenesis of Barrett’s oesophagus, duodenal reflux was induced in Wag/Rij rats and opossums (Monodelphis domestica) by means of an oesophagojejunostomy. Opossums were chosen, because, unlike rats, their gastrointestinal tract has non-
keratinized squamous epithelium in the oesophagus, oesophageal gland ducts and a gall bladder, more resembling the human situation. Animals were sacrificed at either 8 or 12 months thereafter. At 12 months after oesophagojejunostomy all rats showed macroscopic and microscopic Barrett’s oesophagus (length: 16.5 ± 1.6 mm), whereas only 25% of the opossums showed microscopic foci of specialized intestinal metaplasia (length: 0.4 ± 0.3 mm). The duration of reflux correlated well with the length of Barrett’s oesophagus in rats. This is the first study to show macroscopic Barrett’s oesophagus in rats due to continuous duodenal reflux, and in which an oesophagojejunostomy was performed in opossums. Opossums only developed microscopic intestinal metaplasia in a minority of cases, probably because opossums have a different bile acid composition, a gall bladder and a functioning sphincter of Oddi, which results in reflux at only certain times of a day, whereas in rats fluid bile continuously flows into the jejunum. Furthermore it can be concluded that Barrett’s oesophagus does not originate from oesophageal gland ducts because rats lack these structures. In the extensive introduction and discussion part of the present chapter, the results of other studies on the development of an animal model for Barrett’s oesophagus and of studies on the etiology and pathology of Barrett’s oesophagus are evaluated and discussed. The presented rat model provides a unique, reliable animal model for Barrett’s oesophagus.

To investigate the possibilities of PDT for Barrett’s oesophagus an animal model for a fast induction of Barrett’s oesophagus would be more convenient. Therefore, in Chapter 5 several pathogenetic factors and possible synergistic factors that could result in a faster development of Barrett’s oesophagus have been studied. An oesophagojejunostomy was performed in six groups of six rats each. The following additional variables were studied: (1) drinking water acidified to pH 1.8, (2) an oesophagus-specific carcinogen: N-nitrosomethyl-benzylamine, (3) modalities 2 and 3 combined, (4) keratinolysis, and (5) denudation of the mucosa. Animals were sacrificed 18 weeks thereafter. In 80% of animals Barrett’s oesophagus with a length of 1 to 9 mm was found. None of the additional factors hastened the induction of Barrett’s oesophagus. Administration of a carcinogen in combination with an oesophagojejunostomy resulted in squamous or adenosquamous carcinoma in 82% of rats, whereas administration of carcinogen alone did not induce carcinoma. It can be concluded that in rats an oesophagojejunostomy provides a reproducible animal model for Barrett’s oesophagus. Furthermore, in combination with a carcinogen, duodeno-oesophageal reflux induces oesophageal carcinoma.

In Chapter 6 the differential diagnosis of gastric ulceration and perforation as was found in some animals in the rat model for Barrett’s oesophagus, as described
in the previous chapters, is drawn. Infection, gastric ischaemia and acidity could
be excluded as pathogenetic factors. Although the exact pathogenesis remains
unclear, the withdrawal of food away from the bypassed stomach might have been
crucial. Furthermore, vagotomy (inherent to the surgical procedure) leads to
anacidity and a motional impaired stomach unable to evacuate its alkaline contents
leading to pooling at the junction between non-glandular and glandular stomach, the
location where the perforations appeared. The most important lesson from the
presented observation is that the gastric consequences of withdrawal of food should
never be forgotten when experiments (e.g., on the ulcer producing or anti-ulcer
effect of agents) are carried out on the rat stomach.

The first step in making ALA-PDT clinically applicable for the treatment of
Barrett’s oesophagus is a thorough investigation of the pharmacokinetics of ALA
after various routes of administration. Chapter 7, therefore, presents the results of
a pharmacokinetic study in which the biodistribution of ALA and subsequent
accumulation of PpIX was examined in rats. Animals were given 200 mg kg\(^{-1}\) ALA
orally or intravenously and they were sacrificed at 1, 2, 3, 4, 6, 12 or 24 h
thereafter. After ALA administration, ALA and porphyrin concentrations were
measured in 18 tissues and fluids using a chemical extraction method. Liver
enzymes and renal function were measured to determine ALA toxicity. The exact
localization of porphyrins within tissues was determined using laser scanning
microscopy. After both oral and intravenous ALA administration, mild,
short-lasting elevation of creatinine was seen. In both treatment groups, ALA
concentration was highest in kidney, bladder and urine. After oral administration,
high ALA concentrations were also found in duodenal aspirate and jejunum.
Porphyrins, especially PpIX, accumulated mainly in duodenal aspirate, jejunum,
liver and kidney (> 10 nmol per g tissue), less in oesophagus, stomach, colon,
spleen, bladder, heart, lung and nerve (2-10 nmol per g tissue), and only slightly
in plasma, muscle, fat, skin and brain (<2 nmol per g tissue). Peak levels
(typically reached at 2 to 3 h after ALA administration) and total production of
porphyrins were equal after oral and intravenous ALA administration. From
analysis of the sequence of events that took place after ALA administration it
appeared that \textit{in situ} synthesis of porphyrins rather than an enterohepatic circulation
or supply by serum contributed to PpIX accumulation. Laser scanning microscopy
showed selective porphyrin fluorescence in epithelial layers. From this chapter it
can be concluded that administration of 200 mg kg\(^{-1}\) ALA results in accumulation
of photosensitive concentrations of PpIX, 1 to 6 h after ALA administration, in all
tissues but muscle, fat, skin and brain. This knowledge of the time-concentration
relationship should be helpful in selecting dosages, routes of administration and
timing of ALA-PDT.
Chapter 8 focusses in more detail on the pharmacokinetics of ALA and ALA-induced PpIX accumulation in the rat normal and Barrett’s oesophagus. Animals with Barrett’s oesophagus, induced by longstanding duodeno-oesophageal reflux as described in Chapters 4 and 5, and unoperated animals received 200 mg kg\(^{-1}\) ALA intravenously or orally. At different time intervals thereafter (t = 1, 2, 3, 4, 6, 12 or 24 h), ALA and porphyrin concentration in the oesophagus was measured using chemical extraction, and porphyrin localization was determined by laser scanning microscopy. Furthermore, to understand the mechanism of porphyrin accumulation, the activity of the enzyme porphobilinogen deaminase (rate-limiting in PpIX formation) and ferrochelatase (converts PpIX into haem by incorporating ferrous iron) and the iron concentration were determined in the oesophageal mucosa and muscle layer. After chemical extraction it appeared that porphyrin accumulation in the normal oesophageal mucosa was 3.5 fold higher than in the muscularis, with a maximum at 3 h after ALA administration. Laser scanning microscopy also showed maximum porphyrin fluorescence at 3 h after ALA administration. Due to the precise localizing properties of laser scanning microscopy, this study clearly showed selective homogeneous porphyrin fluorescence in the normal oesophageal mucosa and especially in the basal cell layer of the squamous epithelium. The underlaying submucosa and muscularis only showed minor fluorescence. In animals with Barrett’s oesophagus porphyrin fluorescence was heterogeneous but also restricted to epithelial cells. There was no difference in fluorescence intensity between Barrett’s and adjacent squamous epithelium. Porphobilinogen deaminase activity was higher and iron concentration lower in mucosa than in the muscularis (\(P<0.001\)). These results show that ALA-induced porphyrin accumulation selectively occurs in oesophageal mucosa, whether normal or Barrett’s, compared to the muscularis, with a maximum at 3 h after ALA administration. This selectivity may be caused by a different activity of haem synthetic enzymes or a relative iron deficiency in the mucosa. Although porphyrin fluorescence did not selectively occur in Barrett’s epithelium, ALA-induced endogenous photosensibilization seems highly suitable for PDT treatment of Barrett’s oesophagus because for the treatment of oesophageal lesions it is more important to leave the muscle layer intact than the fast-regenerating adjacent normal squamous epithelium. The localization of the laser fibre during treatment will eventually determine the semi-selectivity of ALA-PDT for Barrett’s oesophagus.

