Phototherapy of newborns suffering from hyperbilirubinaemia. An experimental study.

by

Ellen M. Bruzell

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Department of Physics
Norwegian University of Science and Technology
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Preface

This thesis is submitted as a partial fulfilment of the requirements for the degree of *Doctor Scientarium*. It contains work related to preparations for and results from clinical trials and *in vitro* studies on phototherapy of jaundice in newborns. These studies have been performed at the Norwegian Radiation Protection Authority (NRPA), and were financed by the Research Council of Norway and the NRPA.

I would like to thank my supervisor Prof. Terje Christensen for initialising this project together with Prof. Dag Bratlid at St. Olavs Hospital, Trondheim, and for changing the path to cell experiments when the clinical trials were discontinued. I am grateful for Christensen's excellent scientific guidance, creativeness, enthusiasm and motivation.

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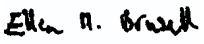
Finally, a big hug to my children who represent a great source of inspiration; to Benjamin who gave me the opportunity to become interested in jaundice 16 years ago and to Maria for demonstrating her fascination with dead and alive cells in the microscope.

Bærum, October 23, 2003



Ellen M. Bruzell

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2 Papers

Part I: Preparation for clinical phototherapy studies

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Paper 2: Ethical aspects of clinical research involving children, exemplified by a research project on phototherapy of jaundice

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1 Presentation of the thesis

1.1 Introduction

The sunshine's fading effect on the yellow skin colour of jaundiced newborns was discovered accidentally by an observant nurse in 1956. The first, controlled treatments of the condition of hyperbilirubinaemia (jaundice) were indeed performed with sunlight as the light source (Cremer et al. 1958), but were very soon followed by the use of blue-light fluorescent lamps. These lamps emitted light in the spectral range of 420-480 nm (visible light) (Fig. 1.1), and the treatment was given interrupted by breaks (intermittently).

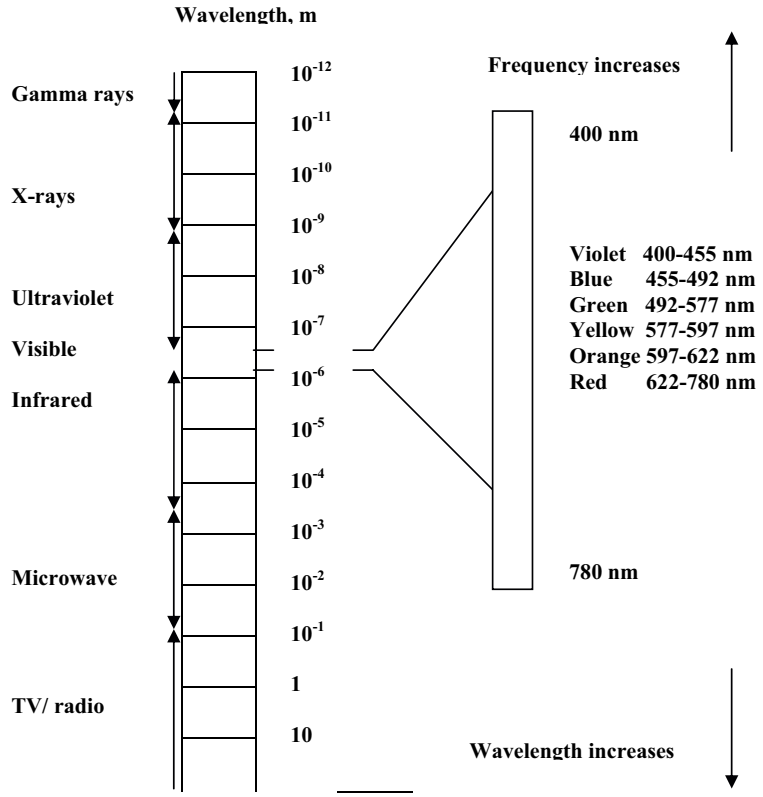

Fig. 1.1 The electromagnetic spectrum (Alonso and Finn 1967)

Two decades prior to the first phototherapy events, scientists had discovered the photoreactivity of bilirubin in *in vitro* systems. Saeki (1932) exposed red blood cells to bilirubin and light, and observed a resulting photohaemolysis. A few years later, Fischer and Herle (1938) found that bilirubin solutions gradually lost their characteristic colour when left in daylight. Cremer et al. (1958) used the latter observation as a model to explain the more rapid recovery of jaundiced newborns after exposure to visible light. The loss of bilirubin observed was thought to be a result of photooxidation or dehydrogenation of the bilirubin pigment to biliverdin (a

breakdown product of bilirubin) or other pigments. Even as late as in the seventies, when phototherapy had become an established and preferred mode of treatment, photooxidation was thought to be the mechanism responsible for the bilirubin loss (McDonagh 1971). However, McDonagh and coworkers were among the first researchers to show that bilirubin can undergo several other photoreactions than photooxidation, notably photoisomerisation (McDonagh et al. 1979). Today, photoisomerisation is considered the main photochemical mechanism behind the effect of phototherapy (see 1.2.2).

Two factors characterising the use of phototherapy of jaundiced newborns today are the lack of standardisation and the unconscious employment. Generally, phototherapy is administered in much the same way as in the early days of treatment. Variations in therapeutic light colour and fractionation regimens have been tried and advocated, but the number of high quality clinical studies is sparse. New types of equipment of varying suitability have been taken into use without thorough clinical follow-up studies. The bulk of phototherapy research has been done on adverse effects, but unfortunately only a small part is relevant for *in vivo* conditions. Not enough attention has been paid to the light physics. Lack of calibration of equipment and radiometers, variability of lamp systems used resulting in considerable differences in irradiance and lack of standardisation of light physics nomenclature are all factors making the comparison of treatment results more difficult. These concerns were expressed by medical physicists in several abstracts presented at the October 2002 meeting of the Institute of Physics and Engineering in Medicine (IPEM 2003).

There is a need for improvement and optimisation of phototherapy. In order to reach these goals, more clinical studies with emphasis both on therapeutic and adverse effects, and systematic *in vitro* studies should be carried out. The studies should always be undertaken prior to changes in the treatment, along with follow-up studies, to assure that newborns are given the best possible treatment with the least adverse effects and to ensure the ethical considerations related to research on children (Roll and Christensen 1997 (paper 2)).

With the above-mentioned goals in mind, a multi-centre clinical study of a newborn, jaundiced cohort was planned and partly carried out in three Norwegian hospitals. Prior to the onset of the study, there was a need for a theoretical investigation of the basis for and perspectives of the trial as well as assessment of the ethical considerations implied. The objectives of the clinical study were as follows: first, to compare differences between therapeutic light colours to determine whether turquoise fluorescent light tubes will give a faster bilirubin clearance in serum than will blue fluorescent light tubes. Second, to attempt to identify the optimal irradiance for overhead phototherapy units equipped with blue fluorescent light tubes (Fig.1.2). Finally, to determine whether an equally good therapeutic effect can be obtained by a smaller total light dose separated by dark periods compared with a continuous treatment. For all the parameters under investigation, the influence of irradiation on light sensitive vitamins in serum was to be assessed.

Fig. 1.2 Overhead phototherapy lamp with light tubes.

Another cohort of hyperbilirubinaemic newborns was subjected to a non-invasive bilirubin measurement technique of reflectance spectroscopy to study differences in skin optical parameters such as melanin and erythema indices of patients undergoing phototherapy. Furthermore, we wished to correlate the bilirubin index found by the non-invasive technique to the total serum bilirubin.

To further evaluate possible adverse effects of phototherapy *in vitro*, we wanted to investigate differences in cytotoxic effects related to variations in the parameters examined in the clinical study described in papers 1, 2 and 4. In addition, the phototherapy effects were to be compared to dark toxicity of bilirubin. These *in vitro* investigations included examination of possible cytotoxic mechanisms; apoptosis and necrosis. Furthermore, studies were performed to reveal possible haematological consequences of phototherapy in jaundiced newborns. The phenomena chosen as end-points were increased osmotic fragility and delayed photohaemolysis of both normal erythrocytes and spherocytes obtained from a donor with hereditary spherocytosis.

On this background, the parts and papers presenting the *in vivo* and *in vitro* studies are as follows (Throughout this thesis the word "paper" has been used equivalent to the word "chapter" when the eight chapters contained in the thesis are presented): Part I consists of two papers related to the planning of clinical studies. The first paper describes the need for optimisation of phototherapy with emphasis on the physical parameters such as wavelength and light intensity, and a thorough evaluation of the therapeutic efficiency and possible side effects of the treatment. A planned epidemiological study that will concentrate on long-term side effects, particularly in relation to cancer development is also described. The second paper deals with the ethical considerations related to research on children using the Declaration of Helsinki and various paediatric directions as references. The ethical aspects needed to be

discussed in connection with the above-mentioned clinical phototherapy study are evaluated.

Results from our clinical studies are given in Part II. Paper 3 contains analyses of reflection spectra of the skin of jaundiced newborns exposed to phototherapy compared to a group not given the treatment. Factors influencing the skin optics of newborns are discussed, as well as the bilirubin kinetics. Paper 4 gives a detailed description of the planned clinical phototherapy study referred to in the papers in Part I as well as a presentation of results of riboflavin (vitamin B_2) and retinol (vitamin A) serum analyses of the newborn patients' blood.

In vitro systems have the advantage of demonstrating effects on a large scale in a short time without the concurrent influence of the whole-body biochemical and physiologic action, especially where such studies will be a major strain on human subjects. Three papers examining effects of bilirubin and visible irradiation on cells are found in Part III. The first evidence for apoptotic cell death of the mouse lymphoma cell line L5178Y-R (LY-R) exposed to bilirubin and blue phototherapy light is presented in paper 5. The finding of apoptosis in cells subjected to bilirubin in the dark has later been confirmed by other groups (Tibbs et al. 2000, Silva et al. 2001). A comparison of fractionated and continuous irradiation on cell death was performed in paper 6, whereas differences in cytotoxicity after blue and turquoise irradiation was the topic of paper 7. In papers 5 and 7, high pressure liquid chromatography analysis was used as an additional method to study the production of photoisomers of bilirubin and the reduction of unconjugated bilirubin to indicate efficiency of the irradiation and the possible formation of photooxidation products.

In the last section of Part III, paper 8 provides a continuation of the first published findings by Saeki in 1932 of photohaemolysis of red blood cells after exposure to bilirubin and blue light. In the study, a possible difference in the sensitivity of normal erythrocytes and spherocytes from a patient with hereditary spherocytosis is addressed. The risk of phototherapy-induced haemolysis is discussed.

The first chapter of this thesis will provide a general background for the most important matters related to phototherapy. Many subjects are thoroughly described in the two papers in Part I, and are also discussed in the remainder of the papers where appropriate. This chapter will, therefore, concentrate on items not covered in the papers and more recent research that has been published after the papers presented in Part I. A short presentation of each paper will be given followed by a general discussion of the results and suggestions for future work.

1.2 Phototherapy of neonatal hyperbilirubinaemia (jaundice)

1.2.1 Physiology of hyperbilirubinaemia

(Main references: Cashore 1998 and Jährig et al. 1993b).

Elevated bilirubin concentration in the serum of newborn infants causing yellow skin colour is due to a normal physiological condition. Concentrations five to seven times higher than those of normal adults occur in more than 50 % of Caucasian newborns (lower in African-Americans and higher in Asians (Maisels 1995)). The term hyperbilirubinaemia refers to concentration levels of bilirubin above defined limits and may be caused by normal physiological influences as well as pathological (e.g., liver diseases, haemolysis).

There are four main causes for the elevated serum bilirubin concentration in the neonate. First, bilirubin, being a catabolite of haem, must be excreted by the newborn itself without the previous fetal help of its mother, giving rise to some novice complications. The erythrocytes (containing haemoproteins) in excess have to be broken down in the newborn baby when the oxygen supply starts to be provided by the infant's own respiration. The breakdown process may occur at a higher rate than does the formation of new erythrocytes. Second, the transport mechanisms for bilirubin in the vascular (albumin) and hepatic (ligandin) systems may not have been fully developed. Third, there may be immaturity of the liver enzymes, especially of the bilirubin uridine-diphosphate glucoronyl transferase (UDGT) responsible for making the lipophilic bilirubin molecule water-soluble and bile-excretable (conjugation). Finally, deconjugated and unconjugated bilirubin may be reabsorbed from the intestine and recirculated in the blood stream contributing to an elevation of the bilirubin concentration (a phenomenon called rebound). Genetic deficiency of the UDGT leads to Gilbert's and Crigler-Najjar syndromes, of which the latter causes chronic hyperbilirubinaemia (Arias et al. 1969).

The reason behind the need for treatment, which is performed by phototherapy in the overwhelming majority of cases, is the neurotoxic effect of unconjugated, unbound bilirubin. In this form, bilirubin causes the often mild and reversible bilirubin encephalopathy, the more severe irreversible neurological damage or the deadly outcome, kernicterus. Normally, the blood-brain barrier is impermeable to albumin and albumin-bound bilirubin. Certain clinical conditions may, however, render the barrier more permeable, thereby increasing the possibility of transport of bilirubin into the brain (see Bratlid 1990 for a review). Bilirubin toxicity is not restricted only to the central nervous system, but may affect many different cell processes, as has been demonstrated in various cell lines *in vitro* (Karp 1979). In combination with visible light, bilirubin can act as a sensitiser with possible phototoxic effects on various cell components (Rosenstein et al. 1983, Christensen 1984).

1.2.2 Bilirubin photochemistry

(Main references: Ennever 1998 and Bonnett 2000).

The formal nomenclature of the native, unconjugated bilirubin is 4Z,15Z-bilirubin IXŭ, being a derivative of protoporhyrin IX opened at the ŭ-carbon bridge and having Z-configurations, as opposed to E-configurations, at the carbon 4 and 15 positions (Fig.1.3). Due to the folded structure of the tetrapyrrole molecule with its internal hydrogen bonds, the polar groups cannot interact with the aqueous environment, rendering the molecule highly lipophilic. This feature complicates biliary and urinary excretion of bilirubin, but facilitates transport through tissue membranes.

The bilirubin molecule can absorb light of certain wavelengths. Bound to albumin, the absorption maximum of bilirubin *in vitro* is 460 nm. When albumin is co-bound to fatty acids *in vivo*, this absorption maximum is shifted to about 480 nm (Malhotra et al. 1987). Upon absorption of a photon, the bilirubin is excited to a higher energy state. The molecule loses its energy to photochemical reactions, which are the desired outcome of phototherapy. Heat production is a more likely event whereas fluorescence (photon emission) is less likely to occur than a photochemical reaction. When photons cause rearrangements (involving a brief cleavage and reforming of a double bond) of the 4Z,15Z-bilirubin molecule (for short: ZZ-bilirubin) around the 4 or 15 carbon positions, configurational photoisomers are formed: 4Z,15E; 4E,15Z and 4E,15E (for short: ZE-, EZ- and EE-bilirubin, respectively). The formation of the isomers is photochemically reversible. The isomerisations cause less internal hydrogen bonding of the molecules, which make them more water-soluble. However, *in vivo* the configurational photoisomers are not the main isomers responsible for the bilirubin clearance as only very small quantities of the ZZ- and the ZE-isomers (isomerising back to ZZ-) are found in the bile and none in the urine of light treated patients (McDonagh and Lightner 1985) (Fig. 1.3).

The principal excretory pathway of bilirubin is through the structural photoisomer, lumirubin. This isomer is formed by binding of two of the pyrrole rings of the molecule, giving rise to a new structure (Fig. 1.4). Lumirubin can be formed in a one-photon process from ZZ-bilirubin or, more likely, in a two-photon step through the EZ-isomer. Lumirubin is more water-soluble than the configurational isomers, and its photoreaction back to ZZ-bilirubin is possible, but slow. Furthermore, lumirubin is rapidly excreted into the bile (Ennever et al. 1987) and urine (Knox et al. 1985, Onishi et al. 1980), and its serum concentration increases with increasing irradiance. Altogether, these characteristics make lumirubin the best candidate for bilirubin elimination.

A third group of photochemical products of bilirubin is the photooxidation products consisting of one or two pyrrole rings. Being polar molecules, they are readily excreted by the kidneys into the urine, but quantitatively they are not as important as lumirubin. In reactions with oxygen, the excited bilirubin molecule may transfer its energy to give singlet oxygen (McDonagh 1971), although this reaction has low quantum efficiency (Sloper and Truscott 1982). In a study using an infrared spectroscopic method, the singlet oxygen production could not be detected (Roll et al. 2001). On the other hand, bilirubin can both interact with singlet oxygen to quench the excited molecule physically, and it can also react with singlet oxygen chemically. It has been shown that irradiated bilirubin can form other reactive oxygen species

such as hydrogen peroxide and other peroxides (Rosenstein et al. 1983, Christensen 1986).

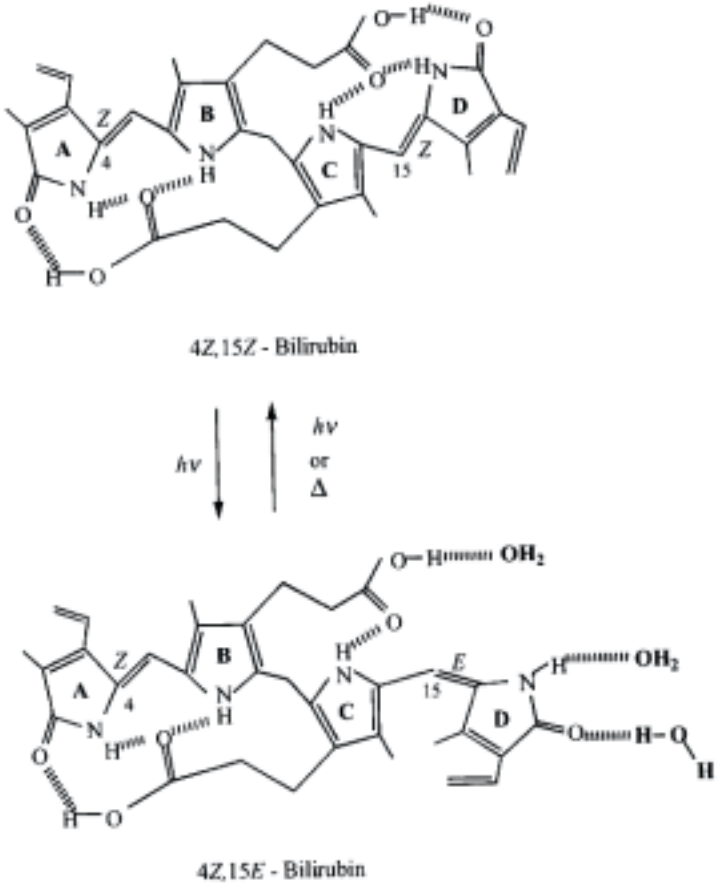

Fig. 1.3 Photoisomerisation of 4Z,15Z-bilirubin to configurational photoisomer 4Z,15E. Hydrogen bonds are denoted with "IIIIII". Copied with permission of Prof. Raymonnd Bonnett (Bonett 2000).

The wavelength of the phototherapeutic light is of importance for the efficiency of the lowering of bilirubin concentration in serum. The *in vivo* shift in absorption spectrum of bilirubin has been mentioned already. The longer wavelength blue-green or green light reaches deeper into the skin than does light with shorter wavelengths, thus reaching more of the skin- and vascular bilirubin. Albumin-bound bilirubin also shows an increase in quantum efficiency of formation of lumirubin with increasing wavelengths in the range of 450-510 nm (Onishi et al. 1986, Vecchi et al. 1986, Greenberg et al. 1987, Itoh et al. 1987, McDonagh et al. 1989).

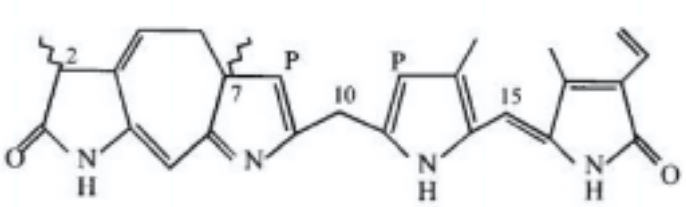

Fig. 1.4 A structural photoisomer of 4Z,15Z-bilirubin, lumirubin. Copied with permission of Prof. Raymonnd Bonnett (Bonett 2000).

1.2.3 Phototherapy today

A presentation on phototherapy practice with regard to equipment, medication and physical parameters, such as wavelength, irradiance and fractionation, is given in paper 1 of this thesis. In this paragraph only additional and new information will be considered.

There is no reason to believe that the present number of newborns undergoing phototherapy for the treatment of jaundice has decreased from the estimated 5-10 % previously reported in Norway. This number is dependent on such factors as changes in inclusion criteria, changes in hospitalisation routines, the number of premature births and changes in the maternal health. For instance, health reports on the increase in the number of neonates born during the nineties, weighing more than 4 kg (Medical Birth Registry of Norway 2003) and the corresponding neonatal complications, such as unstable glucose levels and forceps delivery (Henriksen, National Hospital, personal communication), indicates a potentially higher prevalence of phototherapy. An increase in the number of newborns with high birth weights may also reflect a higher incidence of maternal diabetes, a condition known to increase the risk for hyperbilirubinaemia (Maisels 1988). Furthermore, the early discharge practices in the USA, combined with an increase in the prevalence of breast-feeding, however inadequately established, have increased the number of hospital readmissions and severe cases of hyperbilirubinaemia (Harris et al. 2001, Galbraith et al. 2003).

The conclusions reached in an international survey conducted by Hansen (1996) on approaches to neonatal jaundice coincided with those of Christensen in three previous national studies with respect to the large diversity of phototherapy equipments and regimens in use and the lack of precise procedures (Christensen and Reitan 1987, Christensen et al. 1992). However, the latest national inspection by the Norwegian Radiation Protection Authority (NRPA)(autumn, 2002) of neonatal intensive care and well-baby units, displayed a higher degree of homogeneity of equipments, lamps and procedures compared to previous investigations. Unfortunately, in some hospitals there are still irradiation devices in use that have been advised against (Christensen, unpublished results).

The most frequent phototherapy equipment used in Norwegian hospitals is blue fluorescent tubes in combination with white light. Relatively few units use light mattresses or fiberoptic blankets, and these devices are seldom seen in the neonatal intensive care units. In 1998 one of the major national paediatric units bought a large number of the so-called “Bili-compact” lamps consisting of 10 × 9W1114 (compact)

tubes with a peak emission of 460 nm (blue). The irradiance of the light tubes was measured and found satisfactory, however, the irradiance area was small (a 50 % decrease in maximum irradiance measured 30 cm from the midpoint of the lamp), substantiating less than optimum irradiation to the patient (Bruzell Roll and Riise, unpublished results). This problem is partly solved by increasing the light irradiance by covering the top and sides of the units with white, reflecting and scattering textiles as advised by the NRPA (Eggert et al. 1988, Amundsen 1991).