Chapters 9 to 12 deal with optimization of ALA-PDT for oesophageal lesions. Chapter 9 aimed to optimize the time of illumination after ALA administration. The effectiveness of destroying epithelial cells as well as the possible impact on oesophageal function in the healing phase were evaluated. Animals received 200 mg kg\(^{-1}\) ALA orally and were illuminated at 1, 2, 3, 4, 6 or 12 h thereafter, all with the
same illumination parameters (wavelength 633 nm, radiant energy 25 J per cm diffuser, power output 100 mW per cm diffuser). To deliver homogeneous and circumferential light to the oesophageal wall, a 1 cm cylindrical diffuser was placed in an optically clear balloon catheter. During illumination, PpIX fluorescence spectra and true light fluence at the oesophageal surface were monitored. Animals were sacrificed at 48 h or 28 days after PDT. At day 28, animals were weighed and an oesophagogram was performed to diagnose strictures and dilatations. Damage was scored histologically for each oesophageal layer separately. In accordance with the results of the pharmacokinetic study described in the previous chapter, highest PpIX fluorescence was found at 3 h after ALA administration. Dosimetric results show that, due to the strong light scattering nature of tissue, the \textit{in vivo} fluence rate was 3 times higher than the calculated incident fluence rate. At 48 h after PDT, major epithelial damage was found in all animals illuminated at 2 h, whereas minor epithelial damage was found in animals illuminated at 3, 4 or 6 h after ALA and none at 1 or 12 h. At day 28, in animals illuminated at 4, 6 or 12 h but not at 2 h after ALA, oesophagograms showed severe dilatations. Weight loss of the animals in these groups indicated functional impairment of the oesophagus. Histology showed a loss of Schwann cells, probably because these cells accumulate porphyrins relatively late after ALA administration. The results presented in this chapter demonstrate that the time interval between ALA administration and illumination is critical for achieving epithelial damage without functional impairment. A short interval of 2 to 3 h seems to be most appropriate.

In Chapter 10 the influence of the choice of the laser parameters, including wavelength, power output and total light dose has been evaluated. Animals received 200 mg kg\(^{-1}\) ALA orally and were illuminated with either 633 or 532 nm light through an endo-oesophageal balloon catheter as described in Chapter 9. In all animals illumination was performed at 3 h after ALA administration with either 8.3 or 25 J per cm diffuser, applied with 33, 100, or 300 mW per cm diffuser. During illumination, tissue fluorescence measurements and light dosimetry were performed. Animals were sacrificed at 48 h after PDT and oesophageal damage was scored histologically. During illumination with both wavelengths, PpIX fluorescence (versus illumination time) declined faster when a higher power output was used. Furthermore, due to different optical absorption and scattering of tissue at different wavelengths, fluence rate at the oesophageal surface was highest for 633 nm. This might be the reason why 532 nm light caused less damage to the epithelium and muscle than 633 nm light. In both wavelength groups, illumination with low power output resulted in selective epithelial ablation, whereas illumination with high power output caused muscle damage with minor epithelial damage. Very selective epithelial ablation was achieved by the following laser parameters:
wavelength 633 nm, power output 33 mW per cm diffuser and total light dose 8.3 J per cm diffuser. Probably, rapid PpIX photobleaching and photochemical oxygen consumption during high power irradiances lead to relative hypoxia and therefore to a reduced PDT effect. Increasing the total light dose resulted in more damage, mainly to the muscle layer. The results of the study in this chapter indicate that the selectivity of ALA-PDT for inducing oesophageal epithelial lesions largely depends on the combination of the wavelength, power output and light dose. Most selective epithelial damage was found using low power red light and a low total light dose.

Apart from optimizing the timing of illumination and the laser parameters, the results of ALA-PDT might be further enhanced by fractionating the illumination. In Chapter 11 the hypothesis has been tested that illumination at 1 h after ALA may selectively damage ferrochelatase (the enzyme that converts PpIX into haem), as this enzyme is temporarily bound to PpIX, finally resulting in enhanced PpIX concentrations. Furthermore, the effect of a second ALA dose, administered to ensure that the relative ferrochelatase deficit becomes relevant, was tested. Animals received 200 mg kg\(^{-1}\) ALA orally at \(t=0\) h or at 0 h and at 4 h. The activity of the enzymes ferrochelatase and porphobilinogen deaminase and the PpIX concentration in the oesophageal wall were measured with and without illumination at 1 h after the first ALA administration. In a second study, animals received intra-oesophageal PDT at 4 h after ALA administration with or without illumination at 1 h, or they received a second ALA dose and PDT at 3 h after that second ALA dose, again with or without illumination at 1 h after the first ALA dose. The results of both studies show that illumination at 1 h after ALA caused inhibition of the ferrochelatase activity at 3 and 4 h after ALA. This resulted in a significant increase in the ratio "porphobilinogen deaminase:ferrochelatase" in animals illuminated at 1 h and sacrificed at 4 h after ALA \((P<0.001)\). However, this resulted in neither more PpIX accumulation nor more PDT-induced damage to the oesophageal epithelium or muscle layer. Furthermore, the administration of a second dose of ALA did not result in enhanced porphyrin accumulation or more damage. Therefore, it appears that fractionated illumination in a scheme as studied in this chapter does not enhance ALA-PDT-induced epithelial ablation of the rat oesophagus.

Chapter 12 describes the results of another study in which the effect of fractionating the 633 nm illumination of ALA-PDT of the normal rat oesophagus was studied. In this chapter the hypothesis was tested that fractionation of the illumination could enhance the PDT by: (1) delay of the vascular shutdown or relaxation of the vasoconstriction induced by ALA-PDT or (2) use of newly formed protoporphyrin IX (PpIX), produced between the illuminations. Rats were
randomly allocated to two groups. To study vascular effects, in group 1 illumination with 633 nm was performed at 3 h after oral ALA administration (200 mg kg⁻¹) either continuously with 20 or 40 J per cm diffuser length or fractionated 2x10 J per cm diffuser length with a 150 s interval. Blood flow in the oesophagus was measured with a laser Doppler flowmeter. To study the effect of renewed PpIX formation, animals in group 2 were illuminated continuously at 3 h after ALA with 20 J per cm diffuser length or 40 J per cm diffuser length or fractionated 2x20 J per cm diffuser length with a 3 h interval. In all animals the in vivo fluence rate and PpIX fluorescence were measured during illuminations and animals were sacrificed at 48 h after PDT. ALA-PDT did not cause any significant vasoconstriction. Fluorescence measurements and dosimetric results in group 1 did not differ between animals illuminated continuously or fractionated with a 150 s interval. In group 2, during a 3 h dark interval, PpIX fluorescence increased and bleaching occurred during the second illumination. The tissue optical properties changed during the 3 h dark interval, resulting in a lower in vivo fluence rate (P≤0.001). Fractionation did not result in more oesophageal damage. From this study it can be concluded that a 150 s interval during illumination in ALA-PDT does not increase oesophageal blood flow. During an interval of 3 h new PpIX is formed. In the present study, fractionated illumination using short or long time intervals did not result in more damage. Thus, this study shows no evidence for improved PDT effect with fractionated light delivery.

Chapter 13 includes the general discussion of this thesis and the considerations for clinical application. It provides a review of the results of the studies described in the present thesis, it discusses the consequences of the thesis for the clinical applicability of ALA-PDT for Barrett’s oesophagus and provides recommendations for further research. It is important that whilst the results of both experimental and clinical studies are awaited concerning the completeness of the epithelial ablation, the occurrence of side-effects, the long-term results and in particular the development of adenocarcinoma, the use of ALA-PDT for patients with Barrett’s oesophagus should be limited to randomized clinical studies.

CONCLUSIONS

1. In rats Barrett’s oesophagus can be induced by longstanding duodeno-oesophageal reflux.

2. Barrett’s oesophagus does not originate from oesophageal submucosal glands or gland ducts.
3. 5-Aminolevulinic acid is a safe photosensitizer inducing accumulation of photosensitive concentrations of protoporphyrin IX in the oesophagus.

4. 5-Aminolevulinic acid induces selective protoporphyrin IX accumulation in the oesophageal mucosa compared to the underlaying submucosa and muscle layer. There is no selectivity of protoporphyrin IX accumulation in Barrett’s epithelium in favour of adjacent squamous epithelium.

5. When performing oesophageal ALA-PDT, the choice of the time interval between ALA administration and illumination is crucial to achieve epithelial damage without functional impairment.

6. The combination of wavelength, power output and total light dose largely determines the selectivity of ALA-PDT concerning the induction of epithelial lesions in the oesophagus.

7. Fractionated light delivery in oesophageal ALA-PDT does not result in enhanced PDT-induced epithelial ablation.
SAMENVATTING EN CONCLUSIES

In veel Westerse landen neemt sinds 1970 de incidentie van het adenocarcinoom van de slokdarm sneller toe dan die van enige andere soort kanker. Men neemt aan dat het merendeel van deze adenocarcinomen ontstaat vanuit een Barrett oesofagus. Een algemeen geaccepteerde definitie van een Barrett oesofagus is de aanwezigheid van cilindercel epitheel met intestinale metaplasie en de daarvoor kenmerkende slijnbekercellen in de slokdarm. Fotodynamische therapie met gebruik van 5-aminolevuline zuur is een nieuwe behandelmogelijkheid voor patiënten met een Barrett oesofagus. Hierbij vindt idealiter selectieve endoscopische ablatie van het Barrett epitheel plaats. Alvorens fotodynamische therapie routinematig in de kliniek kan worden toegepast voor de behandeling van patiënten met een Barrett oesofagus dient de techniek te worden geoptimaliseerd teneinde complete ablatie van alle (pre)maligene cellen te bewerkstelligen en het optreden van complicaties te minimaliseren. De preklinische studies in deze dissertatie beogen inzicht te verschaffen in de therapie en vast te stellen op welke wijzen de resultaten ervan verbeterd kunnen worden. Dit proefschrift vormt daarmee de fundamenten voor toekomstige klinische studies betreffende fotodynamische therapie met gebruik van 5-aminolevuline zuur voor slokdarmaandoeningen.