A new blue-green phototherapy lamp was tested in Denmark as part of a multi-centre trial (Agati et al. 2001), showing equal efficiency to the conventional special blue lamp (Ebbesen et al. 2003). One of the newest devices for the treatment of jaundice of newborns utilises solid state lamps consisting of a number of light-emitting diodes (LED). The few reports available so far advocate its use and indicate equal or better efficiency in treating neonatal jaundice (Seidman et al. 2000, Vreman et al. 1998). Another device utilises a laser as the light source. The apparatus is claimed to shorten the course of treatment compared to the use of fluorescent lamps (Mostnikova et al. 2000). A follow-up study of the children who underwent treatment has been conducted.

The administration of medications as an alternative to phototherapy is not widespread. In addition to the types of medications mentioned in paper 1 of this thesis, the elevation of bilirubin concentration in the case of neonatal isoimmunisation can be controlled by the use of intravenous immunoglobulin. Its use is still in the experimental phase (Rubaltelli et al. 1977, Rubaltelli 1998).

From a safety-point of view it has been unsatisfactory that phototherapy has been exempt of an operating guidance with respect to excessive radiation. In 2000 the International Electrotechnical Commission provided an international standard with particular requirements for the safety of infant phototherapy equipment (IEC 2000). The standard includes, among other topics, a section on protection against hazards from unwanted or excessive radiation as well as on accuracy of operating data and protection against hazardous output (measuring principles and uniformity of the irradiance).

1.2.4 Adverse effects of phototherapy

Paper 1 of this thesis addresses risk factors associated with the use of phototherapy. Additional information on and discussions of a number of biochemical (effects on melanin, light sensitive vitamins and haemolysis) and cellular *in vitro* effects (cytotoxicity mechanisms, DNA damage and mutations) are found in papers 3-8. Therefore, only a brief presentation of side effects will be addressed in this paragraph.

Short-term clinical effects
The few reported clinical effects during the use of phototherapy are transient. One of the earliest reports on the issue mentions rash, diarrhoea and lethargy (John 1975). Increased insensible water loss during phototherapy has been described (Bell et al. 1979, Oh and Karecki 1972, Wu and Hodgman 1974). However, newer studies link this observation to heat stress rather than irradiation (Kjartanson et al. 1992a, 1992b). Indeed, halogen lamps without proper filtering will emit infrared radiation and may cause undesired heating of the patients. Jährig et al. (1993a) reported increased body

temperature in 25 % of the cases where such lamps were used. The problem may be relevant in many hospitals since the occurrence of halogen lamps in neonatal intensive care units world-wide has been reported to be 25 % (based on 108 units) (Hansen 1996).

A rare observation of light treated jaundiced newborns is the bronze baby syndrome (Kopelman et al. 1972). The patient develops a greyish-brownish tint as a result of the combination of hyperbilirubinaemia, cholestasis and an increased porphyrin concentration in serum.

Long-term effects
The report by Wu et al. (1992) of increased frequency in sister chromatid exchanges of newborns with Down's syndrome submitted to phototherapy has been confirmed both by a new study (Wu et al. 1996) and by a study on newborns without the chromosomal syndrome (Tsai et al. 1998). Sirota et al. (1999) found that phototherapy affected the function of the immune system in the newborn by altering the cytokine production.

1.3 Synopsis of the papers

A short summary of the various papers with emphasis on the main results is given below. Throughout this thesis the word "paper" has been used equivalent to the word "chapter" when the eight chapters contained in the thesis are presented. In papers 3, 5 and 8 the author of this thesis is responsible for the following parts:

Paper 3: Interpreting the reflectance spectroscopic measurement data with respect to bilirubin and neonatal physiology. Description of the material concerning patients and phototherapy equipment) (Experimental, 2.2.1-2.2.4, except for the description of the instrument in 2.2.3). Writing most of the text concerning biological and physiological aspects. This paper will also be part of the doctoral thesis of Dr. ing. student Lise Lyngsnes Randeberg, NTNU.

Paper 5: HPLC-methodology, experiments, computations, interpretation of results and describing the HPLC-technique in Materials and Methods (2.4). Performing the apoptosis experiments using the TUNEL-method (2.5). Participating in writing and revising the manuscript.

Paper 8: Participating in decisions concerning the methodology, performing some of the experiments in the late phase of the experimental work and writing most of the first version of the paper.

Part I: Preparations for clinical phototherapy studies.

Paper 1: Phototherapy of hyperbilirubinaemia in newborns; the need for a better treatment and follow-up.

Ellen Bruzell Roll and Terje Christensen
The original and abridged Norwegian version is published in the Norwegian Journal of Epidemiology 1997;7 (1):93-8. Translation by the authors.

The paper points out the need for improvement and optimisation of phototherapy of jaundiced newborns based on current knowledge and previous studies. The planned clinical study, which is further described in papers 2 and 4, is presented. Furthermore, the need for long-term follow-up of the treated children, with particular emphasis on cancer development, is addressed. The aetiology of hyperbilirubinaemia is shortly presented, as well as possible adverse effects of the light treatment. Areas of improvement of phototherapy are described and cover the parameters wavelength, irradiance and fractionation treatment. The English version (paper 1) covers the additional topics of treatment devices and medications.

Paper 2: Ethical aspects of clinical research involving children, exemplified by a research project on phototherapy of jaundice.

Ellen Bruzell Roll and Terje Christensen
The original Norwegian version is published in Barn 1997;2:29-45. Translation by Margaret F. Kallevig.

The paper was prepared to assess the ethical considerations essential in the planning and execution of any clinical study, in particular those involving children. The phototherapy optimisation study presented in paper 1 and further detailed in paper 4 is thoroughly examined in relation to the ethical guidelines and assessments addressed in the paper. The article raises questions on the necessity of the currently practiced ethical guidelines and legislation with respect to research on humans, particularly children. Furthermore, whether research on children is a benefit and whether it is inevitable, is discussed herein. Issues on risk concept, informed consent and its ethical implications are also addressed.

Part II: In vivo studies on jaundiced newborns.

Paper 3: In vivo reflectance spectroscopy of jaundiced newborn skin reveals more than a bilirubin index.

Lise Lyngsnes Randeberg, Ellen Bruzell Roll, Lill Tove Norvang Nilsen, Terje Christensen and Lars O. Svaasand
Submitted to Acta Paediatrica

Reflectance spectroscopy is a non-invasive technique used to assess skin optical parameters useful in the monitoring of neonatal hyperbilirubinaemia. The reflectance spectrum obtained is the basis for computations of a bilirubin index, which is correlated to serum bilirubin. Furthermore, analyses of the spectrum enable the assessment of other skin chromophores such as haemoglobin and melanin, and of processes such as bilirubin kinetics and skin changes observed during and after phototherapy. The paper presents reflectance measurements performed on a group of jaundiced and non-jaundiced newborns not subjected to phototherapy and on a group receiving the treatment. The melanin index was higher for the phototherapy group, indicating that pigmentation had occurred. The reflectance measurements strengthened previous arguments for choosing the forehead as a standard measurement site for determination of a transcutaneous bilirubin index. The findings of stagnation in serum bilirubin while the transcutaneous bilirubin index still increased during phototherapy, may aid in the monitoring of the rate of formation and deposition of bilirubin to avoid neurological damage in severe cases.

Paper 4: The effect of phototherapy on light sensitive vitamins in the serum of jaundiced infants.

Ellen Bruzell Roll

Paper 4 is the second of the two papers based on *in vivo* examinations. The jaundiced newborn cohort is unique to the present study. The first two papers of this thesis introduce the clinical investigations presented in this study of various parameters influencing phototherapy and the study of adverse effects related to the irradiation. Further argumentation is given for optimised treatment based on inspection experience and international guidelines. The protocol for the planned and partly accomplished multi-centre study is presented. Blood samples from jaundiced newborns subjected to phototherapy of various durations were analysed for the light sensitive vitamins riboflavin (vitamin B_2), two flavoenzymes and retinol (vitamin A). The serum riboflavin concentration decreased to half its pre-treatment value after few hours of phototherapy, whereas the flavin isoenzymes concentration remained constant. The retinol concentration even increased during phototherapy, indicating that supplementary vitamin was given to most of the infants in the study (most were recruited from intensive care units).

Part III: In vitro cell studies on adverse effects of phototherapy.

Paper 5: Bilirubin- and light induced cell death in a murine lymphoma cell line.

Terje Christensen, Ellen Bruzell Roll, Alicja Jaworska and Gunnar Kinn
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The first *in vitro* article of this thesis presents preliminary results from the combined effects of bilirubin and (blue) phototherapy light on mouse lymphoma LY-R cells. A presentation is given of the cell line and its application in various radiation and cell toxicity studies. Different methods for scoring apoptosis after bilirubin and light-treatment of the cells gave corresponding results and revealed that the cell toxic mechanism apoptosis, occurred early. Experiments performed under less than optimum conditions showed essentially no difference in apoptotic cell toxicity between fractionated and continuous irradiation of equal light dose (radiant exposure). High pressure liquid chromatography analyses of bilirubin solutions showed that the light doses causing death were able to photooxidise bilirubin significantly.

Paper 6: Bilirubin induces apoptosis in the dark, and irrespective of irradiation regimen in mouse lymphoma cells in culture.

Ellen Bruzell Roll
Submitted to Acta Paediatrica

Paper 6 is a continuation and refinement of the previous cell toxicity study. Experimental conditions were improved to keep the background cell toxicity low. LY-R cells were exposed to bilirubin and continuous or intermittent blue phototherapy light at a constant total dose (radiant exposure). The irradiances (I) were either given as I or I/3 every other hour (intermittent treatment) or as I/2 or I/6 (continuous treatment). The three lower irradiance levels were clinically relevant. Cells with bilirubin were also subjected to dark exposure for various durations and incubation times. Like paper 5, cell toxicity was determined by the detection of apoptosis (two methods) and necrosis (one method). There was found dose reciprocity of apoptotic cell death between intermittent and continuous irradiation after both the shorter incubation time applied in the previous paper and after longer. Necrosis, on the other hand, was more pronounced after intermittent treatment. Bilirubin dark toxicity was observed after various exposure conditions and incubation times, and was classified as both apoptotic and necrotic. The paper discusses the three different possible causes for cell toxicity; direct light effects, light-sensitising effects and dark effects. A theoretical explanation of the mechanism of dark toxicity by apoptosis is offered.

Paper 7: Formation of photoproducts and cytotoxicity from bilirubin irradiated with turquoise and blue phototherapy light.

Ellen Bruzell Roll and Terje Christensen
Submitted to Acta Paediatrica

In this study, LY-R cells were exposed to bilirubin and either turquoise or blue phototherapy light of equal and constant irradiance to determine any possible

differences in cell toxicity. The cell toxicity determination methods of the two previous papers were employed. In addition to apoptosis and necrosis, cell damage parameters, such as reduction of mitotic index and inhibited cell growth were observed. Cell suspensions, cell pellets and cell-free solutions were analysed by high pressure liquid chromatography (HPLC) after exposure to bilirubin and irradiation of turquoise or blue light to evaluate the formation of bilirubin photoisomers and the reduction in the native bilirubin. The turquoise lamp caused slightly less cellular damage than did the blue lamp. Evaluation of the HPLC analyses and absorption spectroscopy indicated that photooxidation of bilirubin was more prominent with blue light irradiation, which may explain the higher cell damage observed at this wavelength. Previously reported results of more geometric photoisomers formed by blue irradiation were confirmed. The photoisomer thought to be the most important for the bilirubin clearance, lumirubin, was formed in similar amounts after irradiation with either lamp colour. In the presence of human serum albumin, the bilirubin photoisomers were found to be formed less efficiently from bilirubin bound to cells than from bilirubin in solution

Paper 8: Effects of bilirubin and blue light on the osmotic fragility and the haematoporphyrin-induced delayed photohaemolysis of erythrocytes and spherocytes.

Ellen Bruzell Roll, Terje Christensen and Odrun A. Gederaas
Submitted to Acta Paediatrica

In the last *in vitro* work of this thesis, normal and spherocytic red blood cells were employed as a test system to further study the combined effect of bilirubin and blue phototherapy light in terms of the cells' relative resistance to haemolysis. The blood cells were subjected to tests of both osmotic fragility and haematoporphyrin-induced delayed haemolysis. The osmotic fragility method confirmed that unexposed spherocytes are more fragile than unexposed erythrocytes. However, normal red blood cells exposed to bilirubin and light showed an increased osmotic fragility in contrast to the exposed spherocytes. Delayed haemolysis was not observed in bilirubin-exposed cells in the absence of haematoporphyrin irrespective of pre-irradiation with blue light. The presence of bilirubin increased the degree of delayed photohaemolysis of erythrocytes in a dose dependent manner. However, pre-treatment with blue light had no additional effect. On the other hand, the spherocytes not only showed an increase in the degree of photohaemolysis when exposed to bilirubin, they also succumbed to a significantly larger photohaemolytic effect of bilirubin when the cells were pre-irradiated with blue light.

1.4 General discussion and future work

This thesis contains studies of theoretical and experimental nature. The experimental studies are performed both *in vivo* and *in vitro*. A general discussion is included in this section as more detailed discussions related to each topic are addressed in the following papers.

Preparation for clinical investigations and in vivo studies
The work constituting Parts I and II of this thesis is limited to overhead phototherapy irradiation for treatment of newborn hyperbilirubinaemic patients, although the results may apply to patient groups with liver enzyme defects such as in the Crigler-Najjar syndrome (Arias et al. 1969). Whenever the expression "blue phototherapy light" is used, referral is made to Philips 20W/52 fluorescent light tubes.

Paper 1 of the thesis addresses the need for optimised phototherapy and describes which physical parameters should be studied. This theoretical work offers an update on current phototherapy practices at the time of publication. Follow-up studies of possible cancerous effects are also suggested. The stated need for follow-up studies of skin cell changes is actualised by the finding in paper 3 of increased melanin index after phototherapy. Paper 1 refers to an Australian study (Harrison et al. 1994) linking a high number of naevi (moles) to a risk factor for development of malignant melanoma with UV radiation exposure being a promoter for the later development of the melanomas. If not carefully controlled, phototherapy lamps may contribute to UV exposure (Gies and Roy 1990). Dose limits of UV are given in an international standard (IEC 2000). The increased melanin index (paper 3) may be linked to the tanning processes, immediate pigment darkening and delayed tanning, which may be involved in the development of nevi.

The clinical study presented in paper 4 was assessed with respect to ethical questions and existing ethical and legal guidelines in paper 2. It was concluded that the particular study does neither inflict any unacceptable risk to the patients nor does it raise any serious ethical problems. Furthermore, the study is found to conform to the restrictions of Norwegian legislation and the applicable guidelines. The clinical study was already approved by a national ethical committee at the time of writing. Of the 25 patients enrolled during the recruitment period, the participating hospitals reported of one or two denied consents (hospital staff, personal communication). A problem of uniformity of ethical guidelines occurred during the preparations for the international multi-centre study (Agati et al. 2001), which was incorporated into the protocol presented in paper 4. The problem was solved by letting each participating country act in accordance with their national legislature and guidelines.

The planned clinical study of paper 4 was partly accomplished. The information obtained from the few patients enrolled, however, was not enough to determine whether turquoise fluorescent lamps were superior to blue in the clearance of serum bilirubin. On the other hand, a simultaneously conducted clinical study by our collaborators in the multi-centre trial was able to produce a patient material of acceptable size (Ebbesen et al. 2003). The researchers concluded that the turquoise fluorescent lamp was equally or more efficient in reducing the serum bilirubin concentration. This finding was a confirmation of reports of Donzelli et al. (1995) that a turquoise phototherapy lamp with a similar spectral output shortened the duration of

phototherapy. The irradiance of this lamp was sufficient and comparable to that of blue lamps, unlike previously produced green lamps (Agati et al. 1996). Neither the lamp used in Donzelli's study nor the one used in the multi-centre trial are fabricated commercially. A clinically important advantage of the turquoise lamp is the theoretically less harmful effect to the retina of the patients by a factor of four (Matthes et al. 1999). It may also be speculated that the turquoise light with emission peak of 490 nm could have less photodegrading effect on riboflavin than blue light with an emission peak of 450 nm being almost identical to the absorbance peak of riboflavin (447 nm) as discussed in paper 4. Furthermore, many other endogenous molecules such as porphyrines, vitamins, aromatic amino acids and melanin absorb more strongly in the violet/blue part of the spectrum than at longer wavelengths.

The obtained patient material of paper 4 was inadequate to determine an optimum irradiance level for blue light phototherapy of newborns. According to Tan (1977), there exists a threshold for the irradiance at which there is equilibrium between bilirubin destruction and formation of new bilirubin or transport of ZZ-bilirubin to the skin. He found that no further effect of phototherapy was seen above levels of 1-2 mW/cm^2 (Tan 1982). In paper 3, a point of discussion is the uneven distribution of irradiance emitted to the various body parts of the newborn when receiving phototherapy by a "light bed" and in different distances from an overhead light source. The irradiance received from the "light bed" varied by a factor five from shoulder to toe of the patient. In addition, the irradiance received by a conventional overhead phototherapy device varied by a factor three throughout the front body skin surface (Bruzell Roll, unpublished results).

Radiometric definitions

The term "light intensity" of a lamp is often confused with irradiance. Throughout this thesis the term "irradiance" is used to denote the light effect reaching a unit area and is more precisely defined as "the radiant flux per unit area in a specified surface that is incident on, passing through, or emerging from a point in the specified surface. The defining equation for irradiance, I, is

$I = d\bar{u} / ds,$

where $d\bar{u}$ is an element of radiant flux and ds is an element of area in the surface". The unit of irradiance is W/m^2 (McCluney 1994). The concept of fluence rate is based on the definition of an area being the cross section of a sphere on which the radiation is incident at the point under consideration (Matthes et al. 1999). The term fluence rate is used instead of irradiance in the cell culture studies of paper 5. The term can be used interchangeably with irradiance when none or minute light scattering of the incident light is assumed and the radiation comes from one direction.

All measurements of irradiance were performed with a radiometer (United Detector Technology 371, Hawthorn, CA). To determine the irradiance of the blue fluorescent tubes the photodiode probe 268Blue was used. This probe has a cosine angular response, i.e.,

$I_{measured} = I_{normal} \times \cos \text{ɗ},$

where ɗ is the angle between the surface of the detector and the incident direction of the radiation (within values of ɗ of about 60°). The narrow spectral response of the probe has a peak sensitivity at 450 nm and overlaps the absorbance spectrum of bilirubin. The total light dose of irradiance over time is called radiant exposure, H, and is given by the following relationship:

$H = \int I(t)dt,$

or in the case of constant irradiance which is the case in this thesis:

$H = I \times t.$

In the cell culture studies of paper 5, the term fluence (= fluence rate × t) is used to express the dose.

Although two patient groups were subjected to fractionated and continuous phototherapy in the clinical study outlined in paper 4, other parameters were simultaneously varied and, thus, no conclusions could be drawn. Furthermore, the number of patients enrolled in the study was limited. Discussion of and the basis for fractionated treatment are offered in papers 1, 2 and 4-6. The choice of using light : dark periods of 1 : 1 h in the clinical study was based on findings by Berck et al. (1985) and Jährig et al. (1985) who recommended this cycle as the optimum.

In paper 4 the blue light phototherapy effect on the light-sensitive vitamins riboflavin (vitamin B_2) and retinol (vitamin A) in the serum of jaundiced patients was elucidated. The possible influence of wavelength, irradiance and fractionation regimens were not taken into consideration. The serum riboflavin concentration decreased to half the mean pre-treatment concentration after 7 h of phototherapy. This finding is a confirmation of previously reported results by Sisson et al. (1976) and Gromisch et al. (1977), although they reported on a reduction in the whole blood riboflavin concentration with less decrease and after a longer duration of phototherapy. The concentrations of the coenzyme derivatives of riboflavin, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), although highly sensitive to light, stayed constant throughout 31 h of phototherapy (paper 4). The serum retinol concentration did not decrease during phototherapy. The retinol values of the newborns in the intensive care units were higher than those of the newborns in the well-baby nursery. The finding that the riboflavin coenzymes and retinol concentrations stay relatively constant has not been previously published.

The results presented in paper 3 are, like paper 4, based on a cohort of neonates with hyperbilirubinaemia, of which a part was subjected to phototherapy. The patient material of paper 3 was collected in the fall of 1996. The results of reflectance spectroscopic measurements performed on the neonates were published in a post-graduate thesis (Schmedling 1996) and later in a doctoral thesis (Spott 1999). The results presented in paper 3 are refinements of the algorithms and grouping of the material. New information has been extracted from the original data. The average melanin absorption coefficient, but not the erythema index, was increased for the group subjected to phototherapy compared with the non-treatment group. Tan (1989) describes findings of erythema and skin hardening (tanning) during blue and green light phototherapy. The phototherapy duration of his studies, however, was 300 h

compared to generally 24-48 h in the material presented in paper 3. Leaving out the group receiving phototherapy, the correlation of serum bilirubin to the reflectance spectroscopy based bilirubin index previously obtained by Spott, became weaker. A device for measuring transcutaneous bilirubin based on reflectance spectroscopy technology showed a better correlation (Bhutani et al. 2000). However, neither the size and composition of the newborn cohort nor the serum analysis method can be compared to our study.

In vitro studies

Part III, including papers 5-8, is dedicated to adverse effects of the combination of bilirubin and phototherapy *in vitro*. The three first papers concentrate on cytotoxic effects on the mouse lymphoma cell line, L5178Y-R. This cell line is extensively characterised with respect to various types of radiation, oxidants and other factors that may influence radiation sensitivity. It is found to be relatively sensitive to ultraviolet radiation (Beer et al. 1983, Godar and Lucas 1995, Separovic et al. 1997).

Apoptosis caused by bilirubin in the dark was observed with four different methods in papers 5-7, and confirmation by a fifth method (DNA ladder technique) has been published previously (Roll 1999a). In all studies, early induction of apoptosis was demonstrated. In paper 7, an increase in apoptotic score was seen after 48 h incubation compared to 2 and 18 h. A concentration-dependent early induction of apoptosis and otherwise late apoptosis was observed in studies using bovine endothelial cells (no light exposure) (Tibbs et al. 2000). Studies of rat neural cells using half or less the bilirubin concentration showed early apoptosis (4 h) with a further increase after 16 h (Silva et al. 2001). In the first cell toxicity study, paper 5, similar fractions of the cells succumbed to necrosis and apoptosis in the dark, whereas in papers 6 and 7 necrosis was more pronounced. Compared with the later work, the apoptotic fractions of the first paper were higher as a result of using the less nutritious phosphate buffered saline (PBS) as exposure medium instead of cell medium, as used in the later studies. Paper 6 showed an increase in the number of apoptotic cells after longer dark exposures as measured by two separate methods, whereas the number of necrotic cells was more unaffected by the longer exposure time.