In Hoofdstuk 1 wordt de huidige stand van zaken betreffende de pathofysiologie, diagnose en behandeling van patiënten met een Barrett oesofagus gepresenteerd. Een Barrett oesofagus wordt waarschijnlijk veroorzaakt door langdurige reflux van maag en/of darminhoud in de slokdarm. De diagnose Barrett oesofagus wordt gesteld middels endoscopie, waarbij biopten genomen worden van de distale oesofagus. Waarschijnlijk verloopt de maligne ontanding in een Barrett oesofagus via verschillende stadia. Eerst ontstaat intestinale metaplasie, vervolgens lichte dysplasie, dan ernstige dysplasie en uiteindelijk adenocarcinoom. Er wordt veel onderzoek gedaan naar objectieve markers om het risico op maligne ontanding te kunnen voorspellen. Dit zou op den duur moeten leiden tot efficiënte controle


**Hoofdstuk 3** behandelt het werkingsmechanisme van fotodynamische therapie (PDT) in het algemeen en van PDT met gebruik van 5-aminolevuline zuur (ALA) in het bijzonder. Bij PDT wordt laserlicht gebruikt om een lichtgevoelige stof te activeren. Deze lichtgevoelige stof hoopt zich idealiter selectief op in (pre)maligne weefsels, zoals Barrett oesofagus. Daaropvolgende belichting met laserlicht met een geschikte golflengte veroorzaakt een fotochemische reactie waarbij zuurstofradicalen vrijkomen die schade toebrengen aan het Barrett epitheel. Porfyrines worden het meest gebruikt als lichtgevoelige stof. Deze porfyrines kunnen exogeen worden toegevoegd danwel endogeen worden geproduceerd. ALA, de fotosensitizer
die in dit proefschrift wordt gebruikt, induceert een endogene productie van de lichtgevoelige stof protoporfyrine IX (PpIX). In dit hoofdstuk worden de fysische, chemische en biologische processen die plaatsvinden bij ALA-gemedieerde PDT behandeld.

In Hoofdstuk 4 wordt de ontwikkeling van een diermodel voor Barrett oesofagus beschreven. Ervan uitgaande dat reflux van dunne darm inhoud een causale factor in de pathogenese van Barrett oesofagus is, is reflux van dunne darm inhoud via een oesofagojejunostomie geïnduceerd in zowel Wag/Rij-ratten als opossums (buidelratten, *Monodelphis domestica*). Opossums werden gekozen omdat het maagdarmstelsel van deze dieren, meer dan dat van de rat, lijkt op dat van de mens. Opossums hebben onder andere niet-verhoornend plaveisel epithiel en submucosale kliertjes in de slokdarm. Daarnaast hebben zij een galblaas. De dieren werden op 8 of 12 maanden na de operatie opgeofferd. Na 12 maanden duodeno-oesofageale reflux hadden alle ratten een macroscopische en microscopische Barrett oesofagus (lengte: 16.5 ± 1.6 mm) ontwikkeld, terwijl slechts 25% van de opossums microscopische foci van cilinderepitheel met intestinale metaplasie (lengte: 0.4 ± 0.3 mm) had ontwikkeld. De duur van de reflux correleerde goed met de lengte van het Barrett epitheel in de rat. Dit is de eerste studie waarin ratten een macroscopische Barrett oesofagus ontwikkelden door langdurige duodeno-oesofageale reflux en waarin een oesofagojejunostomie werd uitgevoerd in opossums. De verschillende resultaten in ratten en opossums kunnen waarschijnlijk verklaard worden door het feit dat opossums een galblaas en functionerende sfincter van Oddi hebben, wat resulteert in slechts enkele malen reflux per dag, terwijl in ratten vloeibare gal continu in het jejunum stroomt. Daarnaast hebben opossums een andere galzuursamenstelling dan ratten. Aangezien ratten geen submucosale kliertjes in de slokdarm hebben kan verder worden geconcludeerd dat een Barrett oesofagus niet vanuit deze submucosale kliertjes ontstaat maar waarschijnlijk vanuit epitheliale stemcellen gelokaliseerd in de basale epitheliale cellaag. Tenslotte worden in de uitgebreide introductie en discussie van dit hoofdstuk de resultaten van andere diermodelstudies voor Barrett oesofagus en een aantal studies naar de etiologie en pathogenese van Barrett oesofagus geëvalueerd en bediscussieerd. Het diermodel in de rat zoals dat beschreven wordt in dit hoofdstuk vormt een uniek en betrouwbare model voor Barrett oesofagus.

Om de mogelijkheden van PDT voor Barrett oesofagus te onderzoeken zou een diermodel waarmee sneller een Barrett oesofagus geïnduceerd kan worden praktischer zijn. In Hoofdstuk 5 worden enkele, mogelijk synergistisch, pathogene factoren onderzocht die een snellere inductie van Barrett oesofagus zouden kunnen bewerkstelligen. Hiervoor is bij zes groepen ratten een oesofagojejunostomie
uitgevoerd. De volgende additionele variabelen zijn daarbij onderzocht: (1) aangezuurd drinkwater met een pH van 1.8, (2) de slokdarm-spectifieke carcinogene stof N-nitrosomethyl-benzylamine, (3) een combinatie van beide voorgaanden, (4) keratolyse middels lokale applicatie van salicylzuur en (5) chirurgisch strippen van de slokdarmmucosa. Naar aanleiding van de resultaten uit het vorige hoofdstuk werd dit experiment alleen in ratten uitgevoerd. De dieren werden na 18 weken opgeofferd. In 80% van de dieren werd een Barrett oesofagus met een lengte van 1-9 mm gevonden. Geen van de bijkomende factoren versnelde de inductie van Barrett oesofagus. Toediening van carcinogeen in combinatie met een oesofagojejunostomie resulteerde in 82% van de ratten in een plaveiselcel danwel adenosquameus carcinoom terwijl toediening van alleen de carcinogene stof niet resulteerde in carcinoomvorming. Er kan derhalve worden geconcludeerd dat het solitair uitvoeren van een oesofagojejunostomie in de rat een reproduceerbaar diermodel voor Barrett oesofagus is. Daarnaast induceert duodeno-oesofageale reflux in combinatie met een carcinogene oesofagus carcinoom.