When combining bilirubin and phototherapy light, early apoptosis was demonstrated. A much higher increase in apoptosis was seen 48 h after combined bilirubin and light exposure than after exposure to bilirubin in the dark. A higher fraction of cells were dying from necrosis in the studies of papers 6 and 7 than in paper 5, probably due to improved exposure conditions. Both necrosis (papers 6 and 7) and apoptosis (paper 7) increased with increasing radiant exposure.

Cell toxicity effects observed after the exposure of the combination of bilirubin and phototherapy fractionation were investigated in papers 5 and 6. Although PBS was used as exposure medium in paper 5, similar conclusions were reached in the two papers: there was no difference in apoptotic score between continuous and intermittent irradiation at constant and clinically relevant radiant exposure. A previous *in vitro* study investigating the formation of photoisomers after fractionated and continuous irradiation of cell-free bilirubin solutions concluded that similar amounts were obtained after either irradiation regimen (Roll and Christensen 1999b). The tendency of a higher apoptotic score after bilirubin-incubated fractionated irradiation seen in paper 5 was not confirmed in the later study. On the other hand, the later work

showed that necrosis was more pronounced after bilirubin-incubated fractionated irradiation of lower irradiance and longer duration (same radiant exposure). Still keeping the radiant exposure constant, apoptosis increased when the bilirubin-treated cells received light at lower irradiances lasting longer compared with higher irradiance for shorter times irrespective, of fractionation. If clinically applicable, this observation implies that the phototherapy should be administered with sufficient irradiance to cause a relatively rapid effect instead of lowering the irradiance resulting in longer phototherapy duration.

In paper 7, the non-commercial lamp emitting turquoise light described in the clinical work of paper 4 was compared to blue phototherapy light in identifying possible cytotoxic effects at constant and equal irradiance. The turquoise light caused less cellular damage than did the blue light both in terms of cell toxicity and cell growth. These findings support earlier observations by Sideris et al. (1981), Rosenstein et al. (1983) and Christensen et al. (1988) among others who reported of *in vitro* side effects of blue light. A clinically inclined reason for choosing a longer wavelength lamp is the increase in the formation of the therapeutically efficient photoisomer lumirubin (Onishi et al. 1986) with increasing wavelength. The study of paper 7 concludes that the formation of lumirubin is similar after exposure to either blue or turquoise light.

Papers 5 and 7 confirm previous findings by our group (Christensen 1986, Christensen et al. 1988, Christensen and Kinn 1993) that phototoxicity is more pronounced after light doses that cause photooxidation of bilirubin. The earlier findings by Christensen (1986, 1988) and Rosenstein et al. (1983), together with the results presented in papers 5 and 7, indicate that cell death is preceded by hydrogen peroxide production in the cells and in the medium. Studies performed by McDonagh (1971) indicated formation of singlet oxygen, although of low quantum yield (Sloper and Truscott 1982). In a previous set of experiments we found that the amount of singlet oxygen was below the detection limit of an infrared spectroscopic method (Roll et al. 2001).

Phototoxicity of bilirubin may also be aggravated by sensitisation of endogenous compounds, such as riboflavin, as discussed in paper 4, and porphyrins, which were studied in relation to erythrocytes and spherocytes in paper 8. This paper confirms previous *in vitro* results of increased fragility of erythrocytes exposed to bilirubin and light (Odell et al. 1972), but this effect has not been confirmed *in vivo* (Alpert et al. 1984). When sensitised by porphyrin, only spherocytes show an increased fragility with increasing light dose, indicating that phototherapy may increase haemolysis in jaundiced newborns with hereditary spherocytosis.

Future work

To further approach the optimisation of phototherapy of hyperbilirubinaemic neonates, more clinical studies should be undertaken with focus on the physical parameters investigated in this thesis. The protocol for such investigations has been worked out for the future (paper 4). Clinical results from the use of a new lamp colour have been published, and more results are under way. Follow-up examinations on the development of nevi after phototherapy treatment should also be carried out. *In vitro* phototherapy studies should focus on the formation of reactive oxygen species and the mechanisms leading to cell damage and death.

1.5 References

1. Agati G, Fusi F, Pratesi R, Pratesi S, Donzelli GP. The colorful story of phototherapy for neonatal jaundice. SPIE 1996;2924:2-19.
2. Agati G, Pratesi R, Fusi F et al. Testing a fluorescent lamp for neonatal jaundice phototherapy [Abstract]. In: Book of Abstracts of the 9th Congress of the European Society for Photobiology. Lillehammer: European Society for Photobiology 2001:238, pg.111.
3. Alonso M, Finn EJ. Fundamental university physics. Volume II Fields and waves. Reading, Massachusetts: Addison-Wesley Publishing Company, 1967;764-6.
4. Alpert G, Shohat M, Reisner SH, Danon YL. Osmotic fragility of erythrocytes in newborn infants treated by phototherapy. Acta Paediatr Scand 1984;73:254-7.
5. Amundsen I. Light treatment of infants with hyperbilirubinemia; optimalization and dosimetry. (In Norwegian with English summary) Østerås: Statens institutt for strålehygiene/National Institute of Radiation Hygiene, 1991; SIS report 1991:7.
6. Arias IM, Gartner LM, Cohen M, Ezzer JB, Levi AJ. Chronic nonhemolytic unconjugated hyperbilirubinemia with glucuronyl transferase deficiency. Clinical, biochemical, pharmacologic and genetic evidence for heterogeneity. Am J Med 1969;47(3):395-409.
7. Beer JZ, Budzicka E, Niepokojczycka E et al. Loss of tumorigenicity with simultaneous changes in radiosensitivity and photosensitivity during in vitro growth of L5178Y murine lymphoma cells. Cancer Res 1983;43:4736-42.
8. Bell EF, Neidich GA, Cashore WJ, Oh W. Combined effect of radiant warmer and phototherapy on insensible water loss in low birth weight infants. J Pediatr 1979;94:810-3.
9. Berck A, Jährig D, Meisel P, Jährig K. Die Kalkulation des Phototherapieeffektes unter Berücksichtigung verschiedener Bestrahlungsvarianten. II. Prognostische Beurteilung des Phototherapieeffektes bei intermittierender und kontinuierlicher Phototherapie. Kinderärztl Prax 1985;53:229-34.
10. Bhutani VK, Gourley GR, Adler S, Kreamer B, Dalin C, Johnson LH. Noninvasive measurement of total serum bilirubin in a multiracial predischarge newborn population to assess the risk of severe hyperbilirubinemia. Pediatrics 2000;106(2):e17. http://www.pediatrics.org/cgi/reprint/106/2/e17.pdf Accessed April 2003.
11. Bonnett R. Chemical aspects of photodynamic therapy. Amsterdam: Gordon and Breach Science Publishers, 2000;103-13. (Phillips D, O'Brien P, Roberts S, eds. Advanced chemistry texts; vol 1).
12. Bratlid D. How bilirubin gets into the brain. In: Maisels MJ, ed. Neonatal jaundice. Clin Perinatol 1990;17:449-65.
13. Cashore W. Bilirubin metabolism and toxicity in the newborn. In: Polin RA, Fox WW, eds. Fetal and neonatal physiology. Philadelphia: WB Saunders Company, 1998 (1): 1493-8.
14. Christensen T. Cytotoxicity of bilirubin photoproducts. Photobiochem Photobiophys 1986:10;253-60.
15. Christensen T. Photosensitization of human cells with bilirubin. Photochem Photobiol 1984;39:39.
16. Christensen T, Amundsen I, Kinn G, Kjeldstad B. Phototherapy of newborns with hyperbilirubinemia in Norwegian hospitals. A study of phototherapy units and light conditions. (In Norwegian with English summary). Østerås: Statens institutt

for strålehygiene/National Institute of Radiation Hygiene, 1992; SIS report 1992:2.
17. Christensen T, Kinn G. Bilirubin bound to cells does not form photoisomers. Acta Paediatr 1993;82:22-5.
18. Christensen T, Reitan JB. Phototherapy of hyperbilirubinemia in newborn children. (In Norwegian with English summary) Østerås: Statens institutt for strålehygiene/National Institute of Radiation Hygiene, 1987; SIS report 1987:6.
19. Christensen T, Støttum A, Brunborg G, Reitan JB. Unwanted side effects and optimization of phototherapy. In: Douglas RH, Moan J, Dall'Acqua F, eds. Light in biology and medicine. 1st ed. New York: Plenum Press, 1988;153-9.
20. Cremer RJ, Perryman PW, Richards DH. Influence of light on the hyperbilirubinemia of infants. Lancet 1958;1:1094-7.
21. Donzelli GP, Pratesi S, Rapisardi G, Agati G, Fusi F, Pratesi R. 1-day phototherapy of neonatal jaundice with blue-green lamp. Lancet 1995;346:184-5.
22. Ebbesen F, Agati G, Pratesi R. Phototherapy with turquoise versus blue light. Arch Dis Child Fetal Neonatal Ed 2003;88:F430-1.
23. Eggert P, Stick C, Swalve S. On the efficacy of various irradiation regimens in phototherapy of neonatal hyperbilirubinemia. Eur J Pediatr 1988;147:525-8.
24. Ennever, JF. Phototherapy for neonatal jaundice. In: Polin RA, Fox WW, eds. Fetal and neonatal physiology. Philadelphia: WB Saunders Company, 1998(1);1505-13.
25. Ennever JF, Costarino AT, Polin RA, Speck WT. Rapid clearance of a structural isomer of bilirubin during phototherapy. J Clin Invest 1987;79:1674-8.
26. Fischer H, Herle K. Einwirkung von Licht auf Porphyrine. Überfürung von Ätioporphyrin I in bilirubinoid Farbstoffe. Hoppe Seylers Z Physiol Chem 1938;251:85-96.
27. Galbraith AA, Egerter SA, Marchi KS, et al. Newborn early discharge revisited: are California newborns receiving postnatal services? Pediatrics 2003;111:364-71.
28. Gies HP, Roy CR. Bilirubin phototherapy and potential UVR hazards. Health Phys 1990:58;313-20.
29. Godar DE, Lucas AD. Spectral dependence of UV-induced immediate and delayed apoptosis: the role of membrane and DNA damage. Photochem Photobiol 1995;62: 108-13.
30. Greenberg JW, Malhotra V, Ennever JF. Wavelength dependence of the quantum yield for the structural isomerization of bilirubin. Photochem Photobiol 1987;46:453-6.
31. Gromisch DS, Lopez R, Cole HS, Cooperman JM. Light (phototherapy)-induced riboflavin deficiency in the neonate. J Pediatr 1977;90:118-22.
32. Hansen TWR. Therapeutic approaches to neonatal jaundice: An international survey. Clin Pediatr 1996;35:309-16.
33. Harris MC, Bernbaum JC, Polin JR, Zimmerman R, Polin RA. Developmental follow-up of breastfed and near-term infants with marked hyperbilirubinemia. Pediatrics 2001;107:1075-80.
34. Harrison SL, MacLennan R, Speare R, Wronski I. Sun exposure and melanocytic naevi in young children in Townsville, Queensland. Lancet 1994;344:1529-32.
35. IPEM. http://www.ipem.org.uk/meetings/14oct02abs.pdf Accessed January 2003.
36. IEC. Medical electrical equipment. Part 2-50: Particular requirements for the safety of infant phototherapy equipment. 1st ed. Geneva: International Electrotechnical Commission, 2000; International Standard IEC 60601-2-50:2000(E).

37. Itoh S, Onishi K, Isobe M, Manabe M, Yamakawa Y. Wavelength dependence of the geometric and structural photoisomerization of bilirubin bound to human serum albumin. Biol Neonate 1987;51:10-7.
38. Jährig D, Berck A, Meisel P, Jährig K. Die Kalkulation des Photoeffektes unter Berücksichtigung verschiedener Bestrahlungsvarianten. I. Der Einfluß des Therapiemodus auf den Photoeffekt. Kinderärztl Prax 1985;53:171-5.
39. Jährig K, Jährig D, Meisel P, eds.(a) Clinical issues. In: Phototherapy. Treating neonatal jaundice with visible light. Munich: Quintessenz-Verlags-GmbH, 1993:108.
40. Jährig K, Jährig D, Meisel P, eds. (b) Pathochemical and physiological bases for phototherapy. In: Phototherapy. Treating neonatal jaundice with visible light. Munich: Quintessenz-Verlags-GmbH, 1993:17-20.
41. John E. Complications of phototherapy in neonatal hyperbilirubinemia. Aust Pediatr J 1975;11:53-5.
42. Karp WB. Biochemical alterations in neonatal hyperbilirubinemia and bilirubin encephalophaty. A review. Pediatrics 1979;64:361.
43. Kjartanson S, Hammarlund K, Riesenfeld T, Sedin G. (a) Respiratory water loss and oxygen consumption in newborn infants during phototherapy. Acta Paediatr 1992;81:769-73.
44. Kjartanson S, Hammarlund K, Sedin G. (b) Insensible water loss from the skin during phototherapy in term and preterm infants. Acta Paediatr 1992;81:764-8.
45. Knox I, Ennever JF, Speck WT. Urinary excretion of an isomer of bilirubin during phototherapy. Pediatr Res 1985;19:198-201.
46. Kopelman AE, Brown RS, Odell GB. The “bronze” baby syndrome: a complication of phototherapy. J Pediatr 1972;81:446-50.
47. Maisels MJ. Clinical rounds in the well-baby nursery: treating jaundiced newborns. Pediatr Ann 1995;24(10):547-52.
48. Maisels MJ. Neonatal jaundice. Semin Liver Dis 1988;8:148-62.
49. Malhotra V, Greenberg JW, Dunn LL, Ennever JF. Fatty acid enhancement of the quantum yield for the formation of lumirubin from bilirubin bound to human albumin. Pediatr Res 1987;21:530-3.
50. Matthes R, Bernhardt J, McKinlay A, eds. Guidelines on limiting exposure to non-ionizing radiation. Munich: International Commission on Non-Ionising Radiation, 1999; ICNIRP report 7/99.
51. McCluney R. Fundamental concepts of radiometry. In: Introduction to radiometry and photometry. Boston: Artech House, 1994:1-19.
52. McDonagh AF. The role of singlet oxygen in bilirubin photooxidation. Biochem Biophys Res Commun 1971;44:1306-11.
53. McDonagh AF, Agati G, Fusi F, Pratesi R. Quantum yields for laser photocyclization of bilirubin in the presence of human serum albumin. Dependence of quantum yield on excitation wavelength. Photochem Photobiol 1989;50:305-9.
54. McDonagh AF, Lightner DA: “Like a shriveled blood orange” – bilirubin, jaundice and phototherapy. Pediatrics 1985;75:443-55.
55. McDonagh AF, Lightner DA and Wooldridge TA. Geometric isomerisation of bilirubin IXŭand its dimethyl ester. JCS Chem Comm 1979;1029:110-2.
56. Medical Birth Registry of Norway. http://www.uib.no/mfr/statistikk.html Accessed April 2003.

57. Mostnikova GR, Mostnikov VA, Plavskii VYu, Tret'yakova AI, Andreev SP, Ryabtsev AB. Phototherapeutic apparatus based on an argon laser for the treatment of hyperbilirubinemia in newborns. J Opt Technol 2000;67(11):981-3.
58. Odell GB, Brown RS, Kopelman AE. The photodynamic action of bilirubin on erythrocytes. Pediatrics 1972;81:473-83.
59. Oh W, Karecki H. Phototherapy and insensible water loss in the newborn infant. Am J Dis Child 1972;124:230-2.
60. Onishi S, Isobe K, Itoh S, et al. Demonstration of a geometric isomer of bilirubin IXŬ in the serum of a hyperbilirubinaemic infant and the mechanism of jaundice phototherapy. Biochem J 1980;190:533-6.
61. Onishi S, Itoh S and Isobe K. Wavelength dependence of the relative rate constants for the main geometric and structural photoisomerization of bilirubin IXŬbound to human serum albumin. Biochem J 1986;236:23-9.
62. Roll EB, Christensen T. Ethical aspects of clinical research involving children, exemplified by a research project on phototherapy of jaundice. (In Norwegian with English summary) Barn 1997;2:29-45.
63. Roll EB, Christensen T. (a) Optimization of phototherapy of jaundiced newborns. [Abstract]. Photochem Photobiol 1999;69(suppl):56S.
64. Roll EB, Christensen T. (b) In vitro effects of intermittent vs. continuous light irradiation of bilirubin. [Abstract]. In: Book of Abstracts of the 8th Congress of the European Society for Photobiology. Granada: European Society for Photobiology 1999:P99, pg. 143.
65. Roll EB, Christensen T, Melø TB. Apoptosis in bilirubin- and light treated cells and formation of reactive oxygen species. [Abstract]. In: Book of Abstracts of the 9th Congress of the European Society for Photobiology. Lillehammer: European Society for Photobiology 2001:201, pg. 95.
66. Rosenstein BS, Ducore JM, Cummings SW. The mechanism of bilirubin-photosensitized DNA strand breakage in human cells exposed to phototherapy light. Mutat Res 1983;112:397-406.
67. Rubaltelli FF. Current drug treatment options in neonatal hyperbilirubinaemia and the prevention of kernicterus. Drugs 1998;56(1):23-30.
68. Rubaltelli FF, Piovesan A, Semenzato G, et al. Immune competence in hyperbilirubinemic newborns before and after phototherapy. Helv Paediatr Acta 1977;32:129-33.
69. Saeki K. Jpn J Gastroenterol, Nippon Shokakibyo Gakkai Zasshi 1932;4:153.
70. Schmedling PF. Bilirubinbestemmelse hos nyfødte ved reflektansspektroskopi. Trondheim: Norwegian University of Science and Technology, 1996. Post-graduate thesis.
71. Seidman DS, Moise J, Ergaz Z, et al. A new blue light-emitting phototherapy device: a prospective randomized controlled study. J Pediatr 2000;136:771-4.
72. Separovic D, He J, Oleinick NL. Ceramide generation in response to photodynamic treatment of L5178Y mouse lymphoma cells. Cancer Res 1997;57:1717-21.
73. Sideris EG, Papageorgiou GC, Charalampous SC, Vitsa EM. A spectrum response study on single strand DNA breaks, sister chromatide exchanges, and lethality induced by phototherapy lights. Pediatr Res 1981;15:1019-23.
74. Silva RF, Rodrigues CM, Brites D. Bilirubin-induced apoptosis in cultured rat neural cells is aggravated by chenodeoxycholic acid but prevented by ursodeoxycholic acid. J Hepatol 2001;34:467-70.

75. Sirota L, Straussberg R, Gurary N, Aloni D, Bessler H. Phototherapy for neonatal hyperbilirubinemia affects cytokine production by peripheral blood mononuclear cells. Eur J Pediatr 1999;158:910-3.
76. Sisson TRC, Slaven B, Hamiltion PB. Effect of broad and narrow spectrum fluorescent light on blood constituents. In: Bergsma D, Blondheim SH, eds. Bilirubin metabolism in the newborn (II). New York: American Elsevier for the National Foundation-March of Dimes, 1976; BD:OAS 12(1) 122-31.
77. Sloper RB, Truscott TG. The quantum yield for bilirubin photoisomerisation. Photochem Photobiol 1982;35:743-5.
78. Spott T. Characterization of layered tissue structures with diffusely propagating photon-density waves. Trondheim: Norwegian University of Science and Technology, 1999. Fys. El.-rapport 1999:50. Doctoral thesis.
79. Tan KL. Efficacy of fluorescent daylight, blue and green lamps in the management of nonhemolytic hyperbilirubinemia. J Pediatr 1989;114:132-7.
80. Tan KL. The nature of the dose-response relationship of phototherapy for neonatal hyperbilirubinemia. J Pediatr 1977;90:448-52.
81. Tan KL. The pattern of bilirubin response to phototherapy for neonatal hyperbilirubinemia. Pediatr Res 1982;16:670-4.
82. Tibbs R, Tang J, Zhang J. Bilirubin produces apoptosis in cultured bovine endothelial cells. [Abstract]. 6th Internet World Congress for Biomedical Sciences 2000;24. http://www.uclm.es/inabis2000/symposia/files/024/index.htm Accessed April 2003.
83. Tsai F, Tsai C, Wang T. Sister chromatid exchange in Chinese newborn infants treated with phototherapy for more than five days. Acta Paed Sin 1998;39(5):327-9.
84. Vecchi C, Donzeli GP, Sbrana G, Pratesi R. Phototherapy for neonatal jaundice: clinical equivalence of fluorescent green and “special” blue lamps. J Pediatr 1986;108:452-6.
85. Vreman HJ, Wong RJ, Stevenson DK, et al. Light-emitting diodes: a novel light source for phototherapy. Pediatr Res 1998;44:804-9.
86. Wu FY, Iijima K, Nishida A, Higurashi M. Sister chromatid exchanges in the peripherial lymphocytes of newborns with Down syndrome after in vitro exposure to blue or green light. Mutat Res 1996;367:261-4.
87. Wu F, Iijima K, Takiguchi D, Nishida A, Higurashi M. Effect of phototherapy on sister-chromatid exchange in infants with Down syndrome. Mutation Res 1992;283:65-7.
88. Wu PYK, Hodgman JE. Insensible water loss in preterm infants: changes with post-natal development and non-ionizing radiant energy. Pediatrics 1974;54:704-12.

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Phototherapy of hyperbilirubinaemia in newborns; the need for a better treatment and follow-up

Ellen Bruzell Roll[1,2] and Terje Christensen[1]

[1]Norwegian Radiation Protection Authority,
POB 55, NO-1332 ØSTERÅS, Norway

[2]Department of Physics, Norwegian University of Science and Technology, NO-7491
TRONDHEIM, Norway

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SUMMARY

Treating jaundiced newborns with artificial light has been done for the last three decades without substantial improvements of the method. It can be estimated that between five and 10 % of all newborns receive phototherapy. The standard treatment is the use of artificial, visible light sources for many hours up to several days. The wavelength and intensity of the light are factors influencing the effect of the treatment, however, the risk for long-term side effects should be considered. The results of the treatment need more thorough investigation for two purposes; to evaluate the therapeutic efficiency and to look for potential side effects. Furthermore, recognition of side effects may take more time than three decades. Follow-up studies have raised the question whether these study-periods are sufficiently long and whether a clear distinction between the effects of the light itself and the neurotoxic effects of bilirubin have been made. The aim of our experimental and clinical investigations is to optimise the phototherapy, particularly with respect to light intensity and wavelength. Our epidemiological work will address long- term side effects of phototherapy, particularly in relation to cancer development (lymphocytic leukaemia and malignant melanoma).