In Hoofdstuk 6 wordt de differentiaal diagnose opgesteld van de maagperforatie, zoals deze in sommige dieren in het ratmodel voor Barrett oesofagus, beschreven in de vorige hoofdstukken, optrad. Infectie, ischemie van de maag en "maagzuur" konden worden geëxcludeerd als pathogene factoren. Hoewel de precieze pathogenese onduidelijk blijft, is waarschijnlijk het feit dat er geen voedsel in de maag komt na de chirurgische omleiding cruciaal geweest. Daarnaast induceert de vagotomie, die inherent is aan de chirurgische procedure, anaciditeit en een vermindering van de motoriek van de maag. Dit leidt tot ophoping van alkalische inhoud in de maag met name op de overgang van nonglandulaire en glandulaire maag, exact de locatie waar de perforaties gevonden werden. De belangrijkste conclusie van deze observatie is dat de consequenties van het onthouden van voedsel aan de maag nooit vergeten moeten worden wanneer experimenten (b.v. naar de ulcerogene of anti-ulcerogene werking van stoffen) worden uitgevoerd in de rattenmaag. Alvorens ALA klinisch gebruikt kan worden voor PDT is een diepgaand onderzoek naar de farmakokinetiek van ALA na verschillende toedieningsvormen noodzakelijk. In Hoofdstuk 7 worden daarom de resultaten gepresenteerd van een farmakokinetische studie waarin de biodistributie van ALA en de daaropvolgende accumulatie van PpIX in ratten is onderzocht. De dieren kregen 200 mg kg⁻¹ ALA oraal of intraveneus toegediend en werden op 1, 2, 3, 4, 6, 12 of 24 uur na toediening opgeofferd. Middels een chemische extractiemethode is de concentratie ALA en porfyrine in 18 verschillende weefsels en vloeistoffen bepaald. Om de toxiciteit van ALA te bepalen zijn leverenzymen bepaald en nierfunctietesten
uitgevoerd. De porfyrine lokalisatie in de weefsels is met laser scanning microscopie bepaald. Na zowel orale als intraveneuze toediening van ALA werd een milde, kortdurende stijging van het kreatinine waargenomen. In beide behandelingsgroepen was de ALA-concentratie het hoogst in nier, blaas en urine. Na orale toediening werden eveneens hoge ALA-concentraties gevonden in dunne darm inhoud en jejunum. Porfyrines, met name PpIX, accumuleerden voornamelijk in dunne darm inhoud, jejunum, lever en nier (> 10 nmol per g weefsel), minder in oesofagus, maag, colon, milt, blaas, hart, long en zenuw (2-10 nmol per g weefsel), en slechts minimaal in plasma, spier, vet, huid en hersenen (< 2 nmol per g weefsel). De hoogste concentratie (meestal bereikt op 2 tot 3 uur na ALA-toediening) en de totale produktie van porfyrines waren na orale en intraveneuze toediening identiek. Na analyse van de volgorde van gebeurtenissen die plaatsvonden na ALA-toediening bleek dat in situ synthese van porfyrines, en niet zozeer een enterohepatische kringloop of aanvoer via het serum zorgde voor PpIX accumulatie. Laser scanning microscopie, tenslotte, toonde selectieve porfyrine fluorescentie in de epitheliale lagen van de onderzochte weefsels aan. Uit dit hoofdstuk blijkt dat toediening van 200 mg kg⁻¹ ALA resulteert in accumulatie van fotosensitieve concentraties van PpIX, op 1 tot 6 uur na ALA toediening, in alle weefsels behalve spier, vet, huid en hersenen. Kennis van de ALA en porfyrine concentratie op verschillende tijdstippen na ALA toediening, zoals gepresenteerd in dit hoofdstuk, zal moeten lijden tot inzicht in de optimale dosis, de manier van toediening en het tijdstip van belichting na toediening van ALA.

In Hoofdstuk 8 wordt meer in detail ingegaan op de farmakokinetiek van ALA en van ALA-geïnduceerde PpIX ophoping in de normale en Barrett rattenslokdarm. Aan dieren met een Barrett oesofagus, middels langdurige reflux geïnduceerd zoals beschreven in de hoofdstukken 4 en 5, en aan niet geopereerde dieren werd 200 mg kg⁻¹ ALA oraal danwel intraveneus toegediend. Op verschillende tijdstippen na toediening (t= 1, 2, 3, 4, 6, 12 en 24 uur), werd de ALA- en porfyrine concentratie gemeten in de oesofagus middels een chemische extractie methode. De porfyrine lokalisatie werd met laser scanning microscopie bepaald. Om het mechanisme van porfyrine accumulatie te begrijpen, werd in de mucosa en in de spierlaag van de slokdarm de activiteit van de enzymen porphobilinogene deaminase (dit enzym is snelheidsbeperkend in PpIX aanmaak) en ferrochelatase (dit enzym zet PpIX om in haem door ijzer te incorporeren), alsmede de ijzerconcentratie gemeten. Na chemische extractie bleek dat porfyrine-accumulatie in de normale oesofagus mucosa 3.5 keer zo hoog was als in de spierlaag, met een maximum op 3 uur na ALA toediening. Laser scanning microscopie toonde eveneens maximale porfyrine fluorescentie op 3 uur na ALA toediening aan. Daarnaast kon met deze techniek worden aangetoond dat homogene fluorescentie zich selectief in de mucosa van de
Samenvatting

slokdarm en met name in de basale cellaag van het plaveiselcel epitheel bevond. De onderliggende submucosa en spierlaag lieten slechts zwakke fluoresentie zien. In dieren met een Barrett oesofagus was de porfyrine fluoresentie heterogener, maar beperkte zich eveneens tot de epitheliale cellen. De intensiteit van de fluoresentie verschilde niet tussen Barrett epitheel en aangrenzend plaveisel epitheel. In de slokdarm mucosa was de porphobilinogen deaminase activiteit hoger en de ijzerconcentratie lager dan in de spierlaag ($P<0.001$). De resultaten van de studie in dit hoofdstuk laten zien dat ALA-geïnduceerde porfyrine accumulatie selectief optreedt in de normale en Barrett slokdarm mucosa, met een maximum op 3 uur na ALA toediening; de accumulatie in de spierlaag is in vergelijking hiermee gering. Deze selectiviteit kan berusten op een verschil in activiteit van de enzymen van de haemsynthese of een relatief ijzer tekort in de mucosa. Aangezien het intact houden van de spierlaag voor de behandeling van slokdarmaandoeningen belangrijker is dan het intact houden van het snel regenererende, aangrenzende, normale plaveiselcel epitheel, lijkt ALA-geïnduceerde lichtgevoeligheid zeer geschikt voor PDT van Barrett oesofagus. De plaats van de laserfibre tijdens belichting zal uiteindelijk de semi-selectiviteit van ALA-PDT bepalen.