1.1 INTRODUCTION

The beneficial effect of light on the condition of hyperbilirubinaemia was discovered by an observant nurse in 1956. The observation was done when infants were exposed to normal daylight. Very soon artificial light sources were applied for treatment, and the use of phototherapy spread rapidly after the discovery of Cremer, Perryman and Richards (1). Today it is estimated that 5 - 10 % of all newborns in many countries are given phototherapy for longer or shorter times during the first days of their lives (2, 3). Thus, the total number of persons that have been treated to this day is probably several millions, but there have been no systematic registrations of the total number in any countries. Furthermore, the number of patients undergoing treatment varies with time and with the use of different indications for treatment in various hospitals. A discussion of these aspects can be found elsewhere in this volume (2).

Since the discovery of phototherapy only few improvements in the methodology have been made. Still, the standard treatment is the use of artificial light sources placed at a certain distance from the infant. More modern approaches have been introduced, however, and will be mentioned later in this paper. We do not intend to describe the treatment in detail since general descriptions may be found in several books and journals (e.g. 3-6).

Bilirubin is a tetrapyrrole which is a break-down product of several haemoproteins, although quantitatively the breakdown of haemoglobin plays the dominant role. More than half of all newborns show signs of transient jaundice occurring a few days after birth. There are several reasons for the typical unconjugated hyperbilirubinaemia in newborns. Prenatally the bilirubin is excreted by the mother, and it is believed that the unconjugated bilirubin, being an unpolar substance, can easily cross the placenta. Postnatally the infant must take up the adult mechanism where glucuronic acid residues are coupled to bilirubin to form water soluble bilirubin diglucoronide. The liver enzyme glucoronyltransferase is induced at birth, but its activity is frequently insufficient to conjugate the heavy load of bilirubin occurring due to the rapid turnover of haeme early in life. Several pathological conditions may aggravate the

problem. When unconjugated bilirubin is present in plasma it is normally transported bound to albumin and lipoproteins and the tissue concentration is relatively low. At higher concentrations or if the binding capacity is low the concentration of free bilirubin may increase and the bilirubin may be taken up by other organs, probably mainly as free bilirubin in the form of a mono-anion (7). This process may lead to cytotoxicity, notably in the brain (4, 8). In serious cases the bilirubin will stain certain parts of the brain and the binding is manifested as so-called kernicterus.

Before the introduction of phototherapy the only treatment of severe hyperbilirubinaemia was exchange transfusion, however, this procedure is performed only rarely today. Normally, white or blue light is delivered to the skin of the infant with a relatively high irradiance, ranging from below 0.5 mW/cm^2 to more than 2 mW/cm^2, but the light intensity is seldom measured (9). The light penetrates through the epidermis and is absorbed in the skin or vasculature by bilirubin and other substances. The spectrum of the light is changed on its way through the tissue, and therefore, the more penetrating, longer wavelengths will play a more important role for effects on bilirubin deeper in the tissue (10). The total dose of light is dependent on the duration of treatment and is often expressed as hours of phototherapy, although the physically correct expression should be J/cm^2 or an equivalent unit for light energy. Therefore, it is not straightforward to evaluate the irradiation conditions and their potential therapeutic or adverse effects.

The absorption of light in bilirubin leads to several photochemical reactions in the molecule (see (11) for a review). Probably, the reaction which is mainly responsible for the therapeutic effect is the formation of structural photoisomers being more water soluble than bilirubin itself. Photooxidation of bilirubin, on the other hand, has been suggested as a mechanism that may lead to unwanted side effects (12-14).

The aim of this article is to suggest areas where the therapeutic effect of light may be improved in the future, particularly with respect to light irradiation regimens. The results of the treatment should be followed closely, in order to score both the results of reduced hyperbilirubinaemia and potential side effects. Although a number of follow-up studies have been performed, phototherapy was introduced relatively recently and there may still be effects that have not yet been recognised.

1.2 EVALUATION OF RISK FACTORS AND PREVIOUS FOLLOW-UP STUDIES

The risk of brain toxicity is the reason for treating hyperbilirubinaemia (8, 15). Several follow-up studies have reported on the association between high bilirubin concentrations and different neurological symptoms (see (4, 5, 16) for reviews), but few have been able to demonstrate a clear relationship between phototherapy and a reduced risk of brain toxicity. A different question is whether light treatment may be a risk factor. If so, one should probably look for different types of effects than the classical bilirubin-induced brain damage.

Light may interact directly with molecules in the tissue. The absorption is generally very dependent on the absorbing molecules. Ultraviolet (UV) radiation has been regarded as the biologically most active type of optical radiation because it is absorbed in the DNA and may form photochemical changes in many other biomolecules. The amount of UV radiation in phototherapy lamps is probably very small in most types of equipment (17). The biological

effect of longer wavelength light, present in higher amounts in phototherapy lamps has been studied. Several molecules may absorb in the relevant wavelengths (Table 1) and particularly porphyrins, vitamins, amino acids and bilirubin have been discussed as important light absorbers. Ben-Sasson and Davis (18) hypothesised that an observed increased incidence of acute lymphocytic leukaemia could be partly due to the use of phototherapy in hospitals. Findings of van Steensel-Moll and co-workers in the Dutch Childhood Leukaemia Study Group (19) supported this hypothesis. Photoactivation of endogenous protoporphyrin was suggested as the carcinogenic mechanism. However, the finding that bilirubin is a photosensitiser capable of inducing DNA damage and mutations in vitro (12-14, 20) indicates that bilirubin may play a role as well because of its presence in much higher concentrations in newborns and its higher blue light absorption than protoporphyrin. In our work with in vitro photoeffects of bilirubin it has been shown that bilirubin can produce photoproducts with relatively long lifetimes. Therefore, these products may diffuse from the site of formation to other organs. Rosenstein and colleagues (12) showed that H_2O_2 (hydrogen peroxide) was formed upon irradiation of bilirubin together with other unidentified photoproducts. These products may be organic peroxides (14) or results of light induced cross-linking between bilirubin and other biomolecules (21). It is interesting to notice that photochemical formation of H_2O_2 also is known to take place in parenteral solutions containing riboflavin and amino acids when exposed to phototherapy lamps (22).

It has been debated whether phototherapy can lead to long lasting clinical side effects. In a study of the frequency of sister chromatid exchanges it was concluded that in genetically normal infants the therapy did not increase the frequency. Nevertheless, a significant increase in the frequency was found in infants with Down's syndrome (23). Among other important topics are eye damage (24-26), depletion of essential nutrients (vitamins and amino acids) and a number of short-term clinical side effects (27). Psycho-social effects on the relationship between parents and the infant due to separation during long-lasting phototherapy have been indicated as a side effect that should be taken into consideration (28).

1.3 AREAS OF IMPROVEMENTS

Despite the high number of newborns receiving phototherapy and the 30 years of experience with this form of treatment there is still need for improvements and optimisation.

The manner in which the phototherapy is «administered» to the hyperbilirubinaemic newborns vary internationally as well as nationally. Criteria for treatment are set up and followed within hospitals, but vary between them, and are currently heavily debated. (See (2)). The thorough work of some researchers has led to a better understanding of the effect of phototherapy (5), but there is still no standardisation with respect to irradiance, wavelength, fractionation and type of equipment. Thus, there is no consistency in the treatment given to certain groups of jaundiced newborns.

1.3.1 Irradiance

Irradiance is defined as the power of light reaching a unit area, often expressed in mW/cm^2. Multiplied by the *duration* of irradiation the *radiant exposure* (J/cm^2) is obtained, of which the photoeffect is a function. There is assumed to exist an optimum irradiance energy in phototherapy of jaundiced newborns where a further increase above this value induces photoconversion of bilirubin less efficiently (29, 30). Likewise, there exists a threshold dose for

the irradiance at which there is equilibrium between bilirubin destruction and formation of new bilirubin (30). In Tan's work there was no further effect of phototherapy above levels of 1-2 mW/cm^2 (31). In spite of this knowledge we have found levels of irradiance below and above this irradiance range in Norwegian hospitals (9).

A common error in reporting irradiance values in the literature is the lack of specifying the band width of irradiance. The value is often given in $\mu W/cm^2 \cdot nm$ leaving it to the reader's guesswork to find the total irradiance. Attempts are made to give irradiance values in mW/cm^2 from radiometer measurements, but often leaving out information on the spectral sensitivity of the probe used. This inconsistency makes it complicated to compare various types of equipment and more importantly, to determine the total light dose (radiant exposure) received by the patient. The total duration of the phototherapy is not recorded at most hospitals, also contributing to the unknown light dose uncertainty.

1.3.2 Wavelength

The wavelength of the light source is another variable factor in the phototherapy treatment of hyperbilirubinaemia. White and blue light is most commonly used, the blue light being thought to match the peak of the bilirubin absorption spectrum more closely. Unfortunately, we have also indications that daylight-simulating fluorescent tubes, emitting wavelengths in the UV region, have been used in phototherapy.

The use of blue versus green or blue-green lamps is still a subject of controversy (32-37). There are several factors favouring the longer wavelength lamps, taking into account not only the absorption of bilirubin, but also the formation of the more water soluble photoproduct, lumirubin, which has an increase in quantum yield in the green wavelength region (38, 39). Also, there is a shift in the bilirubin absorption in this part of the spectrum in vivo due to skin constituents and the binding of fatty acids to albumin (40, 41). A third factor favouring green light is that due to lower absorption coefficients of the main skin pigments in this part of the spectrum green wavelengths reach deeper into the skin tissue than do the blue wavelengths (Table 1). In vitro studies of cells in the presence of bilirubin and light show the largest cellular effects (cell death, mutations and DNA damage) at 450 nm (blue) (42). Also worth mentioning in this respect is the research done by Setlow's group on the melanoma induction of the fish Xiphophorus. The group found that UVA radiation induced melanomas most efficiently and furthermore, that visible light as well was capable of induction of melanomas in that particular fish model (43).

It is important to note that in comparing lamps the difference in spectral irradiance for all wavelengths must be considered. Some clinicians have found that the duration of phototherapy was significantly shorter using blue lamps (observing, however, less phototherapy rebound for the green light treatment) (44), but this fact may be attributed to the lower irradiance of the green lamp, not to the wavelength itself. Pratesi's group in Italy has introduced a new blue-green phototherapy lamp which proves to be more efficient compared to a special-blue lamp (45). Further testing, also in vitro, of this blue-green lamp, is now being carried out by our group in Norway.

1.3.3 Fractionation

It is known that the effectiveness of irradiation depends exponentially on the initial bilirubin concentration (5). During an interruption of phototherapy a rebound of bilirubin into the skin occurs, improving the effectiveness of continued treatment. From an energy dose viewpoint

fractionation of the treatment will be advantageous to a continuous treatment. Even though the intermittent phototherapy increases the time before a certain lowering of bilirubin is achieved the total light dose to the patient would be less (5). Jährig et al. recommend an optimal form of treatment as light : dark periods of 1 : 1 hours (accompanied by regular position changes of the infant) (5).

There are other factors favouring intermittent phototherapy aside from the lower energy dose to the newborn. The dark periods both give the parents better opportunity to closeness with their baby and it makes nursing easier. It is our opinion that the fractionation regimen recommended by Jährig et al. is not widely followed in Norwegian hospitals. The infants receiving phototherapy are taken out of the light for nursing and diapering «now and then» without control of the time spent outside the phototherapy unit.

1.3.4 New equipment

During the past seven years three new phototherapy equipments have arrived on the market. Two of these apply a similar principle; the fiberoptic blanket, which can be wrapped around the jaundiced infant or the infant can be laid directly onto it. Compared to conventional phototherapy, the use of the blanket device improves the contact between baby and parents. The «Bili-bed», on the other hand, consisting of a single fluorescent lamp, illuminates the infant through a transparent mattress. Measurements of this latter unit earlier this year led us to conclude that the «Bili-bed» gives relatively high irradiance values (3.1-3.5 mW/cm^2) locally on the mattress, but the irradiance varied from less than 0.5 to 3.5 mW/cm^2 throughout the bed area (Roll and Christensen, unpublished results). A «Biliblanket» had a relatively homogenous irradiance between 1.4 and 2.1 mW/cm^2, although the measurement involved several technical difficulties (UDT-detector 371, probe «268 BLUE») (9).

It is our impression that these types of equipment are not being used in Norwegian hospitals for treatment of the higher bilirubin values due to the paediatricians' experience that the light-bed and -blanket are less efficient than conventional phototherapy.

High intensity double-surface phototherapy on a fluid bed has been reported (46, 47) concluding that this form of treatment is more effective in reducing bilirubin than conventional phototherapy. The authors recommend this high intensity therapy during critical periods of hyperbilirubinaemia demanding rapid control of the condition.

1.3.5 Medication

There are two major types of medication in use for the control of hyperbilirubinaemia, both of which may be combined with phototherapy. Medication is given shortly after birth. The first medication induces the synthesis of bilirubin conjugating enzyme (uridine-diphosphate glucoronyl transferase) and of the ligandin (the hepatic transport protein). Substances used for this purpose are mainly the barbiturates phenobarbitone and methylphenobarbitone, but also nikethamide (48, 49). The second type of medication is based on pharmacological inhibition of haeme oxygenase, thereby limiting the formation of new bilirubin. The drug used is metalloporphyrin (50, 51), some of which may have a photosensitising effect (Christensen and Kinn, unpublished results)*.

In addition, several inert absorbents have been tried with the capacity to bind bilirubin in the intestinal lumen. Agar, charcoal and cholestyramine have all been administered with varying

success, although the combination with phototherapy improves the therapeutic outcome (52, 53).

The need for optimisation of the physical parameters of phototherapy is perhaps even more urgent as the trend towards a later treatment onset is advocated in several hospitals. A jaundiced infant who is allowed high bilirubin values will be at increased risk if submitted to a treatment regimen of imprecise character due to lack of control of irradiance and duration and a suboptimum wavelength. George (54) concluded in his article that perhaps more attention should be drawn to intensity instead of colour. We would like to add that more attention should be drawn to physics in general.

**Christensen T, Kinn, G, Reitan J B. Photosensitizing effects of metalloporphyrins in connection with hyperbilirubinemia. First Internet Conference on Photochemistry and Photobiology, Internet Journal of Science, Biological Chemistry, 1997(3). http://www.photobiology.com/v1/contrib.htm*

1.4 WHAT TO LOOK FOR IN EPIDEMIOLOGICAL STUDIES

In a follow-up study three years after phototherapy Valkeakari and coworkers (55) found no differences between the phototherapy group and the control group when a number of clinical and biochemical parameters were compared. The same trend can be found in a number of other follow-up studies. These findings seem reassuring, but it is pertinent to inquire into whether the previous studies focused on the relevant parameters of phototherapy or whether the follow-up periods have been sufficiently long to reveal possible side effects of the treatment. The first law of photobiology states that only absorbed light can have a biological effect. Therefore, a first approach will be to look for effects in light accessible organs, first of all in the skin and the eyes. Transport of photoproducts from the light exposed field to other organs may also take place, and the blood may be exposed to a certain degree in the capillaries of the dermis. On this background one may expect both local and systemic effects, but it is important to be able to separate effects of light and effects of bilirubin toxicity.

Increased incidence of cancer is a possible effect in persons treated with light as newborns. In this respect we have established a cooperation project with the Medical Birth Registry in Bergen with the aim to match their register with the Norwegian Cancer Registry. After 1985 the information on the use of phototherapy has been collected systematically by the birth registry. We have applied for funds to establish a cohort of phototherapy treated persons between 1967 and 1985 from a group of more than 10 000 persons reported with hyperbilirubinaemia during this period. The studies of cancer in general are interesting, but it is our hope to be able to put particular emphasis on lesions in the skin and to test the hypothesis of Ben-Sasson and Davis (18) that phototherapy can lead to increased risk of acute lymphocytic leukaemia.

Other skin diseases and conditions are also of interest. It has been shown that the number of pigmented moles (nevi) at early age is influenced by UV irradiation, (56) and we are interested in testing whether the number of nevi may be increased by phototherapy. If so, this finding could indicate a higher risk of melanoma at older age, since a high number of nevi is a risk factor for development of malignant melanoma (with UV radiation exposure being a promoter for the later development of the melanomas) (57).

As mentioned earlier, eye damage from phototherapy light is clearly a possibility if the eyes are not properly shielded, but we would hesitate to regard this as a general problem for the children exposed to phototherapy. To reduce the risk of eye damage, the evaluation of cases where the eye shields have not been used properly should be done on an individual basis. Of course, it should be stressed that eye protection must be applied on all infants undergoing conventional phototherapy.

Finally, it is desirable to evaluate different phototherapy intensities and different wavelengths in the future, but at present the records of these parameters are too weak to assign individual infants to specific treatment categories.

Molecule	Main absorption wavelengths	Biological effect
Endogenous		
Porphyrins	UVA and visible	Photosensitisation by singlet oxygen
Vitamins (Riboflavin)	UVA and blue	Formation of hydrogen peroxide and other peroxides
Aromatic amino acids	UVB and UVA	Lost function, photosensitistion
Melanin	UV and visible	Photoprotection, photosensitistion
Bilirubin	Blue	Photosensitisation (may also act as anti-oxidant)
Exogenous Drugs (e.g. tetracyclins, psoralens from plants)	All optical wavelengths	Photoallergy and -toxicity

Table 1. Some light absorbing molecules and probable biological effects of light absorption.

1.5 REFERENCES

1. Cremer RJ, Perryman PW, Richards DH. Influence of light on the hyperbilirubinemia of infants. Lancet 1958;1:1094-7.
2. Meberg A. Hyperbilirubinemia - how many newborn infants need treatment? Experiences with new guidelines for phototherapy and exchange transfusion. (In Norwegian with English summary) Nor Epid 1997;7:93-102.
3. Christensen T, Reitan JB. Phototherapy of hyperbilirubinemia in newborn children. (In Norwegian with English summary) Østerås: Statens institutt for strålehygiene/National Institute of Radiation Hygiene, 1987; SIS report 1987:6.
4. Maisels MJ, ed. Neonatal jaundice. Clin Perinatol 1990;17(2).
5. Jährig K, Jährig D, Meisel P, eds. Phototherapy. Treating Neonatal Jaundice with Visible Light. München: Quintessenz-Verlags-GmbH, 1993.
6. Bratlid D. Fototerapi ved neonatal hyperbilirubinemi. (In Norwegian) Tidsskr Nor Lægeforen 1986;106:1830-3.

7. Wennberg RP. The importance of free bilirubin acid salt in bilirubin uptake by erythrocytes and mitochondria. Pediatr Res 1988;23:443-7.
8. Hansen TWR. Bilirubin and brain toxicity. Oslo: University of Oslo, 1988. Thesis.
9. Christensen T, Amundsen I, Kinn G, Kjeldstad B. Phototherapy of newborns with hyperbilirubinemia in Norwegian hospitals. A study of phototherapy units and light conditions. (In Norwegian with English summary). Østerås: Statens institutt for strålehygiene/National Institute of Radiation Hygiene, 1992; SIS report 1992:2.
10. Agati G, Fusi F, Donzelli GP, Pratesi R. Quantum yield and skin filtering effects on the formation rates of lumirubin. J Photochem Photobiol B 1993;18:197-203.
11. McDonagh AF, Lightner DA. «Like a shrivelled blood orange» - Bilirubin, jaundice, and phototherapy. Pediatrics 1985;75;443-55.
12. Rosenstein BS, Ducore JM, Cummings SW. The mechanism of bilirubin-photosensitized DNA strand breakage in human cells exposed to phototherapy light. Mutat Res 1983;112: 397-406.
13. Christensen T. Photosensitization of human cells with bilirubin. Photochem Photobiol 1984;39:39.
14. Christensen T. Cytotoxicity of bilirubin photoproducts. Photobiochem Photobiophys 1986;10:253-60.
15. Bratlid D. Criteria for treatment of neonatal jaundice. J Perinatol 1996;16:83-8.
16. Van de Bor M, Ens-Dokkum M, Schreuder AM, Veen S, Brand R, Verloove-Vanhorick SP. Hyperbilirubinemia in low birth weight infants and outcome at 5 years of age. Pediatrics 1992;89:359-64.
17. Gies HP, Roy CR. Bilirubin phototherapy and potential UVR hazards. Health Phys 1990; 58:313-20.
18. Ben-Sasson SA, Davis DL. Neonatal exposure to protoporphyrin-activating lighting as a contributing cause of childhood acute lymphocytic leukemia. Cancer Causes Control 1992;3:383-7.
19. Van Steensel-Moll HA, van Duijn CM, Valkenburg HA, van Zanen GE. Predominance of hospital deliveries among children with acute lymphocytic leukemia: speculations about neonatal exposure to fluorescent light. Cancer Causes Control 1992;3:389-90.
20. Christensen T, Støttum A, Brunborg G, Reitan JB. Unwanted side effects and optimization of phototherapy. Light Biol Med 1988;1:153-9.
21. Lightner DA. New photochemical transformations of bilirubin. [Abstract]. 12th International Congress on Photobiology. Vienna: September 1996: S50.
22. Brawley V, Bhatia J, Karp B. Hydrogen peroxide generation in a model pediatric parenteral amino acid solution. Clin Sci 1993; 85:709-72.
23. Wu F, Iijima K, Takiguchi D, Nishida A, Higurashi M. Effect of phototherapy on sister-chromatid exchange in infants with Down's syndrome. Mutat Res 1992;283:65-7.
24. Sisson TRC, Glauser SC, Glauser EM, Tasman W, Kuwabara T. Retinal changes produced by phototherapy. J Pediatr 1970;77:221-7.
25. Glass P, Avery GB, Subramanian KNS, Keys MP, Sostek AM, Friendly DS. Effect of bright light in the hospital nursery on the incidence of retinopathy of prematurity. N Engl J Med 1985;313:401-4.
26. Stiris TA, Hall C, Christensen T, Bratlid D. Effect of different phototherapy lights on retinal and choroidal blood flow. Dev Pharmacol Ther 1991;17:70-8.
27. John E. Complications of phototherapy in neonatal hyperbilirubinemia. Aust Pediatr J 1975;11:53-5.
28. Christensen T, Hodt S, Jacobsen PL, Reitan JB. Mother-infant interactions and hospital conditions related to the treatment of hyperbilirubinemia in newborns. (In Norwegian with

English summary) Østerås: Statens institutt for strålehygiene/National Institute of Radiation Hygiene, 1992; SIS work document 1992:3.
29. Bergsma D, Blondheim SH, eds. Bilirubin metabolism in the newborn (II). Birth defects Orig Artic Ser 1976;12:2.
30. Tan KL. The nature of the dose-response relationship of phototherapy for neonatal hyperbilirubinemia. J Pediatr 1977; 90:448-52.
31. Tan KL. The pattern of bilirubin response to phototherapy for neonatal hyperbilirubinemia. Pediatr Res 1982;16:670-4.
32. Ayyash H, Hadjigeorgiou E, Sofatzis J. Green or blue light phototherapy for neonatal hyperbilirubinemia. Arch Dis Child 1987;62:843-5.
33. Ayyash H, Hadjigeorgiou E, Sofatzis J, et al. Green light phototherapy in newborn infants with ABO hemolytic disease. J Pediatr 1987;111:882-7.
34. Hegyi T, Graff M, Zapanta V, et al. Transcutaneous bilirubinometry. III. Dermal bilirubin kinetics under green and blue-light phototherapy. Am J Dis Child 1986;140:994-7.
35. Romagnoli C, Marrocco G, De Carolis MP. Phototherapy for hyperbilirubinemia in preterm infants: Green versus blue or white light. J Pediatr 1988;112:476-8.
36. Sbrana G, Donzelli GP, Vecchi C. Phototherapy in the management of neonatal hyperbilirubinemia: Efficacy with light sources emitting more than 500 nm. Pediatrics 1987;80:395-8.
37. Tan KL. Efficacy of fluorescent daylight, blue and green lamps in the management of nonhemolytic hyperbilirubinemia. J Pediatr 1989;114:132-7.
38. Greenberg JW, Malhotra V, Ennever JF. Wavelength dependence of the quantum yield for the structural isomerization of bilirubin. Photochem Photobiol 1987;46:453-6.
39. McDonagh AF, Agati G, Fusi F, Pratesi R. Quantum yields for laser photocyclization of bilirubin in the presence of human serum albumin. Dependence of quantum yield on excitation wavelength. Photochem Photobiol 1989;50:305-19.
40. Kapoor L, Murti KRK, Bajpaj PC. Uptake and release of bilirubin by skin. Biochem J 1973;136:35-9.
41. Malhotra V, Greenberg JW, Dunn LL, Ennever JF. Fatty acid enhancement of the quantum yield for the formation of lumirubin from bilirubin bound to human albumin. Pediatr Res 1987;21:530-3.
42. Christensen T, Kinn G, Granli T, Amundsen I. Cells, bilirubin and light: Formation of bilirubin photoproducts and cellular damage at defined wavelengths. Acta Paediatr 1994:83;7-12.
43. Setlow RB. Action spectrum for melanoma induction in hybrid fish of the genus Xiphophorus. [Abstract]. 12th International Congress on Photobiology. Vienna: September 1996;S89.
44. Amato M, Inaebnit D. Clinical usefulness of high intensity green light phototherapy in the treatment of neonatal jaundice. Eur J Pediatr 1991;150:274-6.
45. Donzelli GP, Pratesi S, Rapisardi G, Agati G, Fusi F, Pratesi R. 1-day phototherapy of neonatal jaundice with blue-green lamp. The Lancet 1995;346:184-5.
46. Garg AK, Ward OC. Double surface phototherapy on a fluid bed. Ann Trop Pediatr 1994;14:81-4.
47. Garg AK, Prasad RS, Al Hifzi I. A controlled trial of high-intensity double-surface phototherapy on a fluid bed versus conventional phototherapy in neonatal jaundice. Pediatrics 1995;95:914-6.
48. Wallin A, Boréus LO. Phenobarbital prophylaxis for hyperbilirubinaemia in preterm infants. Acta Paediatr Scand 1984;73:488-97.

49. Grauel L, Almanza M, Alvarez R, et al. Induction of bilirubin-eliminating processes by methylphenobarbital in mature newborn babies. J Perinat Med 1988;16:431-5.
50. Kappas A, Drummond GS. Control of heme metabolism with synthetic metalloporphyrins. J Clin Inves 1986;77:335-9.
51. Valaes T, Petmezaki S, Henschke C, Drummond GS, Kappas A. Control of jaundice in preterm newborns by an inhibitor of bilirubin production: Studies with tin-mesoporphyrin. Pediatrics 1994;83:1-10.
52. Kemper K, Horowitz RI, McCarthy P. Decreased neonatal serum bilirubin with plain agar: a metaanalysis. Pediatrics 1988;82:631-8.
53. Davis DR, Yeary RA, Lee K. Activated charcoal decreases plasma bilirubin levels in hyperbilirubinemic rats. Pediatr Res 1983;17:208-9.
54. George P, Lynch M. Ohmeda Biliblanket vs Wallaby Phototherapy System for the reduction of bilirubin levels in the home-care setting. Clin Pediatr 1994;(March):178-80.
55. Valkeakari T, Anttolainen I, Aurekoski H, Björkqvist SE. Follow-up study of phototreated fullterm newborns. Acta Paediatr Scand 1981;70:21-5.
56. Harrison SL, MacLennan R, Speare R, Wronski I. Sun exposure and melanocytic naevus (mole) development in young Australian children. [Abstract]. 12th International Congress on Photobiology. Vienna: September 1996; P238.
57. Westerdahl J. Malignant melanoma - risk factors. Lund: University of Lund (Sweden), 1995. Thesis.

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Paper 2

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Ethical aspects of clinical research involving children, exemplified by a research project on phototherapy of jaundice

Ellen Bruzell Roll[1,2] and Terje Christensen[1]

[1]Norwegian Radiation Protection Authority, POB 55, NO-1332 ØSTERÅS, Norway
[2]Department of Physics, Norwegian University of Science and Technology, NO-7491 TRONDHEIM, Norway

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2.1 INTRODUCTION

Researchers without previous clinical experience are confronted by many ethical considerations when initiating a project which involves newborn human test subjects. A number of guidelines exist for research involving children, but these are not necessarily in complete agreement with each other. We will in the following article discuss the need for ethical guidelines in general, and for research on children in particular. Questions concerning research justification, risk factors involved and informed consent will be elucidated. The present research project, which aims to improve phototherapy of newborns with jaundice, will be described. The ethical considerations with regard to the research protocol will be pointed out, and the project will be discussed in relation to some basic issues which seem relevant for clinical research involving children.

2.2 THE NEED FOR ETHICAL GUIDELINES

Aside from the fact that the research in itself must be justifiable, it is also important that the correct type of research is chosen and that it is conducted according to ethically acceptable principles.

The so-called descriptive normative systems for ethics are usually divided into two main groups (Kragh and Pedersen 1991): *Consequence ethics* and *deontic ethics*. The consequential theory is that an action is morally correct if it leads to the best possible result – "benefit greater than harm". Thus, in medical research one must assess what benefit (i.e., alleviation of pain or improved life-quality) a certain project will have for the research patients and future patients, and if this benefit will be passed on to all patient groups.

Justification and freedom are the main principles in deontic ethics. A deontic theory central to the existent guidelines for medical research, is individual autonomy. According to the philosopher Emmanuel Kant, individual autonomy is a necessary attribute for a free-acting person and should, therefore, be respected without reservation.

The need for ethical guidelines has increased due to the fact that research in general has achieved a far larger scope. New technology is constantly being introduced, and mankind has experienced that research can also be used to the researchers' advantage and carried out in a blameworthy manner. The demand for ethical guidelines played a prominent part in the Nuremberg Proceedings after the Second World War. The Nuremberg Code was drafted in 1947 in response to this demand. An attempt was, thereby, made to formulate the basic conditions for which medical-scientific experiments using human subjects is ethically justifiable (Rossel 1985). The Declaration of Helsinki was first composed in 1964, and later revised in 1975 and 1983 (Declaration of Helsinki 1983, Riis 1985). This declaration aims to regulate the ethical aspects of research in an international context, "in agreement with science's boundless forms for collaboration and exchange of information". The Declaration of Helsinki applies both deontic ethics and consequence ethics. An example of consequence ethics is the references to the research subjects' safety, while an example of deontology is found in the Declaration's Part III, paragraph 4: "In research on man, the interest of science and society should never take precedence over considerations related to the wellbeing of the subject". The Declaration has evolved from a comparatively general recording of ethical principles in the form of imagined situations or research projects. Naturally, it can neither cover all situations and projects, nor all patient groups. Furthermore, it is not binding in a Norwegian court of law. Thus, on basis of the Declaration of Helsinki, one is provided with some national guidelines for certain areas within medical research.

2.3 GUIDELINES FOR RESEARCH INVOLVING CHILDREN

Similar to research with humans, research with animals undoubtedly raises ethical problems. Ethical guidelines for the use of test animals are legalized (*Act concerning the welfare of animals* of 20.12.1974, *Regulations for biological experiments with animals*, Norwegian Department of Agriculture 22.12.1977), and the *Law for medical use of biotechnology* of 1.9.1994). On the other hand, humans used as test objects have a very weak position in Norwegian law. Test activities are based on voluntary participation, grounded on respect for personal integrity. Thus, one is protected to a certain extent by criminal laws' paragraphs concerning bodily damage, illegal force, deprivation of freedom and consent (*The General Civil Penal Code, paragraphs* 229, 231, 222, 223, 235 (subclause 2). There are also laws that provide advice on what is permissible, such as the *Law for Doctors* (19.06.69) and the *Hospital Law* of 13.06.80 (doctor's professional secrecy and journal instructions). Furthermore, regulations exist for limited areas (clinical trials, research in psychiatric institutions and research on the mentally handicapped), but supplementary guidelines are absolutely necessary to protect the test patients as much as possible. These guidelines are especially important for test persons who are minors, unconscious, mentally retarded, mentally disturbed or senile (Riis 1985).

As test persons, children are an exposed group for several reasons. They are psychologically and physically less robust than adults. They are physiologically more vulnerable, since they are still growing, and the risk of future complications is greater. Furthermore, the youngest groups do not have the prerequisites to understand what it may imply to be a test subject.

The Declaration of Helsinki indirectly opens for research involving children in Part I, paragraph 11, which deals with informed consent from a legally authorized representative. On the other hand, there is no direct encouragement. In 1982 the World Health Organization (WHO) in collaboration with the Council of International Organizations of Medical Sciences (CIOMS) prepared a set of guidelines which elaborates certain points in the Declaration of Helsinki (Proposed International Guidelines for Biomedical Research Involving Human Subjects. CIOMS, Geneva, 1982). In one chapter concerning informed consent, there is a separate section dealing with children. Here it is pointed out that research on children should not be performed if similar research can be just as effectively performed on adults. In addition, it is stated that children's participation is necessary in research on children's diseases and conditions to which children are particularly vulnerable.

These attitudes are also expressed in "Guidelines for the ethical conduct of medical research involving children", published by the British Paediatric Association in 1992. Many Norwegian pediatric researchers follow these guidelines. One of the principles found herein actually encourages research with children with the wording: "Research involving children is important for the benefit of all children and should be supported, encouraged and conducted in an ethical manner" (Søvik 1993). In the case of nontherapeutic research, i.e., research that does not necessarily benefit the patient directly, but has a general value, the CIOMS guidelines are more cautious than the British ones, but clearly open for this type of research: "Children should in no circumstances be the subjects of research holding no potential benefit for them unless with the objective of elucidating physiological or pathological conditions peculiar to infancy and childhood". Earlier British guidelines have warned against nontherapeutic research with minors, while the latest now actually support this activity. Nevertheless, they include an explanation as to what this type of research encompasses: "We therefore support the premise that research that is of no potential benefit to the child subject is not necessarily unethical or illegal. Such research includes observing and measuring normal

development, assessing diagnostic methods, the use of 'healthy volunteers' and of placebos in controlled trials" (British Paediatric Association 1992).

2.4 THE NECESSITY FOR RESEARCH INVOLVING CHILDREN

Unfortunately, the results from medical research performed on adults cannot be directly transferred to children. Therefore, research on children is a necessity for a better diagnosis, therapy, prophylaxis and prognosis (Søvik 1975).

Infancy is commonly considered to be an age with a great need for research. In this age group special illnesses and conditions arise which have no relation to adult physiology. Concrete examples are a condition such as jaundice, as well as lung illnesses and congenital heart defects.

Other needs include establishing normal findings for the various age groups of children, and trying out new medicines. In addition, there is the need for knowledge about typical children's illnesses which seldom, if ever, occur in adults (Søvik 1975).

2.5 LACK OF RESEARCH – ETHICALLY JUSTIFIABLE?

Both hospital personnel and the child patients' parents may, of course, be skeptical to research involving children. This expression of care may, however, be in contrast to the importance of the up-to-date medical knowledge which is necessary to render the best treatment or to make the most correct diagnosis. The British guidelines from 1993 value the educational aspect: "An enquiring mind disciplined to test hypothesis by the approved canons of research while sensitive to the vulnerability of child patients *should be seen as a valued professional asset in a paediatrician*" (British Paediatric Association 1992).

Another important point to consider is the consequences of a lack of research. There is the risk of both a complete stop in the development of methods of treatment, and that general medical advice will be followed without being tested or controlled.

An example of changes that have been made in the treatment of jaundice in newborns without previous systematic testing, are the criteria for the limit to the degree of jaundice (concentration of bilirubin, see under "What is jaundice?") considered acceptable before treatment is initiated. These limits have been changed several places in the world on the recommendation of certain American pediatricians (Newman and Maisels 1992). Accordingly, the newborn is allowed a higher bilirubin concentration before phototherapy or exchange of blood is initiated. It is quite certain that this change in boundary criteria has not been systematically tested out and controlled, and that the group for which the criteria has been changed is large (5-10% of all newborns). After the change in these criteria was introduced several cases of kernicterus – the deadly result of an excessive bilirubin concentration – have been recorded in the USA (Hansen 1996).

There are other medical advice not necessarily based on randomized, controlled studies. For example, parents were earlier advised to place newborns on their stomachs to reduce the risk of crib death. They were later advised to lay the child on its back (Markestad 1992). The latter advice is based on literature and epidemiological studies. It is difficult, or course, to randomize newborns to one position or another both for practical and ethical reasons, the latter because researchers on crib death consider that they have convincing reasons to recommend the back-position (Rognum 1997). However, all changes in criteria and advice involving children's health require research both before and after the introduction of a change.

2.6 THE RISK CONCEPT IN CLINICAL RESEARCH

A central factor in the ethical assessment of a research project is the estimated risk for the test person. The Declaration of Helsinki is rather general in this area, but emphasizes that medical research involving human subjects should only be conducted if the importance of the objective outweighs the inherent risks and burdens to the subject (Declaration of Helsinki, Part 1, paragraph 4). Again there is a need for more detail. An argument that the harm factor must be more extensive in research with children is presented in *Ethical guidelines for medical research on children* (*Etiske retningslinjer ved biomedisinsk forskning på barn*) published by the Norwegian Research Council (NAVF) in 1983 (Utvalget for forskningsetikk 1983). It is pointed out that a child's personal integrity is more vulnerable than an adult's, and that taking part in a research project may affect them permanently, even without physical or mental injury.

Therapeutic and nontherapeutic research are handled separately in the risk assessment in NAVF's guidelines. On the basis of the Declaration of Helsinki, the Norwegian guidelines conclude that risk *per se* is not acceptable in nontherapeutic research with children. When a protocol both includes treatment and research aspects, the risk must be substantiated in relationship to the benefits of the treatment. Furthermore, it is stated that the risk analysis shall be absolute, and made without consideration of the anticipated benefit of the research. An increased risk is not acceptable even when the expected benefit is large. Thus, the question is what comprises the risk concept. The British Paediatric Association has rated risk in three categories: Minimal, low and high risk. Examples of minimal risk are questioning, observations and blood tests taken in connection with another routine examination. The higher risk categories are only of interest in therapeutic research, and then only with children with serious illnesses. In nontherapeutic research it is considered unethical to expose children to more than the minimal risk.

2.7 INFORMED CONSENT

During the Nuremberg processes after the Second World War, German Nazi doctors defended themselves by contending that by exposing a relatively small number of people to experimentation, future generations could be spared. This argumentation was rejected on the grounds of the requirement for informed consent already integrated in the Nuremberg code. The first point in the code demands voluntary informed consent from a test subject prior to participation in a project. This principle is prominent in the Declaration of Helsinki as well.
It is quite obvious that this principle cannot be applied to children, certainly not small children. Even if the children are mature enough to understand the contents and reason for the research, they are not necessarily capable to evaluate possible risks involved. According to the Declaration of Helsinki, informed consent must be obtained from a legal guardian when the test subject is a minor, and from the nearest responsible relative when the child in question is underage. Furthermore, it is stated that "Whenever the minor child is in fact able to give consent, the minor's consent must be obtained in addition to the consent of the minor's legal guardian". Thus, it is the child, when it is mature and intelligent enough to understand the situation, who primarily makes the decision. In this case, the parents' acceptance alone is insufficient.

This principle is also emphasized in the guidelines from the British Paediatric Society from 1992 and in NAVF's guidelines from 1983, both based on the Declaration of Helsinki from 1975. In the Norwegian guidelines, reference is made to paragraph 31 in the children's law: "When a child has reached 12 years of age, the child shall be given the opportunity to express its own opinion before any decision is made regarding its personal affairs".

Furthermore, the child's opinion shall always be heard and seriously considered when the child is able to express its own opinion. This fact may also apply to children under 12 years of age. These issues in the Norwegian guidelines concur with CIOM's guidelines. The chapter regarding consent states that "To the extent that is feasible, which will vary with age, the willing cooperation of the child should be sought, after it has been frankly informed of any possible discomfort and inconvenience. Older children may be assumed to be capable of giving informed consent, preferably also with the consent of the parent or other legal guardian" (point 8). It should, however, be noted that the Declaration of Helsinki provides an opening for *not* procuring informed consent in certain cases (Part II, point 5). This viewpoint is also expressed in the European Convention for Bioethics, article 6 (Council of Europe 1994): "Additional informed consent from parents is unnecessary if the research is to the child's advantage". It is difficult to find evidence of this viewpoint in the interpretation of Norwegian law. According to Kjønstad's (1983) article *Requirements regarding consent from the test subject/patient in medical research* (*Krav om samtykke fra forsøksperson/pasient i medisinsk forskning*), it appears to be disputable that parents have the competence to consent to research on their children. In the article it is claimed that this option should be more acceptable in therapeutic research than in nontherapeutic research, to which only limited acceptance is suggested. The ethical committee that approves the nontherapeutic research project will set strict requirements for the project (Mjølhus 1994).

Very concrete and detailed guidelines for informed consent have recently been published from the American Academy of Pediatrics (Committee on Bioethics 1995). The guidelines are most relevant for information and consent in the treatment situation. Also these guidelines indicate a trend in the direction of increased influence from the child. Concrete proposals are outlined as to when an older child should be allowed to make the treatment decision on his own.

2.8 ETHICAL PROBLEMS WITH INFORMED CONSENT

In spite of all the work and good intentions invested in these various guidelines, certain difficult ethical aspects remain with regard to informed consent.

One of these problems is that the person who provides information often has a personal interest in the project and may, therefore, with difficulty evaluate the risk objectively. This problem can be solved by recruiting a person, with no authority for clinical decision, who will inform the test subject or parents. Another problem is similarly associated with the researcher's or doctor's interpretation of risk. It may even be impossible to provide concrete information on the risk of treatment because the person involved, perhaps due to lack of expertise in the field, is not aware of all possible consequences of a certain treatment. A third difficulty is connected with the test subject's assessment of risk. In a child's case, one may be able to judge that the child is mature enough to give informed consent, but unable to determine how much the child understands about the possible risk, nor whether the child approves due to outside influence.

Furthermore, information about an experiment may not be easy to grasp by adult test subjects or guardians. Lack of comprehension may be due to experience and/or education or for emotional reasons. Ingelfinger (1972) states the following about interpretation of information: "Comprehension of medical information given to untutored subjects is inversely correlated with the elaborateness of the material presented".

For example, the parents of a sick newborn child have many feelings to handle and may have problems understanding the implications of a possibly advanced method of treatment.

Or, as written in an editorial in "The Lancet" (1995): "Informed consent from poorly educated parents entering a complex trial in stressful conditions is a sham."
It is possible that many parents will give their consent for fear of coming in conflict with the attending physician. In certain cases a decision must be made quickly, and there is no time to assess the information critically enough. Levene and coworkers (1996) report that more parents agreed to participation in a study of sick newborns when they were asked two hours after the birth (76 %) than one to two weeks after birth (43 %). This fact could be due to a lack of time for consideration, but also to the fact that many mothers are not necessarily as strongly bound to their children the first days after birth and, thus, assess the elements of risk in the investigation less carefully (Christensen *et al.* 1992b).

Requesting informed consent from a family in a difficult situation can seem so unpleasant for some clinicians that they prefer to abandon the project.

2.9 IMPROVEMENT OF PHOTOTHERAPY OF NEWBORNS

When one shall initiate a clinical investigation which involves children, it is necessary to assess both the conventions and aspects discussed above, and the general plan for the investigation. An evaluation must be made as to whether the current demands in the area have been fulfilled or whether adjustments must be made in the planned investigations. Perhaps the investigations should be abandoned entirely. Although approval from a committee of ethics is required, the researchers themselves must make responsible assessments, especially since sufficiently clear regulations in the area are not always found.

The following is a concrete description of the phototherapy project and the measures for improvement which are to be tried out. Finally, a conclusion will be drawn on the ethical justification for the clinical trials.

2.9.1 What is jaundice?

Jaundice is the term used for a yellowish tint to the skin which is caused by an accumulation of the pigment bilirubin in the skin and other organs. Bilirubin is a breakdown product of hemoglobin and similar substances in the blood. All individuals normally produce several milligrams of bilirubin daily due to a constant replacement of red blood cells. This replacement is initiated immediately after birth. In older individuals bilirubin is cleared through an enzymatic conversion in the liver and further transport into the bile. At birth, the enzyme system in the liver is not necessarily sufficiently developed enough to handle the conversion of large amounts of bilirubin. More than half of all newborns show signs of high bilirubin levels, and five to ten per cent are treated by phototherapy for jaundice (hyperbilirubinemia) (Christensen and Reitan 1987). The treatment is performed to stop the child from acquiring excessive concentrations of bilirubin in the blood and tissues, a situation which can lead to brain damage and at worst death. In most instances the jaundice disappears by itself within a week after birth. Therefore, it may be difficult for the doctor to decide whether the jaundice is serious enough to require treatment or whether it is safe to let nature take its course and reduce the bilirubin level via ripening of the enzymatic processes.