De hoofdstukken 9 tot en met 12 handelen over het optimaliseren van ALA-PDT voor slokdarmaandoeningen. In Hoofdstuk 9 wordt het tijdstip van belichting na toediening van ALA geoptimaliseerd. De effectiviteit in mate van epitheel ablatie alsmede het mogelijke gevolg voor de slokdarmfunctie in de herstelfase is onderzocht. De dieren kregen 200 mg kg$^{-1}$ ALA oraal toegediend en werden belicht op 1, 2, 3, 4, 6 of 12 uur daarna, allemaal met identieke laserparameters (golflengte: 633nm; totale lichtdosis: 25 J per cm diffuser; uitgangsvermogen: 100 mW per cm diffuser). Om de slokdarm homoge en circumferentieel te belichten werd een 1 cm-lange cilindrische diffuser geplaatst in een doorzichtige balloncatheter. Tijdens de belichting werden PpIX-fluorescentiespectra en de daadwerkelijke lichthoeveelheid op het slokdarmepitheel gemeten. De dieren werden 48 uur of 28 dagen na PDT opgeofferd. Op dag 28 werden de dieren gewogen en werd een slikfoto gemaakt om stricturen en dilataties te diagnostiseren. De schade werd voor iedere weefsellaag apart, histologisch vastgesteld. In overeenstemming met de resultaten van de farmakokinetische studie beschreven in hoofdstuk 8, werd de sterkste PpIX-fluorescentie gemeten op 3 uur na ALA-toediening. De resultaten van de dosimetrie toonden aan dat, door de sterk licht-weerkaatstende eigenschappen van weefsels, de in vivo lichtsterke gemeten op het slokdarm epitheel drie maal hoger was dan de theoretische lichtsterkte. Op 48 uur na PDT werd ernstige schade aan het epitheel gevonden in dieren die belicht waren op 2 uur na ALA, terwijl slechts lichte schade aan het epitheel werd gevonden in dieren belicht op 3, 4 of 6 uur en geen schade in dieren belicht op 1 of 12 uur. Op
dag 28 toonden de slikfoto’s van dieren die op 4, 6 of 12 uur na ALA-toediening werden belicht in tegenstelling tot dieren die op 2 uur belicht werden, ernstige dilataties en duidde het gewichtsverlies van de dieren in deze groepen op een verminderde slokdarmfunctie. Histologisch onderzoek liet in deze groepen een verlies van Schwannse cellen zien, mogelijk omdat in deze cellen pas relatief laat na ALA-toediening porfyrines accumuleren. De resultaten die in dit hoofdstuk gepresenteerd worden laten zien dat de keuze van het tijdsinterval tussen ALA-toediening en belichting cruciaal is om epitheelschade zonder functionele achteruitgang te krijgen. Een relatief kort interval van 2 tot 3 uur na ALA-toediening lijkt optimaal.

In Hoofdstuk 10 wordt het effect van de keuze van de verschillende laserparameters, te weten golflengte, uitgangsvermogen en totale lichtdosis onderzocht. De dieren kregen 200 mg kg\(^{-1}\) ALA oraal toegediend en werden belicht met ofwel 633 nm ofwel 532 nm licht via een endo-oesofageale balloncatheter, zoals beschreven in hoofdstuk 9. Bij alle dieren vond de belichting plaats op 3 uur na ALA toediening met 8.3 of 25 J per cm diffuser geleverd met 33, 100 of 300 mW per cm diffuser. Tijdens de belichting werden weefselfluorescentie metingen verricht en licht dosimetrie uitgevoerd. De dieren werden op 48 uur na PDT opgeofferd en de schade aan de slokdarm werd histologisch geclassificeerd. Tijdens belichting met beide golflengtes daalde de PpIX-fluorescentie uitgezet tegen de belichtingstijd sneller bij een hoger uitgangsvermogen. De lichtdosis op het slokdarmoppervlak was het hoogste bij 633 nm. Dit kan verklaard worden door het feit dat de optische absorptie en weerkaatsing van weefsels verschilt bij verschillende golflengtes. Dit is mogelijk de reden waarom 532 nm licht minder schade aan het epitheel en de spierlaag veroorzaakte dan 633 nm licht. In beide groepen veroorzaakte belichting met een laag uitgangsvermogen selectieve epitheliale ablatie terwijl belichting met een hoog uitgangsvermogen resulteerde in spierschade met minimale schade aan het epitheel. Zeer selectieve epitheel ablatie werd geïnduceerd met de volgende laser parameters: golflengte 633 nm, uitgangsvermogen 33 mW per cm diffuser en een totale lichtdosis van 8.3 J per cm diffuser. Waarschijnlijk zal snelle PpIX-fotobleking en fotochemische zuurstofconsumptie tijdens belichting met hoog vermogen leiden tot relatieve hypoxie en daardoor tot een verminderd PDT-effect. Verhoging van de totale lichtdosis resulteerde in meer schade, met name aan de spierlaag. Deze resultaten laten zien dat de selectiviteit van ALA-PDT om epitheel laesies van de slokdarm te veroorzaken sterk afhankelijk is van de combinatie van gebruikte golflengte, uitgangsvermogen en lichtdosis. De meest selectieve epitheel ablatie werd verkregen met laag vermogen rood licht en een lage totale lichtdosis.
Naast het optimaliseren van het tijdstip van belichting en de belichtingsparameters is onderzocht of de resultaten van ALA-PDT verder verbeterd kunnen worden door de belichting te fractioneren. In Hoofdstuk 11 is de hypothese getoetst dat belichting op 1 uur na toediening van ALA selectieve schade aan het enzym ferrochelatase (het enzym dat PpIX omzet in haem) kan toebrengen omdat dit enzym tijdelijk aan PpIX gebonden is, wat uiteindelijk lijdt tot de beoogde hogere PpIX-concentratie. Om ervoor te zorgen dat het relatieve ferrochelatase tekort ontstaan door de eerste belichting, benut wordt, is een studie uitgevoerd waarbij het effect van het toedienen van een tweede dosis ALA is onderzocht. Ratten kregen oraal 200 mg kg⁻¹ ALA op 0 uur toegediend of 200 mg kg⁻¹ ALA op zowel 0 uur als 4 uur. De activiteit van de enzymen ferrochelatase en porphobilinogeen deaminase en de PpIX concentratie in de slokdarmwand werden gemeten, met of zonder belichting op 1 uur na (de eerste dosis) ALA. In een tweede studie kregen de dieren intra-oesofageale PDT op 4 uur na ALA toediening, met of zonder belichting op 1 uur, of zij kregen een tweede dosis ALA en PDT op 3 uur na deze tweede dosis ALA, eveneens met of zonder belichting op 1 uur na de eerste ALA dosis. De resultaten van deze studie tonen aan dat belichting op 1 uur na ALA de ferrochelatase activiteit op 3 en 4 h na ALA kan verminderen. Dit resulteerde in een significante toename van de ratio "porphobilinogeen deaminase:ferrochelatase" in dieren die op 1 uur waren belicht en op 4 uur werden opgeofferd (P < 0.001). Dit resulteerde niet in een hogere PpIX accumulatie noch in meer PDT- schade aan slokdarmepitheel of spierlaag. Ook een tweede dosis ALA leidde niet tot meer porfyrine ophoping of tot meer schade. Een gefractioneerde belichting volgens het toepast tijdschema lijkt derhalve niet te resulteren in betere ALA-PDT-geïnduceerde abalatie van het slokdarmepitheel van de rat.