2.9.2 Phototherapy

The mode of operation and practice of phototherapy have been described earlier (e.g. Jährig *et al.* 1993). The effect of light on children with jaundice was discovered accidentally by a nurse in England in 1956 (Cremer *et al.* 1958). It was observed that children that lay near a window recovered more quickly, and that children with jaundice benefited from a trip to the park in daylight. These observations led to a series of studies that explained to a large extent how the

treatment works. At the present time one is not sure about all details in the effect mechanisms, and it is hoped that further research will uncover details that can be used to improve the treatment. Knowledge of the effect mechanism is particularly pertinent with regard to what light dose is required to convert sufficient amounts of bilirubin and prevent a concentration that may cause brain damage (Tan 1982). Similar to excessive doses of other medicines or unnecessarily high exposure to radiation in connection with x-ray diagnostics, an overdose of phototherapy is not recommended or of advantage.

The color of the light can also have a great influence on the reactions that take place in the bilirubin, and several researchers recommend the use of long wavelengths (green instead of blue light). The reason for this recommendation is that the effect can be greater in the green area, and also that blue light has a greater effect on other biomolecules (closer to ultraviolet radiation). Blue light can, therefore, involve a greater risk for undesirable reactions in the exposed tissue than green light.

The treatment normally begins one to two days after birth. The child lies naked with its eyes protected under white or blue light provided by overhead therapy lamps. Alternatively, a fiber optic "blanket" can be wrapped around the child's stomach and back, or the child can lie on a transparent mattress with light underneath. The intensity of the light (irradiance, measured in mW/cm^2) and the distance from the subject vary (Christensen *et al.* 1992a). The duration of the treatment is one or more days. Blood tests of the child are taken regularly to determine if the concentration of bilirubin indicates a termination or continuation of the phototherapy. If the bilirubin concentration has increased, it may be necessary to employ exchange transfusion (replacement of blood). It is common practice in Norwegian hospitals to remove the child from the phototherapy unit for nursing and care at nonsystematic periods of time.

2.9.3 Planned clinical studies

In our study of phototherapy we will explore three parameters of treatment in relation to their ability to reduce the bilirubin concentration in the blood of newborns. Only overhead phototherapy lamps will be used in this project. Firstly, we will determine if a *continuous* or *fractionated* treatment is optimal. (In this case, continuous treatment is defined as three hours under light for every hour away from the lamp, and fractionated treatment is defined as every other hour under and away from the lamp.) Secondly, we will investigate how effectively the bilirubin concentration is reduced with relation to the irradiance of the light meaning that the child will lie at different distances from the lamp. Thirdly, we will study the influence of the wavelength of the light on the breakdown of bilirubin. Blue light tubes or a blue-green lamp will be used as phototherapy devices. The blue-green lamp has been newly developed for this purpose and has previously been used in limited clinical studies (Donzelli *et al.* 1995). In addition, we will look into three biochemical parameters with regard to possible side effects of the treatment, namely the light-sensitive vitamins A and B_2 (riboflavin) and the hormone melatonin (which varies according to daily rhythm, Kennaway *et al.* 1996). About 300, otherwise healthy newborns with jaundice comprise the basis for different treatment groups randomized to four hospitals in Norway. A summary of the various treatment groups is presented in Figure 1.

Color	Intensity	Regimen	1 h	1 h	1 h	1 h	1 h	1 h	etc.
BLUE	HIGH	CONTINUOUS							
BLUE	MIDDLE	CONTINUOUS							
BLUE	LOW	CONTINUOUS							
BLUE	HIGH	FRACTIONATED							
BL-GR	HIGH	CONTINUOUS							
BL-GR	HIGH	FRACTIONATED							

Fig. 1 The distribution of light and dark periods in the planned clinical investigation (BLUE: Blue light tubes, BL-GR: Blue-green lamp). In the dark time periods the light is turned off, the eye protection removed, and the parents are free to be with the child for care and nursing. Radiation continues according to the same schedule until the bilirubin value has fallen to a predetermined standard level. For control, blood tests of the child are taken every seventh or eighth hour, performed in the same way as in routine treatment.

2.9.4 Practical consequences of the difference between conventional and research treatment

Only blue light tubes and the blue-green lamp shall be used in the project, not white light, fiber optics or the transparent mattress that are otherwise widely used in maternity wards at hospitals. Psychologically, these blue and green wavelengths can appear to be more unnatural and less comfortable to look at than white light. The present study omits the above-mentioned alternative devices, the use of which considerably facilitates nursing, care and bodily contact with the infant. The so-called "mother-child friendly alternative" includes the infant being in the mother's room day and night, always accessible for nursing, if the mother so wishes. The aim is to provide good conditions for nursing and to increase the bond between mother and child. For practical reasons this alternative cannot always be performed for infants who need medical treatment, an argument which also applies to infants with the need for phototherapy. For the children in this particular study, the periods of time the child lies under or outside the overhead therapy lamps will be controlled. The child cannot be removed from the lamps without consideration. The necessity of this scheme must be evaluated individually - for example, if the child cries and obviously needs a rest. The children participating in the project will, however, receive a total of more and systematically light-free periods than what is usual with the use of overhead therapy lamps. As mentioned earlier, the child must wear eye protection while under the lamps, despite possible discomfort and inconvenience.

As noted previously, the irradiance on the child is dependent upon the child's distance from the lamp, and how many and which type of light tube is used. This equipment has already been adapted for use in the test treatments. The irradiance is adjusted by varying the numbers of lamps turned on and by modifying the dispersion of light by hanging a sheet or paper in the light area. The "low" irradiance described in the tests is no lower than that which is in general use at many hospitals (Christensen *et al.* 1992a). The test treatment requires the same number of blood tests as conventional treatment. Somewhat more blood will be taken than in routine tests, but in the same manner. The blood tests will be taken at predetermined intervals.

2.9.5 Ethical aspects of the test treatment

This project is supported by the Norwegian Research Council and is approved by the ethical committee in Health Region IV. All the various treatments to be tested have been or are in use, although not systematically and not necessarily in Norway. Even the irradiance termed as "low" in our study, is within the values routinely used in Norwegian hospitals. Therefore, it cannot be claimed that the treatment offered in our study is inadequate compared to standard treatment. We add no new forms of treatment, but utilize some that have seldom been

practiced in spite of researchers' recommendations – for example, systematic fractionated treatment or the use of blue-green light (Jährig *et al.* 1995). Blood tests are taken of these children, regardless of whether they are receiving conventional or test treatment, and the small extra amount of blood drawn causes the child no discomfort. On the other hand, it may be ethically questionable that the blood samples are presently stored for an unknown period of time. Information about the test subject will be received and kept on file according to standard regulations at a health care institute (Norwegian Radiation Protection Authority, Radiation Medicine Dept.).

As with other test treatments, general ethical questions arise with the use of children, especially newborns, in research. The parents of the test subject must give their informed consent to allow the child to participate in the study. As mentioned earlier, it is especially difficult to request informed consent from parents in a possibly stressful situation shortly after a birth. This problem may be left unsolved because it is impossible to delay the decision when the child needs immediate treatment. The question to the parents will not be whether or not the newborn shall receive treatment, but which type of light equipment shall be used.

Equipment with very different technical design is found in Norwegian hospitals (Christensen *et al.* 1992a). We assume that the doctor, midwife or nurse chooses the equipment to be used in conventional treatment without consulting the parents. This aspect is, therefore, not particularly different when comparing conventional treatment and test treatment.

In all events, the treatment will be terminated when the bilirubin level is reduced to justifiable levels. Possible complications or the need for supplementary treatment will be handled in the same way, regardless of whether or not the child participates in the test project.

The test treatment is so similar to the conventional one that the risk involved is minimal compared with the already very limited risk associated with the customary implementation of phototherapy and accompanying collection of samples, etc.

By consequential analysis, it can be concluded that carrying out the research project may be of great benefit for three main reasons: Based on research knowledge of phototherapy the investigation will not lead to less effective treatment of the newborns than will the conventional treatment. The results from the experiment may lead to improved treatment of a large group of newborns (approx. 2500 children in Norway annually), since the treatment time can be shortened so that contact with parents and environment becomes more natural. Furthermore, there is the possibility that the use of blue-green light may be more gentle with respect to biological damage than pure blue light, and that the possibilities for side effects are less, accordingly.

2.10 CONCLUSION

The described project "Optimizing phototherapy of newborns with hyperbilirubinemia" has a valid justification for research: There are great variations between recommended and practiced treatment, there is a large group of children that are affected by phototherapy, and the research cannot be carried out as effectively on adult test subjects or test animals. The study can be characterized as therapeutic research. It is beneficial for the individual receiving a treatment which, if different from the conventional treatment, is probably superior. Similar to conventional treatment, the risk involved in the test treatment is minimal.

On the other hand, as discussed in the chapter "Ethical problems with informed consent", there are a number of problems connected with informed consent when the research involves children, especially newborns. These aspects must be taken into careful consideration, and it

is, therefore, important that both written and verbal information are presented to the parents in the best possible manner.

The project raises few or no serious ethical problems, and the regional committee of ethics that reviewed the test protocol had no objections. Thus, the project conforms to the restrictions of Norwegian legislation and the applicable guidelines.

Generally, it is a problem for the ethical considerations that Norway lacks concrete, updated guidelines in conformity with the country's legislation. NAVF published its *Ethical guidelines for biomedical research on children* (*Etiske retningslinjer ved biomedisinsk forskning på barn*) fifteen years ago. There the problem is described in the following way: "According to Norwegian legislation, it appears clear that one cannot on another's behalf give consent to operations which involve a real risk for physical or psychic damage, nor does the legislation instruct a citizen to run such a risk on another's behalf under conditions that are relevant for the research situation".

However, it seems clear that research, also on children, can be justified by its necessity. The performance of the research should make great demands on the researcher's and the ethical committee's responsibility.

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2.12 REFERENCES

1. British Paediatric Association. 1992: Guidelines for the ethical conduct of medical research involving children. Bull Med Eth 80:13-20.
2. Christensen T, Amundsen I, Kinn G, Kjeldstad B. 1992. Phototherapy of newborns with hyperbilirubinemia in Norwegian hospitals. A study of phototherapy units and light conditions. In Norwegian with English summary. SIS-rapport, Statens institutt for strålehygiene, Østerås, 1992:2.
3. Christensen T, Hodt S, Jacobsen PL, Reitan JB. 1992b. Mother-infant interactions and hospital conditions related to the treatment of hyperbilirubinemia in newborns. In Norwegian with English summary. SIS arbeidsdokument, Statens institutt for strålehygiene, Østerås, 1992:3.
4. Christensen T, Reitan JB. 1987. Phototherapy of hyperbilirubinemia in newborn children. In Norwegian with English summary. SIS-rapport, Statens institutt for strålehygiene, Østerås, 1987:6.
5. Committee on Bioethics. 1995. Informed Consent, Parental Permission, and Assent in Pediatric Practice. Pediatrics 95:314-7.
6. Council of Europe, Directorate of Legal Affairs, Strasbourg. 1994. Draft. Convention for the protection of Human Rights and the dignity of the human being with regard to the application of biology and medicine: Bioethics Convention and Explanatory Report.
7. Cremer RJ, Perryman PW, Richards DH. 1958. Influence of light on the hyperbilirubinemia of infants. Lancet 1:1094-7.
8. Declaration of Helsinki. Amended by the 35th World Medical Assembly, Venice, Italy, October 1983.

9. Donzelli GP, Pratesi S, Rapisardi G, Agati G, Fusi F, Pratesi R .1995. 1-day phototherapy of neonatal jaundice with blue-green lamp. Lancet 346:184-5.
10. Editorial. 1995. Your baby is in a trial. Lancet 345:805-6.
11. Hansen TW Ruud. 1996. Treatment of neonatal jaundice. Norwegian guidelines in an international perspective. In Norwegian with English summary. Tidsskr Nor Lægeforen 27:3215-8.
12. Declaration of Helsinki. Amended by the 29th World Medical Association Assembly i Tokyo, Japan 1975. Norwegian translation by William D. Shepard and Erik Enger.
13. Ingelfinger FJ. 1972. Informed (but uneducated) consent. N Engl J Med 287:465-6.
14. Jährig K, Jährig D, Meisel P (eds.). 1993. Clinical issues. In: Phototherapy. Treating Neonatal Jaundice with Visible Light: 89-156. München: Quintessenz-Verlags-GmbH.
15. Kennaway DJ, Goble FC, Stamp GE. 1996. Factors influencing the development of melatonin rhythmicity in humans. J Clin Endocrinol Metab 81:1525-32.
16. Kjønstad A. 1983. Krav om samtykke fra forsøksperson/pasient i medisinsk forskning. In Norwegian. Lov og Rett: 403-31.
17. Kragh H, Pedersen SA. 1991. Videnskabsteoriens aktualitet. In Danish. In: Naturvidenskabens teori. En indføring i naturvidenskabernes og teknologiens filosofiske problemer: 303-351. København: Nyt Nordisk Forlag Arnold Busck.
18. Levene M, Wright I, Griffiths G. 1996. Is informed consent in neonatal randomised controlled trials ritual? Lancet 347:475.
19. Markestad T. 1992. Sleeping position and crib death in Norway. In Norwegian with English summary. Tidsskr Nor Lægeforen 11:1427-9.
20. Mjølhus K. 1994. Juridiske spørsmål ved forskning på mennesker. In: Forskning på mennesker. Lover, regler og retningslinjer. In Norwegian. Den nasjonale forskningsetiske komité for medisin. Oslo/Tromsø, 16-20.
21. Newman TB, Maisels MJ. 1992. Evaluation and treatment of jaundice in the term newborn: a kinder, gentler approach. Pediatrics 89:809-18.
22. Riis P. 1985. Medicinsk videnskabsetik. In Danish. In: Andersen D, Mabeck CE, Riis P (eds.). Medicinsk Etik: 556-579. København: FADL's Forlag.
23. Rognum TO. 1997. Bruken av forskningsmidler. In Norwegian. Aftenposten 15.11.97.
24. Rossel P. 1985. Det informerede samtykke. In Danish. In: Andersen D, Mabeck CE, Riis P (eds.). Medicinsk Etik: 252-62. København: FADL's Forlag.
25. Søvik O. 1975. Etiske problemer ved klinisk forskning i barneavdelinger. In Norwegian. Tidsskr Nor Lægeforen 22:1197-1200.
26. Søvik O. 1993. Etiske retningslinjer for medisinsk forskning som omfatter barn. In Norwegian. Tidsskr Nor Lægeforen 113:2726-7.
27. Søvik O. 1995. Metabolsk sykdom - screening av nyfødte. In Norwegian. Tidsskr Nor Lægeforen 115:582-3.
28. Tan KL. 1982. The pattern of bilirubin response to phototherapy for neonatal hyperbilirubinemia. Pediatr Res 16:670-4.
29. Utvalget for forskningsetikk, Rådet for medisinsk forskning. Pediatrisk forskningsetikk. Etiske retningslinjer ved biomedisinsk forskning på barn. In Norwegian. Norges Almenvitenskapelige forskningsråd, Oslo, 1983.

Part II: *In vivo* studies on jaundiced newborns

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Paper 3

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In vivo spectroscopy of jaundiced newborn skin reveals more than a bilirubin index

Lise Lyngsnes Randeberg (MSc) (a), Ellen Bruzell Roll (MSc) (b, c)
Lill T. Norvang Nilsen (PhD) (c), Terje Christensen (PhD) (c)
Lars O. Svaasand (PhD) (a)

(a) Department of Physical Electronics
Norwegian University of Science and Technology, Trondheim, Norway

(b) Department of Physics
Norwegian University of Science and Technology, Trondheim, Norway

(c) Norwegian Radiation Protection Authority, Østerås, Norway.

Short title: Reflectance spectroscopy and jaundice

Correspondence to:
Lise Lyngsnes Randeberg
Department of Physical Electronics
Norwegian University of Science and Technology
N-7034 Trondheim
Norway

E-mail: Lise.Randeberg@fysel.ntnu.no
Fax: +47 73591440

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Paper 4

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The effect of phototherapy on light sensitive vitamins in the serum of jaundiced infants.

Ellen Bruzell Roll

Norwegian Radiation Protection Authority, POB 55, NO-1332 ØSTERÅS, Norway
and
Department of Physics, Norwegian University of Science and Technology, NO-7491 TRONDHEIM, Norway

URN:NBN:no-7250

ABSTRACT

There is a need for clinical investigations of various parameters influencing phototherapy. The study of adverse effects related to the irradiation is based on inspection experience and few international guidelines. The protocol for a planned and partly accomplished multi-centre study is presented. Blood samples from jaundiced newborns subjected to phototherapy of various durations were analysed for the light sensitive vitamins riboflavin (vitamin B_2), flavoenzymes and retinol (vitamin A). The serum riboflavin concentration showed a significant decrease to half its pre-treatment value after seven hours of phototherapy, whereas the flavoenzymes concentration remained constant. The retinol concentration even increased during phototherapy.

4.1 INTRODUCTION

Despite the frequent use of phototherapy as treatment of hyperbilirubinemia there have been relatively few controlled clinical studies aimed at optimising the therapy with respect to irradiance, fractionation regimens, wavelength and the simultaneous assessment of adverse effects (Bruzell Roll and Christensen, 1997 (Paper 1)). The need for optimisation is based on the knowledge that phototherapy is, unlike most administration of medication, not a standard procedure, neither internationally nor nationally. Investigations of the national practices of phototherapy in 1986 (Christensen and Reitan 1987), 1989 and 1991 (Christensen et al. 1992), and internationally in 1996 (Hansen 1996), in addition to our experience after inspection of several hospitals nation-wide throughout the period 1996-2002, revealed the existence of a variety of equipment for the treatment of hyperbilirubinemia. The lamps inspected emitted a wide range of wavelengths including UVA, and irradiances varied between 0.2 and 3.3 mW/cm^2 in the blue wavelength region (Christensen and Reitan 1987). In general, there was no systematically planned fractionation or control of the dark-and light periods.

In Norway, optical radiation in medicine is regulated through the Act on Radiation Protection and Use of Radiation (Act No. 36 of 12 May 2000. In Norwegian). Section 13 expresses that the use of radiation in medicine must be justified and optimised. However, the law offers no dose limits. In fact, the only organ dose limits for blue light in phototherapy apply to the eyes (Matthes 1999), resulting in the requirement of eye protection of the treated infants. For shorter wavelengths, on the other hand, there are dose limits prepared by the International Electrotechnical Commission in an International Standard (IEC 2000) drawn up for infant phototherapy equipment.

Taking the widespread use of phototherapy (5-10 % of all newborns) into account systematic studies on the effect on clinical and haematological parameters are few (Jährig et al. 1993). Among the studies listed are the bilirubin/albumin binding, oxygen binding capacity of erythrocytes, spherocyte formation, osmotic fragility, amino acid metabolism, fatty acids concentration and serum enzymes activity. A decline in the riboflavin (vitamin B_2) concentration in serum of patients receiving phototherapy has been observed (Gromisch et al. 1977). However, a deficit is made up within days (Jährig et al. 1993). A phototherapy effect on another light-absorbing vitamin, retinol (vitamin A) has, to our knowledge, not been studied.

On this background, we planned and partly carried out a multi-centre trial including jaundiced newborns to be subjected to a phototherapy protocol for investigation of variation in irradiance, fractionation regimen and wavelength. The aim was to monitor the formation of photoisomers of bilirubin and the simultaneous reduction in ZZ-bilirubin in addition to the total serum bilirubin (TSB) concentration during phototherapy as a measure of the efficacy of the various treatments. The serum samples taken to determine TSB and photoisomers were also analysed to determine the variations in riboflavin and vitamin A concentrations before, during and after phototherapy for the various treatments. For several reasons we did not succeed in accomplishing the entire study. Three hospitals participated in the study in a way that produced material for analysis, however, the number of patients was less than a tenth of the planned inclusion number. The irradiation regimens of the participating hospitals were not comparable. Nevertheless, we were able to obtain serum samples from 25 patients, and results from riboflavin and vitamin A analyses are presented.

4.2 PROTOCOL FOR CLINICAL TRIALS

4.2.1 Prepared protocol for multi-centre clinical trials

Clinical trials were prepared in four Norwegian hospitals. The number of children to be included in the trial was approximately 250-300. The treatment regimens, as well as the order, were randomised between hospitals.

4.2.1.1 Treatment regimens (Table 4.1)

I. Comparison of wavelengths

a) Blue light (Four Philips 20W/52 tubes. Emission peak: 450 nm. Tubes must be covered with plexiglass)

b) «Turquoise light» (Four Osram tubes. Emission peak: 490 nm. Tubes must be covered with plexiglass). The turquoise fluorescent tubes were a gift from Dr. Giovanni Agati, CNR, Firenze, Italy. Not commercially available (Ebbesen et al. 2003). (See 4.2.1.2. Phototherapy units, irradiance and calibration)

II. Comparison of continuous and intermittent treatment

a) Continuous: 3 h light on, 1 h light off

b) Intermittent: 1 h light on, 1 h light off

III. Comparison of irradiance

a) High: 2 mW/cm^2

b) Normal: 1 mW/cm^2

c) Low: 0.5 mW/cm^2

Therapeutic light colour	Irradiance level	Irradiation regimen
Blue	1. Low 2. Medium 3. High	Continuous
Blue	High	1. Continuous 2. Intermittent
1. Turquoise 2. Blue	High	Continuous

Table 4.1. Clinical trial irradiation regimen plan.

4.2.1.2 Phototherapy units, irradiance and calibration

Equipment: Air-Shields PT 533-2E (Fluoro-lite^{R}) equipped with four light tubes (Philips 20W/52 or the non-commercial turquoise Osram tubes) with draping (white, thin cotton-sheets) or the overhead phototherapy equipment available at the hospital.

Irradiance was measured using a UDT (United Detector Technology 371, Hawthorn, CA) radiometer. To determine the irradiance of the fluorescent tubes the probe 247 (similar sensitivity at 490 and 450 nm) was used. The readings were adjusted for the cosine angular response by measurements of the irradiance of the blue light tubes with probe 268Blue (peak sensitivity of 450 nm). The tubes were placed at a distance from the bed (or incubator) giving irradiances of 1.3 ± 0.03 mW/cm^2 as measured by the probe 247 and corrected to 2 ± 0.04 mW/cm^2 ("high irradiance") by the probe 268Blue.

The photodiode was recently calibrated prior to the onset of the studies by the supplier and again in our optical lab in advance of the study period. The irradiance of the phototherapy units was checked regularly by the instrument.

4.2.1.3 Blood samples

a) Total serum bilirubin concentration analysed by the diazo reaction method performed routinely in the hospital labs.
b) Relative concentration of photoisomers of bilirubin in serum determined by high pressure liquid chromatography analysis.
c) Riboflavin (vitamin B) and retinol (vitamin A) analysed commercially.