Hoofdstuk 12 beschrijft de resultaten van een volgende studie waarbij het effect van een gefractioneerde belichting wordt onderzocht. Twee hypotheses die het versterkte PDT-effect van een gefractioneerde belichting zouden kunnen verklaren zijn getoetst: (1) fractioneren vermindert de vasoconstrictie die veroorzaakt wordt door ALA-PDT en (2) bij een gefractioneerde belichting kan nieuw gevormd PpIX (geproduceerd tussen de belichtingen in) gebruikt worden. De ratten werden verdeeld in twee groepen. Om de vasculaire effecten te bestuderen werden de dieren in groep 1 op 3 h na ALA (200 mg kg⁻¹) continue belicht met 20 J per cm diffuser danwel gefractioneerd belicht met een pauze van 150 s tussen 2 maal 10 J per cm diffuser. De doorbloeding van het belichte gebied werd gemeten met een laser Doppler stroomsnelheidsmeter. Om het effect van nieuw gevormd PpIX te bestuderen werden de dieren in groep 2 belicht op 3 h na ALA (met 20 of 40 J per cm diffuser) of op zowel 3 als 6 h na ALA met 20 J per cm diffuser. In alle dieren werd de in vivo lichtsterkte op het slokdarm epitheel en de PpIX-
fluorescentie gemeten tijdens belichting. De dieren werden op 48 uur na PDT opgeofferd. Uit de metingen bleek dat ALA-PDT geen significante vasoconstrictie veroorzaakte. Zowel de fluorescentie metingen als de dosimetrer resultaten in groep 1 waren niet verschillend tussen dieren die continue of gefractioneerd belicht waren. Tijdens de 3 uur durende pauze in groep 2 steeg de PpIX fluorescentie en deze daalde tijdens de belichting op 6 h na ALA. De optische eigenschappen van het weefsel veranderden tijdens de 3 h durende pauze hetgeen resulteerde in een lagere *in vivo* lichtdosis op 6 h (*P*<0.001). Een gefractioneerde belichting met zowel korte als lange tijdsintervalen resulteerde niet in meer schade aan de slokdarm. Uit deze studie kan geconcludeerd worden dat bij ALA-PDT een pauze van 150 s tussen de belichtingen niet resulteert in een verbetering van de doorbloeding van de slokdarm. Tijdens een 3 uur durende pauze wordt nieuw PpIX gevormd. Dit resulteerde echter niet in meer schade. Deze studie levert derhalve geen bewijs voor een versterkt PDT-effect door een gefractioneerde belichting.

Met de algemene discussie in Hoofdstuk 13 wordt dit proefschrift besloten. Het geeft een overzicht van de resultaten van de in dit proefschrift beschreven studies, bespreekt de gevolgen ervan voor de kliniek en geeft suggesties voor verder onderzoek. Het is belangrijk dat, zolang de uitkomsten van experimentele en klinische studies nog geen uitkomst hebben geboden ten aanzien van de volledigheid van de epitheel ablatie, het optreden van bijwerkingen, het resultaat op lange termijn en met name het optreden van adenocarcinoom, ALA-PDT voor Barrett oesofagus slechts in trial verband plaatsvindt.

**Conclusies**

1. In de rat kan een Barrett oesofagus worden geïnduceerd door langdurige reflux van dunne darm inhoud in de slokdarm.

2. Barrett oesofagus ontstaat niet vanuit submucosale klieren of klierbuizen.

3. 5-Aminolevuline zuur is een veilige fotosensitizer die accumulatie van lichtgevoelige concentraties van protoporfyrine IX induceert in de slokdarm.

4. 5-Aminolevuline zuur induceert selectieve protoporfyrine IX accumulatie in de slokdarm mucosa in vergelijking met de onderliggende submucosa en spierlaag. Er treed geen selectieve accumulatie van protoporfyrine IX op in Barrett epithiel in vergelijking met het aangrenzende plaveisel cel epitheel.
5. Bij ALA-PDT van de slokdarm is de keuze van de tijd tussen ALA toediening en belichting essentieel om selectieve schade aan het epitheel toe te brengen zonder functionele achteruitgang.

6. De combinatie van golflengte, uitgangsvermogen en totale lichtdosis bepaalt in belangrijke mate de selektiviteit van ALA-PDT om epitheel schade in de slokdarm te induceren.

7. Gefractioneerde belichting bij ALA-PDT van de slokdarm resulteert niet in meer PDT-geïnduceerde epitheel ablatie.
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DANKWOORD

Dit proefschrift is tot stand gekomen met de hulp en inzet van velen. Graag dank ik een aantal van hen met name.

Mijn promotor prof.dr. H.W. Tilanus. Reeds in het tweede jaar van mijn studie leerde ik u als clinicus (toen nog geen professor) kennen in de periode dat ik werkzaam was in het studententeam op "10 Midden". Dat u nu, jaren later, mijn promotor bent doet mij genoegen. Dank voor het vertrouwen dat u in mij heeft gesteld en de vrijheid die u mij heeft gegeven bij het uitvoeren van het onderzoek.

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Beste Ron, samen met Richard vormde jij één van de beste begeleiders die een promovendus zich kan wensen. Jij was er altijd. Dit proefschrift is eens te meer het bewijs dat de volgorde van de auteurs bij de artikelen niet altijd de inspanningen van betrokkenen weerspiegelt. Ik heb het als bijzonder prettig ervaren dat ik met al mijn vragen altijd bij jou terecht kon. Jouw hulp bij de praktische uitvoering is eigenlijk een apart hoofdstuk waard. Daarnaast hebben de inhoudelijke discussies, naarmate ons beider kennis over fotodynamische therapie toenam, regelmatig gezorgd voor nieuwe impulsen leidend tot het verrichten van nog niet eerder
uitgevoerde experimenten. Het feit dat ik mijn ambitie om thorax chirug te worden met jou besprak lang voordat anderen hiervan wisten zegt denk ik genoeg over de band die wij hebben opgebouwd. Ik hoop van harte dat wij in de toekomst gezamenlijk onderzoek kunnen blijven doen.