In addition to the blood samples the tissue bilirubin concentration will be measured with non-invasive reflectance spectroscopy technology.

4.2.2 Practical protocol check-list

4.2.2.1 Inclusion criteria

Where hyperbilirubinemia is suspected, a blood sample is taken. The serum bilirubin concentration indicates treatment. In one hospital the bilirubin concentration criteria for phototherapy are as follows: Full-term (∅37 weeks) at day 3: 350 σM Premature ({ 37 weeks) at day 3: 180 σM. For the three other hospitals the criteria are (day 3): { 1.5 kg: 175 σM, 1.5-2.5 kg: 225 σM, } 2.5 kg: 275 σM.

Assess whether the infant is eligible to be included in the phototherapy project according to the following inclusion criteria:

- not too premature (∅1500 g, ∅32 weeks of gestation)
- no other substantial illnesses
  -negative Coombs' test
  -no RDS (respiratory distress syndrome), sepsis, serious hypoglycaemia, serious birth-
  defects
  -no babies in their first day of life
- babies on parenteral diet due to lack of nursing can be part of the study if otherwise healthy

Babies needing more than one treatment can enter the trial again, but as «new» patients.
Babies not included in the project at their first treatment may be included at a later treatment. However, parents are not asked a second time to include their baby in the project after first having refused.

4.2.2.2 Information to parents: informed consent
Norwegian guidelines for paediatric clinical trials follow the Helsinki declaration and the guidelines from the British Paediatric Association. The project is approved by a national ethical committee. A pamphlet was prepared called «Information to parents of children participating in the phototherapy project» as a supplement to personal communication between parents and health personnel.

4.2.2.3 Phototherapy initiation procedures
Blood samples are obtained by heel stick.
A zero-point blood sample must be drawn as close to the treatment onset as possible. This zero-point blood sample is taken in addition to those taken on routine basis for checking the bilirubin level. Subsequently, samples are taken 7, 15 and 23 hours after treatment onset, and then every eight hours.
A timer is connected to the phototherapy unit, and is set to the 3+1 h or the 1+1 h regimen.
Make sure the hospital staff (nurses/midwives/doctors) is aware of the following:
- the baby is supposed to stay under the light when the light is on
- the baby must be turned around (back/front) after every «dark»-period
- routine observations to be written in the baby's protocol is also to be written on the phototherapy form placed on the bed

4.2.2.4 Phototherapy termination
Duration of the phototherapy is normally between one and three days.
The treatment is terminated according to the hospital cessation criteria.

4.3 MATERIALS AND METHODS

Serum samples were obtained from three different irradiation regimens: 16 patients received "Blue light, normal irradiance, continuous treatment", four patients received "Blue light, high irradiance, intermittent treatment" and five patients received "Turquoise or blue light, high irradiance, continuous treatment". Samples were collected according to the study protocol during the years 1998-2001 and kept frozen at -80°C until time of analysis (2001). The samples were analysed for riboflavin

(vitamin B_2) and all-trans retinol (vitamin A) concentrations by a commercial laboratory (Vitas AS, Forskningsparken, Oslo. www.vitas.no). In addition to the riboflavin concentration the concentrations of the flavocoenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) were measured.

4.3.2 Vitamin analysis procedure

Retinol (vitamin A, all-trans retinol):

Human plasma was diluted with 2-propanol containing the internal standard TMMP-retinol and BHT as an antioxidant. After thorough mixing (15 min) and centrifugation (10 min, 4000 g at 10 °C), supernatant was injected into the HPLC system. HPLC was performed with a HP 1100 liquid chromatograph (Agilent Technologies, Palo Alta, CA, USA) with a HP1100 single wavelength UV detector operated at 325 nm. (Column temperature: 40 °C). Retinol was separated from the matrix and internal standard on a 4.6 mm x 25 mm reversed phase column. A two-point calibration curve was made from analysis of plasma calibrators with known retinol concentrations. Recovery is > 95 %, the method is linear from 0.1-10 µM at least and the limit of detection is 0.01 µM. RSD is 4.9 % (1.2 µM) 5.8 % (1.7 µM)).

Riboflavin (vitamin B_2) and flavocoenzymes:

Human plasma was added to 20 % trichloroacetic acid . The acid conditions hydrolysed FAD to FMN. After thorough mixing (15 min) and centrifugation (10 min, 4000 g at 10 °C), the sample was incubated at 70°C for 30 minutes. Supernatant was then injected into the HPLC system described above, except in this analysis the HPLC was equipped with a HP1100 fluorescence detector (emission: 520 nm, excitation: 450 nm). B_2 vitamers were separated on a 2.1 mm x 50 mm reversed phase column with a methanol –water gradient. A four-point calibration curve was made from analysis of a 3 % albumin solution enriched with known concentrations of the B_2 vitamers. Recovery is > 95 %, the method is linear from 2-42 µg/L at least and the limit of detection is 0.3 µg/L.

4.3.3 Statistics

Wilcoxon Signed Ranks test were used as appropriate (software package: SPSS 11.0).

4.4 RESULTS AND DISCUSSION

The vitamin concentrations were grouped according to exposure-time of phototherapy irrespective of treatment regimen due to the low number of samples from two of the groups. Samples taken up to and including 31 h of light treatment were included. The sample material consisted of less than six samples in each time-group after 31 h, and hence, these samples were not included. In a few instances, when the sample collection times followed the hospital routine rather than the protocol, the samples were grouped with the times of best correspondence (approximately ± 3 h).

After 7 h of phototherapy the riboflavin concentration decreased to half the mean zero-time concentration (Fig. 4.1) and stayed at this lower concentration level throughout the treatment period. The reference values for riboflavin in plasma of infants (6-37 months) have been reported in the literature to be 12.7 - 53.4 nM (4.8 - 20 µg/l) (Capo-chichi et al. 2000). These numbers are lower than the mean riboflavin concentrations of the newborns in our study prior to phototherapy (22.7µg/l, SDEV ± 29). This finding indicates that evaporation of (some of) the samples may have occurred.

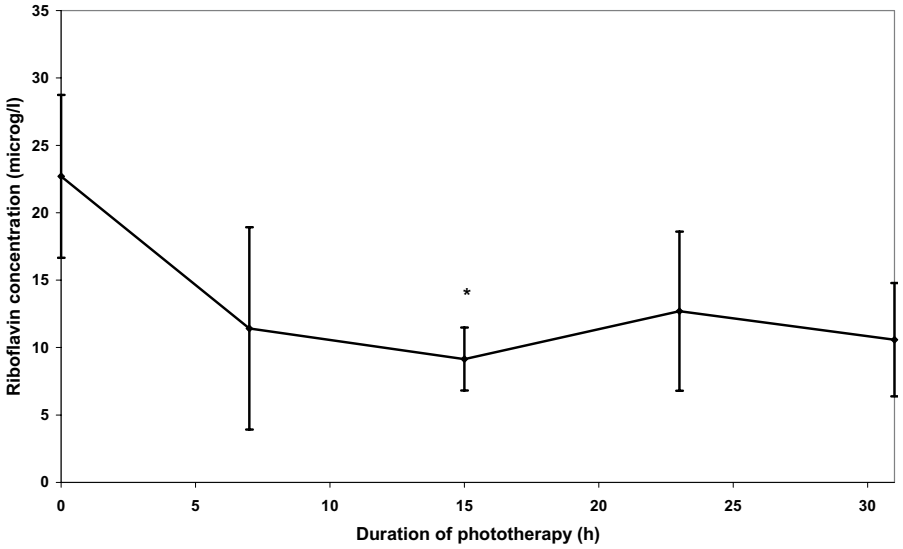

Fig. 4.1 Mean serum riboflavin concentration of jaundiced newborns before and during phototherapy (Philips 20W/52 or non-commercial turquoise Osram lamp). Irradiance: 1-2 mW/cm^2. ±SEM n_{0h}=23, n_{7h}= 20, n_{15h}= 15, n_{23h}=9, n_{31h}=5.*: Different from [riboflavin] before phototherapy, $p<0.05$.

Riboflavin has one of four absorption maxima at 447 nm (in aqueous solution). This absorption minimum enables the molecule to undergo photoreduction caused by wavelengths from blue phototherapy light. Flavins are known to catalyse several substances by phototsensitisation. In these processes, triplet flavin transfers energy to oxygen to yield singlet O_2 (Sisson 1987). For this reason, riboflavin is not recommended as a photosensitiser to increase the effect of phototherapy (Jährig et al. 1993).

A reduction in the riboflavin concentration in infant serum during phototherapy has been described by Sisson et al. (1976) and Gromisch et al.(1977) among others. There have been speculated that the riboflavin shortage would cause a greater haemolysis, disturbing the phototherapy effect and possibly lead to anaemia (Jährig et al. 1993). However, Jährig (1980) found no greater frequency of anaemia during long-term follow up. The riboflavin deficit was determined by Tan et al. (1978) to be prevented by 0.3 mg riboflavin daily, indicating no need for general riboflavin substitutions. However, more recently it has been suggested to increase the daily recommended riboflavin dose from 60 µg/100 kcal to 200-300 µg/100 kcal in preterm infants receiving prolonged phototherapy (Hansen 1993).

The sample size of the group comparing blue and turquoise wavelengths was too small to detect any possible differences between the two irradiation colours. It may be speculated that the turquoise light with emission peak of 490 nm could have a lesser photodegrading effect on riboflavin than blue light with an emission peak (450 nm) being almost identical to the absorbance peak of riboflavin (447 nm). Elucidation of any correlation between wavelength and riboflavin concentration clearly requires a wavelength comparison study.

Coenzyme derivatives of riboflavin are FAD and FMN. Together with riboflavin these are among the biologically most important flavins. The sum of FAD and FMN is expressed as flavocoenzyme concentration in Fig. 4.2. Although the flavocoenzymes are highly sensitive to light their concentrations stay constant throughout 31 h of phototherapy, perhaps due to a lesser distribution in the body tissues and fluids compared to riboflavin. Reference values of FAD and FMN in infant plasma as determined by Capo-chichi et al. (2000) are 53.5 – 108.2 nM and 9 - 25.1 nM, respectively. For comparison, based on the mean value of the molecular weights of the two enzymes, the mean concentration of the sum of FAD and FMN prior to phototherapy in our study is 11.6 nM. Taking evaporation of the samples into consideration as discussed in the case of riboflavin, the real concentration may be even lower, pointing to increased instability of the enzymes during storage, thawing and freezing.

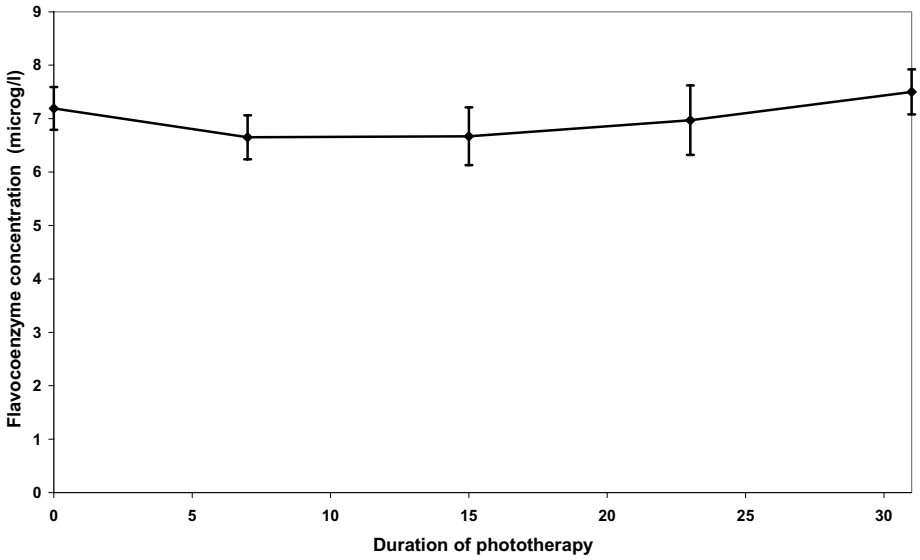

Fig. 4.2 Mean serum flavoenzyme concentration (sum of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN)) of jaundiced newborns before and during phototherapy (Philips 20W/52 or non-commercial turquoise Osram lamp). Irradiance: 1-2 mW/cm^2. ±SEM n_{0h}=25, n_{7h}= 20, n_{15h}= 15, n_{23h}=9, n_{31h}=6.

The retinol concentration shows a significant increase after 15 h of phototherapy, with a further increase after 31 h (Fig. 4.3), thus being unaffected by irradiation of the applied wavelengths in our material. The mean concentration obtained prior to phototherapy was 0.5 µM, which is lower than the reference level for infants (0.62-1.7 µM) 0-1 months old (Aruplab 2003) and lower than the concentration of 0.7 µM considered to indicate vitamin A deficiency (Moran and Greene 1998). The low retinol values may be caused by the long storage.

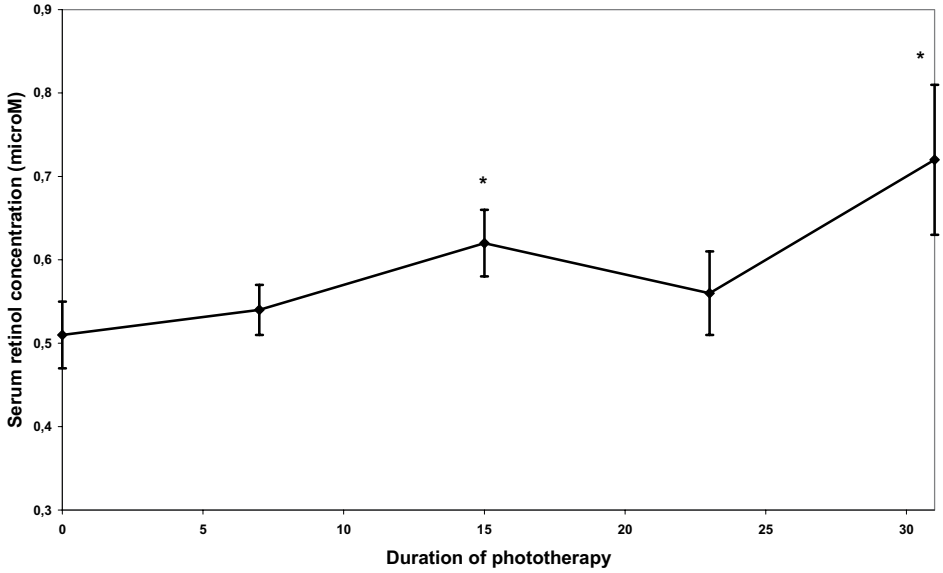

Fig. 4.3. Mean (all-trans) retinol concentration of 25 jaundiced newborns before and during phototherapy (Philips 20W/52 or non-commercial turquoise Osram lamp). Irradiance: 1-2 mW/cm^2. ±SEM n_{0h}=25, n_{7h}= 19, n_{15h}= 15, n_{23h}=9, n_{31h}=6.*: Different from [retinol] before phototherapy, $p<0.01$.

Although unsupplemented preterm infants have shown low retinol levels (Moran and Greene 1998), it is evident that the preterm neonates of the intensive care units in our study receive more vitamin A than do their term "peers" in the maternity ward (Fig. 4.4). The latter group does most likely receive adequate levels despite the low concentrations shown in the figure, as breast-fed infants of well-nourished mothers do not show signs of vitamin A deficiency (Olson 1987). Any supplementation of vitamin A may have caused a masking of a possible photodegradation of the vitamin.

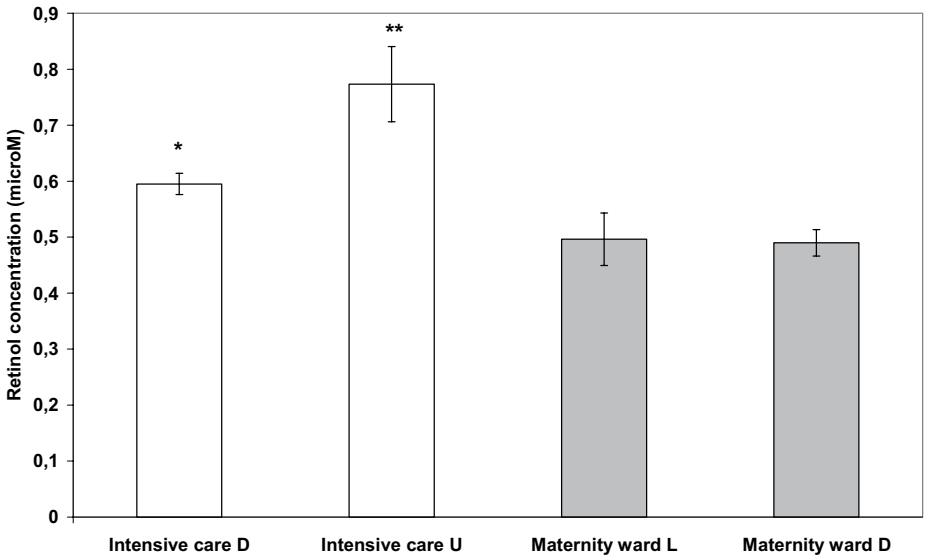

Fig. 4.4 Mean (all-trans) retinol serum concentration of jaundiced newborns irrespective of phototherapy in three hospitals (D, U and L) and two types of units; intensive care (white rods) or well-baby (grey rods). Bars: ±SEM. *: Sign. different from maternity wards L and D, $p<0.05$ **: Sign. different from intensive care D and maternity wards L and D, $p< 0.005$. From left to right: n (patients) = 10, 5, 4 and 4 and n (serum samples) = 51, 15, 11 and 12.

In summary, the serum samples collected from four hospital wards show a decrease in the riboflavin (vitamin B_2) concentration to half the initial concentration after seven h of phototherapy (various regimens) followed by a relatively constant concentration level throughout the 31 h irradiation period. On the other hand, retinol (vitamin A) in serum samples appears to be unaffected by phototherapy.

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4.6 REFERENCES

1. Aruplab.www.Aruplab.com. Accessed March 2003.
2. Capo-chichi CD, Gueant JL, Feillet F, Namour F, Vidailhet M. Analysis of riboflavin and riboflavin cofactor levels in plasma by high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl 2000;739(1):219-24.
3. Christensen T, Amundsen I, Kinn G, Kjeldstad B. Phototherapy of newborns with hyperbilirubinemia in Norwegian hospitals. A study of phototherapy units and light conditions. (In Norwegian with English summary). Østerås: Statens institutt for strålehygiene/National Institute of Radiation Hygiene, 1992; SIS report 1992:2.
4. Christensen T, Reitan JB. Phototherapy of hyperbilirubinemia in newborn children. (In Norwegian with English summary) Østerås: Statens institutt for strålehygiene/National Institute of Radiation Hygiene, 1987; SIS report 1987:6.
5. Ebbesen F, Agati G, Pratesi R. Phototherapy with turquoise versus blue light. Arch Dis Child Fetal Neonatal Ed 2003;88:F430-1.
6. Gromisch DS, Lopez R, Cole HS, Cooperman JM. Light- (phototherapy-) induced riboflavin deficiency in the neonate, J Pediatr 1977;90:118-22.
7. Hansen JW. Consensus recommendations. Niacin, panthothenic acis, and biotin. In: Tsang RC et al., eds. Nutritional needs of the preterm infant. Baltimore: Williams & Wilkins, 1993;288-91.
8. Hansen TWR. Therapeutic approaches to neonatal jaundice: An international survey. Clin Pediatr 1996;35:309-16.
9. IEC. Medical electrical equipment. Part 2-50: Particular requirements for the safety of infant phototherapy equipment. 1st ed. Geneva: International Electrotechnical Commission, 2000; International Standard IEC 60601-2-50:2000(E).
10. Jährig D. Untersuchungen zur Effektivität und zu den Nebenwirkungen der Phototherapie mit der Halogenmetalldampflampe NC-400-04 sowie zum Einsatz von Photosensibilisatoren in vitro und in vivo. Greifswald: University of Greifswald, 1980. Dissertation (B).
11. Jährig K, Jährig D, Meisel P, eds. Phototherapy. Treating neonatal jaundice with visible light. Munich: Quintessenz-Verlags-GmbH, 1993;105-116, 132-139.
12. Matthes R, Bernhardt J, McKinlay A, eds. Guidelines on limiting exposure to non-ionizing radiation. Munich: International Commission on Non-Ionising Radiation, 1999; ICNIRP report 7/99.
13. Moran JR, Greene HL. Vitamin requirements. In: Polin RA, Fox WW, eds. Fetal and neonatal physiology. Philadelphia: WB Saunders Company, 1998;1:344-53.
14. Olson JA. Recommended dietary intakes (RDI) of vitamin A in humans. Am J Clin Nutr 1987;45:704.
15. Roll EB, Christensen T. Phototherapy of hyperbilirubinemia in newborns; the need for a better treatment and follow-up. (In Norwegian with English summary). Nor Epid 1997;7(1):93-8.

16. Sisson TRC. Photodegradation of riboflavin in neonates. Proceedings of the 70th annual meeting of the Federatuon of American Societies for Experimental Biology. St- Louis: Federation Proc 1987;46:1883-7.
17. Sisson TRC, Slaven B, Hamiltion PB. Effect of broad and narrow spectrum fluorescent light on blood constituents. In: Bergsma D, Blondheim SH, eds. Bilirubin metabolism in the newborn (II). New York: American Elsevier for the National Foundation-March of Dimes, 1976; BD:OAS 12(1) 122-31.
18. Tan KL, Chow MT, Karun SMM. Effect of phototherapy on neonatal riboflavin status. J Pediatr 1978;93:494-5.

Part III: *In vitro* cell studies on adverse effects of phototherapy

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Bilirubin- and light induced cell death in a murine lymphoma cell line☆

Terje Christensen[a,b,*], Ellen B. Roll[a,b], Alicja Jaworska[a], Gunnar Kinn[a]

[a]*Radiation Medicine Department, Norwegian Radiation Protection Authority and Institutes of Radiology and Pathology, Norwegian National Hospital, University of Oslo, Oslo, Norway*

[b]*Institute of Physics, Norwegian University of Science and Technology, Trondheim, Norway*

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Abstract

Cells from the mouse lymphoma cell line L5178Y-R were exposed to blue light from phototherapy lamps in the presence of solutions of 160 μM bilirubin supplemented with serum albumin. HPLC analysis showed that the bilirubin solution was photooxidised as a function of increasing light dose. The cells were stained with trypan blue to score necrosis, and apoptosis was assayed by the terminal deoxynucleotide transferase assay (TdT) or by studying the nuclear structure in cells stained with propidium iodide. A rapidly developing apoptosis was observed after light doses killing 60–80% of the cells as judged from the trypan blue exclusion test. The fraction of apoptotic cells was smaller than the fraction of necrotic cells. Exposure of the cells to fractions of light at a high dose rate was compared to the effect of the same total dose at a lower dose rate given as a single fraction. No large differences were found, however, there was a tendency of a higher degree of necrosis as well as apoptosis in the cells receiving the light in fractions at a high dose rate.