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Een aantal collega’s van de afdeling Gastroenterologie en Hepatologie wil ik danken voor hun samenwerking. Dr. P.D. Siersema, beste Peter, dank voor jouw klinische input tijdens de PDT-werkgroep, jouw betrokkenheid bij het onderzoek en voor het doornemen van de manuscripten. Jij corrigeerde de stukken altijd snel en vakkundig. Ik hoop dat de toekomstige gezamenlijke onderzoeken even plezierig en succesvol verlopen. Dr. F.W.M. de Rooij en Annie Edixhoven-Bosdijk wil ik bedanken voor alle bepalingen die zijn verricht op het Lab Interne II. Beste Felix, jouw interesse in en kennis van porfyrines en gelzuren heeft tot goede ideeën geleid die de impact van de manuscripten hebben vergroot. Beste Annie, jouw hulp bij de bepalingen in het lab Interne II is onmisbaar geweest. Dank je voor jouw niet aflatende inzet.

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Met de PDT-groep van de Daniël den Hoed Kliniek zijn meerdere inspirerende
gesprekken gevoerd. Zeer nauwe samenwerking vond plaats met dr. H.J. van Staveren. Beste Hugo, jouw inbreng in de PDT experimenten is voor de resultaten van deze studies van groot belang gebleken. Jij had de kennis om met dat hele kleine isotrope bolletje zowel de fluorescentie als de lichtdosis in de rattenslokdarm te meten. Omdat ik vaak "vergat" een koffie- of rookpauze in te lassen waren de experimentele dagen lang en intensief. En na afloop ging jij naar huis met een computer vol data waarvan ik de uitwerkingen graag al de volgende dag had willen hebben. Dank je voor jouw (fysische) input en de enthousiaste, prettige samenwerking.

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voorbij periode bij menig hockeytourenoi in de praktijk gebracht, zal alles 8 december gladjes verlopen.

De thoraxchirurgen en collega arts-assistenten van de afdeling Cardio Thoracale Chirurgie dank ik voor de prettige sfeer op de afdeling. Het plezierige dagelijkse werk in de kliniek heeft het in de avonduren completeren van dit boekje verlicht. Bedankt voor jullie collegialiteit en getoonde interesse.

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CURRICULUM VITAE

Jolanda van den Boogert-Kluin was born on May 20, 1971 in Rotterdam, the Netherlands. In 1989 she graduated from the City College St. Franciscus (gymnasium β) in Rotterdam. In the same year she started her medical training at the Erasmus University Rotterdam, where she graduated in May 1994. During her medical studies she worked in the "studentteam" at the Department of General Surgery from the University Hospital Rotterdam Dijkzigt. As part of her 4th year she spent six months in Ivory Coast, West-Africa, working on a research project concerning drug resistance of malaria among children. In June 1996 she obtained her medical degree cum laude.

From August 1996 she worked as a PhD student on the project "Photodynamic therapy for Barrett's oesophagus with use of 5-aminolevulinic acid" which was supported for two years by the Netherlands Digestive Diseases Foundation (applicant dr. R. van Hillegersberg). The studies described in this thesis were performed at the Laboratory for Experimental Surgery from the Erasmus University Rotterdam (dr. R.L. Marquet). The supervision was conducted by dr. R. van Hillegersberg and dr. R.W.F. de Bruin.

For the experiment described in chapter 8 she received the "Resident Award of the American Society for Laser Medicine and Surgery" and with the study described in chapter 10 the "Prix de Communication Scientifique d'Université de Nantes". During her last year as a PhD student she was offered a post-doctoral position at the Laser Centre from the University College London.

However, her ambition led her to the Department of Cardio Thoracic Surgery from the University Hospital Rotterdam Dijkzigt where she works as a house officer since October 1998. In January 2000 she will start her residency in general surgery at the Ikazia Hospital, Rotterdam (dr. W.F. Weidema) as part of her specialty training in cardio thoracic surgery (prof.dr. A.J.J.C. Bogers).
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABPC</td>
<td>argon beam plasma coagulation</td>
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<tr>
<td>ALA</td>
<td>5-aminolevulinic acid</td>
</tr>
<tr>
<td>ALAT</td>
<td>alanine aminotransferase</td>
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<tr>
<td>ASAT</td>
<td>aspartate aminotransferase</td>
</tr>
<tr>
<td>ASC</td>
<td>adenosquamous carcinoma</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BO</td>
<td>Barrett's oesophagus</td>
</tr>
<tr>
<td>CR</td>
<td>complete remission</td>
</tr>
<tr>
<td>DHE</td>
<td>dihaematoporphyrin ester/ether</td>
</tr>
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<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>FU</td>
<td>follow-up</td>
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<tr>
<td>H&amp;E</td>
<td>haematoxylin &amp; eosin</td>
</tr>
<tr>
<td>HGD</td>
<td>high-grade dypslasia</td>
</tr>
<tr>
<td>HpD</td>
<td>haematoporphyrin derivative</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenously</td>
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<tr>
<td>KTP</td>
<td>potassium titanium phosphate</td>
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<tr>
<td>LGD</td>
<td>low-grade dysplasia</td>
</tr>
<tr>
<td>LDF</td>
<td>laser Doppler flowmeter</td>
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<tr>
<td>MGD</td>
<td>moderate-grade dypslasia</td>
</tr>
<tr>
<td>MPEC</td>
<td>multipolar electrocoagulation</td>
</tr>
<tr>
<td>ND</td>
<td>no dysplasia</td>
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<tr>
<td>Nd:YAG</td>
<td>neodymium:yttrium-aluminium-garnet</td>
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<tr>
<td>NMBA</td>
<td>N-nitrosomethyl-benzylamine</td>
</tr>
<tr>
<td>PAS</td>
<td>periodic acid-Schiff</td>
</tr>
<tr>
<td>PBG</td>
<td>porphobilinogen</td>
</tr>
<tr>
<td>PBGD</td>
<td>porphobilinogen deaminase</td>
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<tr>
<td>PDT</td>
<td>photodynamic therapy</td>
</tr>
<tr>
<td>p.o.</td>
<td>per os (orally)</td>
</tr>
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<td>PpIX</td>
<td>protoporphyrin IX</td>
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<tr>
<td>SCC</td>
<td>squamous cell carcinoma</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SF</td>
<td>selectivity factor (for epithelial damage)</td>
</tr>
</tbody>
</table>
Een stuk is voldaan als de meester
zijn voornemen daar in bereikt heeft

(Rembrandt)