Keywords: Phototherapy; Newborn; Bilirubin; Apoptosis; Necrosis; Blue light; Mouse lymphoma cells

1. Introduction

Phototherapy of neonatal hyperbilirubinemia has been performed for more than thirty years. In most hospitals the light treatment is given as a continuous irradiation only interrupted by short periods of feeding, and the light intensity and colour of the light being under poor control [1]. Several methods for improving the effect of phototherapy have been suggested, e.g. the use of more optimal wavelengths [2], more optimal irradiance [3] or fractionation of the light dose [4].

When choosing the right physical parameters it must be taken into consideration that blue light itself or in combination with bilirubin may cause photochemical tissue damage. The most serious risk is retinal damage if the eyes are exposed [5].

It has been shown that bilirubin may induce photosensitising effects on cells in culture [6–9].

Cells from several different cell lines may be killed by bilirubin and light, most efficiently when the cells are present in the light field suspended in a solution of bilirubin, but toxicity is also occurring when unexposed cells are added to bilirubin solutions having been exposed to high light doses prior to addition of the cells. It is assumed that the toxic effects are caused by hydrogen peroxide and other peroxides [7,8]. The present article deals with the mode of cell death, and it is our intention to further elucidate the possibility that apoptosis, or programmed cell death, may be induced. Previous observations of damage to DNA, induction of mutations, inhibition of cell multiplication and inhibition of the progression through the cell cycle [10] are of particular interest. Studies of the line 308 mouse epidermal cells indicated that double strand breaks in the DNA were formed, but the morphology of the cells after light irradiation in the presence of bilirubin did not indicate changes typical for late apoptosis [11].

Bilirubin is an antioxidant and has been reported to be a scavenger of peroxyl radicals generated chemically [12–14].

L5178Y-R (LY-R) murine lymphoma cell line was

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*Corresponding author. Norwegian Radiation Protection Authority, P.O. Box 55, N-1332 Østerås, Norway. Internet: http://www.nrpa.no. Fax: +47-22461304.

E-mail address: Terje.Christensen@nrpa.no (T. Christensen).

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isolated from a methylcholantrene-induced lymphoma in a DBA/2 mouse at the end of the 1950s. This cell line, together with its variant (LY-S), is extensively characterised with respect to sensitivity to different types of radiation (ionising-, UV- and visible-), oxidants and other factors which may influence radiation sensitivity [15–17]. Several studies on DNA damage and -repair have been performed and apoptosis has been the subject of studies in the last decade [16,18]. The enzymatic antioxidant system has been extensively studied in this cell line as well [19]. Briefly, LY-R cells are relatively sensitive to UV [20] and H_2O_2, relatively deficient in H_2O_2 scavenging enzymes activity, but not deficient in superoxide dismutase activity.

The aims of the present study are:

- To find out whether LY-R cells are killed by an apoptotic mechanism when subjected to light irradiation in the presence of bilirubin.
- To determine how fast the apoptotic process proceeds.
- To study the influence of intermittent light exposure compared to continuous irradiation.

2. Materials and methods

2.1. Cell culture

Suspension cultures of mouse lymphoma LY-R cells were cultivated in RPMI 1640 medium (GIBCO) containing 10% foetal calf serum. 500.000 cells/ml were dispensed in 25 cm^2 tissue culture flasks prior to treatment.

2.2. Experimental solution

Bilirubin (BR) (mixed isomers from bovine gall stones, Sigma) was used without further purification. The bilirubin was dissolved in 0.01 or 0.1 N NaOH as a stock solution of 1600 μM and used within 4 h. It was filtered through a sterile filter and adjusted to pH 7.2–7.4 with 0.1 N HCl if necessary. The BR stock solution was added to a final concentration of 160 μM BR in solutions of phosphate buffered saline (PBS) with supplements of serum albumin. Two different concentrations of serum albumin were used: Either a dilution of foetal calf serum yielding approximately 50 μM bovine serum albumin (BSA) or a solution of 200 μM human serum albumin (HSA, Sigma, essentially fatty acid free). It should be noted that in the former solution the BR concentration was higher than the concentration of albumin while it can be assumed that most of the BR was bound to HSA in the latter due to the higher molar concentration of HSA compared to BR.

2.3. Light irradiation and dosimetry

Irradiation of the cells was performed in flasks on a plexiglass stand placed above two blue fluorescent tubes (Philips TL20W/52 commonly used in phototherapy). The lamps emit blue light with a spectral distribution peaking at 450 nm. The spectra do not include ultraviolet radiation to any significant extent. The spectrum of the lamps and dose measurements have been published previously [21]. The irradiances used in the present experiments were either 5.5 mW/cm^2 (termed "I") or half this value (2.7 mW/cm^2, "I/2") depending on the distance between the lamps and the plexiglass stand. There was a certain variation in irradiance (5–10%) as a function of the position of the flasks in the longitudinal direction of the lamps. To avoid any systematic effect of this variation, the flasks were placed on the stand in random order and the positions of the flasks were exchanged throughout the experiment.

2.4. High pressure liquid chromatography

Cell culture flasks (25 cm^2) containing 5 ml of bilirubin solution (160 μM BR, 200 μM HSA in PBS) were placed on top of two fluorescent light tubes as described above. Mean irradiance along the tubes was 5.5 mW/cm^2. Samples for HPLC-analysis at a detection wavelength of 450 nm were taken after 0, 1, 2.5, 5, 10 and 15 min respectively and subsequently every 15 min for a total period of 120 min. The samples were diluted 1:4 with mobile phase and centrifuged for 5 min at 5000 rpm. The supernatant was gassed with helium prior to HPLC-detection. The HPLC instrument consisted of a Shimadzu LC-10 AT liquid chromatograph, SPD-10 AV spectrophotometric detector, equipped with a Nucleosil 120-5C18 reversed-phase column (250×4.6 mm) (Macherey–Nagel AG). Integration was done with Class LC-10 software. The mobile phase consisted of methanol (HPLC-grade, Rathburn), acetic acid (puriss.p.a., Fluka) and destilled water, and pH was adjusted to 7.7 with dioctylamine (for synthesis, Merck). It was continuously gassed with helium gas during analysis, and the flow was set to 1 ml/min. The areas under the peaks of the chromatograms were used in calculating the relative concentrations of the bilirubin isomers. The sum of the areas of the 4Z,15Z and the photoisomers (4Z,15E), (4E,15Z), E- and Z-lumirubin in a non-irradiated bilirubin solution was normalised to 1.0. The integrated peaks were corrected for differences in isomer absorption at 450 nm relative to 4Z,15Z. The relative extinction coefficients for 4Z,15Z; 4Z,15E; 4E,15Z; 15Z-lumirubin; 15E-lumirubin at 450 nm are 1;0.8;0.98;0.59 and 0.49, respectively [22].

2.5. Measurements of apoptosis

The procedure of terminal deoxynucleotide transferase (TdT) assay was taken from Gorczyca et al. [23] with slight modification. Briefly, cells were fixed in 1% paraformaldehyde in PBS followed by 100% methanol at −20°C. To detect apoptosis the fixed cells were incubated in TdT-solution (5 units in 50 μl) with biotinylated deoxyuridinetriphosphate (Boehringer, Mannheim) as substrate for 30 min at 37°C and washed, and the cell pellets were

resuspended in PBS with streptavidin-fluorescein (Amersham), 0.1% Triton X-100 and 3% fat-free milk powder. After 30 min at 4°C, and washing with PBS the cells were treated with 100 μg/ml RNAase (Boehringer, Mannheim), and stained with propidium iodide (PI) (5 μg/ml) for total DNA content. Red fluorescence from the dye propidium iodide (PI) and green fluorescence from labelling DNA-ends in the TdT assay were measured for 10^4 cells in a Vantage laser flow cytometer (Becton-Dickinson, USA).

Apoptosis in the mouse lymphoma cells was also evaluated in the fluorescence microscope. At least 100 cells were counted in each sample.

Morphological changes in the nuclei were studied in samples stained with propidium iodide as described above. The nuclei were evaluated by counting the percentage of the cells with fragmented nuclei. In the samples stained for the TdT assay, the percentage of the cells with green fluorescence was determined.

Viable and dead cell numbers were determined based on exclusion of trypan blue dye 18 h after light treatment.

3. Results

Fig. 1 indicates that a fraction of the cells treated with bilirubin and light showed signs of apoptosis very rapidly after the end of light treatment. The percentage of apoptotic cells did not increase substantially for incubation periods longer than 3 h, but a tendency towards disintegration was observed in the microscope after incubation times longer than approx. 18 h. In the later experiments an incubation time of 3 h after the end of irradiation was chosen as standard.

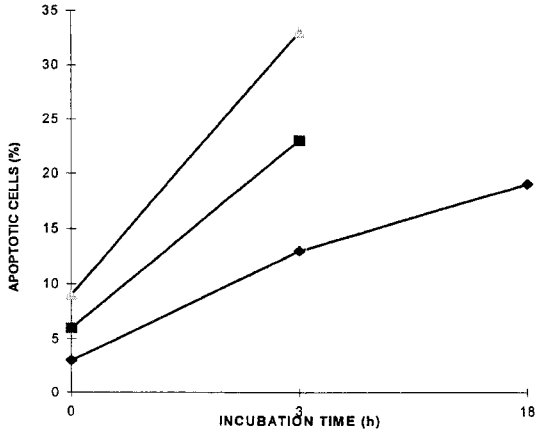

Fig. 1. The induction of apoptosis assayed by the formation of fragmented nuclei in mouse lymphoma cells stained with propidium iodide at 0, 3 and 18 h after blue light irradiation for 1.5 h of 5.5 mW/cm^2 irradiance in a solution of bilirubin containing BSA. Results from three independent experiments are shown.

Table 1
Apoptosis in mouse lymphoma cells assayed with three different methods[a]

Treatment	Method	Apoptosis (%)	Standard deviation
Control	PI	2.4	1.3
Control	TdT, microscopy	6.7	*: two experim.
Control	TdT, flow cytom.	4.2	3.8
1.5 h light, BR	PI	20	8.0
1.5 h light, BR	TdT, microscopy	40	23
1.5 h light, BR	TdT, flow cytom.	35	25

[a] The cells were suspended in a bilirubin solution containing BSA and irradiated with blue light (5.5 mW/cm^2). The cells were fixated 3 h after the end of irradiation.

The different methods for observing apoptosis gave corresponding results as shown in Table 1. It can also be observed that about 60% of the cells were non-viable cells as indicated by trypan blue staining 18 h after irradiation for 1.5 h in the presence of BSA and that the percentage of apoptotic cells is smaller than the fraction that can be scored as necrotic by trypan blue staining (Table 1, Fig. 2). This was also the case when the cells were irradiated in the presence of HSA (Table 2). By comparing the sensitivity to cell death, either by necrosis (Fig. 2) or by apoptosis (Tables 1 and 2) with the doses leading to significant photooxidation of bilirubin (Fig. 3) [11], one may draw the conclusion that the cell killing is taking place at light doses causing observable photooxidation of bilirubin.

Table 2 shows the comparison between the effects of intermittent vs. continuous irradiation. Due to long time incubation in PBS the toxicity in the controls was relatively high compared to the values seen in Table 1 and Fig. 2 (values for samples treated for 2 h or less in the presence of BSA). No significant difference between cells treated with continuous and split light doses, respectively, can be

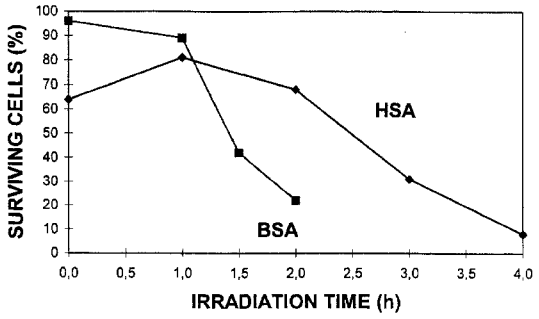

Fig. 2. Induction of necrosis in mouse lymphoma cells irradiated with blue light (5.5 mW/cm^2) for 0–4 h in solutions of bilirubin and BSA or HSA. Exclusion of trypan blue was assayed after 18 h incubation.

Table 2
Cell necrosis assayed with trypan blue exclusion and apoptosis assayed with two different methods in mouse lymphoma cells[a]

Sample	Necrosis, trypan blue (%)	S.E.	Apoptosis, PI (%)	S.E.	Apoptosis, TdT (%)	S.E.
I, control, split	34	12.4	42.3	3.8	39.4	6.1
I/2, control, continuous	26	9.3	35.4	5.1	26.4	5.3
I BR, split	71.3	10.7	67.8	6.1	69.5	5.5
I/2 BR, continuous	61	9.6	54.9	11.5	44.9	3.9
Dark control	20.3	5.1	25.3	6.3	24.5	4.6
Dark BR	19	2.1	25.0	8.4	25.3	5.9

[a] The cells were suspended in a bilirubin solution containing HSA and irradiated with blue light, either 5.5 mW/cm^2 (I) for a total of 3 h in three fractions of 1 h each, interrupted by two 1 h periods in the dark, or continuously for 6 h at irradiance of 2.7 mW/cm^2 (I/2). In both cases the total light fluence was the same (5.9 J/cm^2). The cells were fixated 3 h after the end of irradiation. Mean of three separate experiments with two parallel samples and corresponding standard error are shown.

observed, although there is a tendency of higher toxicity and more apoptosis after split light exposures.

4. Discussion

The present study has shown that mouse lymphoma cells of the line L5178Y-R can undergo apoptosis and that the development of the reaction is relatively rapid (Fig. 1). Early apoptosis in cells in vitro is also induced by other photosensitised reactions, e.g. by photoactivated protoporphyrin [24]. However, the development of apoptosis is dependent on the sub-cellular localisation of the photosensitising drugs [25].

Three different methods have been used to measure the fluorescence from the cells, and two different biological characteristics, namely the presence of free DNA-ends and morphological changes of the nuclei, give rise to the differences in fluorescence observed. In a previous paper

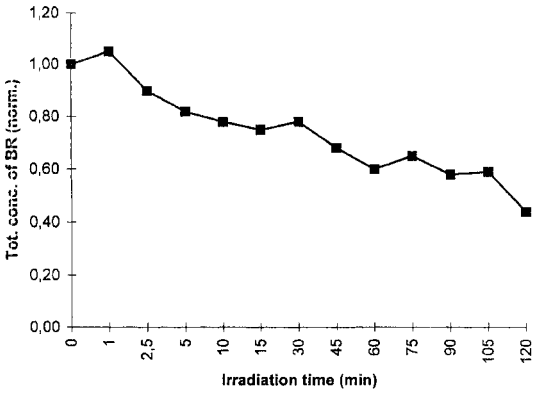

Fig. 3. The sum of conc. of ZZ-BR and photoisomers E-lum, Z-lum and ZE/EZ-BR after irradiation times of 0–120 min. Total conc. is normalised to 1.0 prior to irradiation.

[11] an indication of double strand breaks in the DNA of mouse 308 epitheloid cells was found, but no gross morphological changes were observed. The choice of LY-R cells in the continuation of our studies was based on the fact that they are sensitive to ultraviolet radiation and hydrogen peroxide, and it has been shown by the authors [11] and by Rosenstein et al. [7] that this species and other peroxides may be important for the phototoxic effects of bilirubin. It was shown previously that the phototoxicity is prominent after light doses that cause photooxidation of bilirubin, which is supported by the data in Figs. 2 and 3. Furthermore, it was shown that a fraction of the phototoxic products leading to cellular damage was formed in the medium outside the cells, and that addition of catalase, but not superoxide dismutase, to the medium reduced the cytotoxicity [8,9]. Therefore, it is reasonable to assume that the fact that the LY-R cells undergo apoptosis is a result of hydrogen peroxide production in the cells and in the medium.

The toxicity of bilirubin in the dark is not fully elucidated, but several mechanisms are possible [26]. It is probable that the dark toxicity and the phototoxicity of bilirubin act by different mechanisms and that some of the phototoxic reactions are a «type III»-photosensitising effect arising as a result of the toxicity of the photoproducts of bilirubin acting together with the production of H_2O_2 [7]. In cells treated with photoactivated bilirubin, it is to be expected that the cellular damage is a mixture of responses to bilirubin and its photoproducts. The present paper indicate that apoptosis as well as necrosis are possible modes of cell death in both cases.

Our data show that the presence of free bilirubin (not bound to albumin) increases the efficiency of the photosensitisation. In Fig. 2 and Tables 1 and 2 it can bee seen that the cells are killed by a lower light dose when suspended in 50 μM BSA instead of a solution of 200 μM HSA. Both solutions contained 160 μM bilirubin, however, the latter containing HSA had a higher molar concentration and most of the bilirubin was bound to the HSA since bilirubin is bound reversibly to at least two

binding sites on the albumin molecule with high binding constants (see [27] and Refs. therein).

The split dose experiments show that there is a possibility that delivery of light at a high dose rate in fractions may give slightly more cellular damage than continuous light exposure, but these experiments are technically difficult to perform and the variance between individual experiments is large. Furthermore, the observed toxicity in the controls is relatively high. The temperature of the cell suspensions were measured during a typical experiment and was found to be below 37°C. In the future more detailed studies will be performed to see if the tendency observed is significant, and to attempt to explain a possible split dose effect or a dose rate effect. The dose rate during phototherapy may influence the formation of bilirubin isomers. Probably there exists an optimal dose rate where the therapeutic effect is maximum [3] and the detrimental effects of light exposure are of limited significance for the patient.

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References

[1] T. Christensen, J.B. Reitan, Fototerapi av hyperbilirubinemi hos nyfødte. SIS Rapport 1987, p. 6 (in Norwegian).
[2] C. Vecchi, G.P. Donzelli, M.G. Migliorini, G. Sbrana, R. Pratesi, New light in phototherapy, Lancet 14 (1982) 390.
[3] K.L. Tan, The pattern of bilirubin response of phototherapy for neonatal hyperbilirubinemia, Pediatr. Res. 16 (1982) 670–674.
[4] D. Jährig, A. Berck, P. Meisel, K. Jährig, Die Kalkulation dic Phototherapieeffektes unter Berücksichtigung verschiedener Bestrahlungsvarianten. I. Der Einfluss des Therapiemodus auf den Photoeffect, Kinderärzl. Praxis 53 (1985) 171–175.
[5] T.R.C. Sisson, S.C. Glauser, E.M. Glauser, W. Tasman, T. Kuwabara, Retinal changes produced by phototherapy, J. Pediatrics 77 (1970) 221–227.
[6] T. Christensen, J.B. Reitan, G. Kinn, Single strand breaks in the DNA of human cells exposed to visible light from phototherapy lamps in the presence and absence of bilirubin, J. Photochem. Photobiol. B: Biol. 7 (1990) 337–346.
[7] B.S. Rosenstein, J.M. Ducore, S.W. Cummings, The mechanism of bilirubin-photosensitized DNA strand breakage in human cells exposed to phototherapy light, Mutation Res. 112 (1983) 397–406.
[8] T. Christensen, Cytotoxicity of bilirubin photoproducts, Photobiochem. Photobiophys. 10 (1986) 253–260.
[9] T. Christensen, A. Støttum, G. Brunborg, J.B. Reitan, Unwanted side effects and optimization of phototherapy, in: R.H. Douglas, J. Moan, F. Dall'Acqua (Eds.), Light in Biology and Medicine, Vol. 1, Plenum Press, New York and London, 1988, pp. 153–159.
[10] T. Christensen, G. Kinn, T. Granli, I. Amundsen, Bilirubin and light effects on cells. 12th International Congress on Photobiology, Wien 1–6. Sept., 1996 (abstract nr. O 46)
[11] T. Christensen, G. Kinn, I. Gradzka, A. Jaworska, E.B. Roll, Photosensitising effects of bilirubin First Internet Conference on Photochemistry and Photobiology, November 17–December 12, 1997, Internet Journal of Science Biological Chemistry, Vol. 3, 1997, http://www.photobiology.com/v1/contrib.htm
[12] R. Stocker, B.N. Ames, Potential role of conjugated bilirubin and copper in the metabolism of lipid peroxides in bile, Proc. Natl. Acad. Sci. USA 84 (1987) 8130–8134.
[13] R. Stocker, A.N. Glazer, B.N. Ames, Antioxidant activity of albumin-bound bilirubin, Proc. Natl. Acad. Sci. USA 84 (1987) 5918–5922.
[14] R. Stocker, Y. Yamamoto, A.F. McDonagh, A.N. Glazer, B.N. Ames, Bilirubin is an antioxidant of possible physiological importance, Science 235 (1987) 1043–1046.
[15] J.Z. Beer, E. Budzicka, E. Niepokojczycka et al., Loss of tumorigenicity with simultaneous changes in radiosensitivity and photosensitivity during in vitro growth of L5178Y murine lymphoma cells, Cancer Res. 43 (1983) 4736–4742.
[16] D.E. Godar, A.D. Lucas, Spectral dependence of UV-induced immediate and delayed apoptosis: The role of membrane and DNA damage, Photochem. Photobiol. 62 (1995) 108–113.
[17] D. Separovic, J. He, N.L. Oleinick, Ceramide generation in response to photodynamic treatment of L5178Y mouse lymphoma cells, Cancer Res. 57 (1997) 1717–1721.
[18] H. Tauchi, S. Sawada, Analysis of mitotic cell death by radiation in mouse leukaemia L5178Y cells: Apoptosis is the ultimate form of cell death following mitotic failure, Int. J. Radiat. Biol. 65 (1994) 449–455.
[19] E. Bouzyk, T. Iwanenko, N. Jarocewicz, M. Kruszewski, B. Sochanowicz, I. Szumiel, Antioxidant defense system in differentially hydrogen peroxide sensitive L5178Y sublines, Free Radical Biology and Medicine 22 (1997) 697–704.
[20] J.Z. Beer, K.M. Olvey, S.A. Miller, D.P. Thomas, D.E. Godar, Non-nuclear damage and cell lysis are induced by UVA, but not UVB or UVC, radiation in three strains of L5178Y cells, Photochem. Photobiol. 58 (1993) 676–681.
[21] T. Christensen, G. Kinn, T. Granli, I. Amundsen, Cells, bilirubin and light. Formation of photoproducts and cellular effects at defined wavelengths, Acta Paediatr. 83 (1994) 7–12.
[22] G. Agati, F. Fusi, S. Pratesi, P. Galvan, G. P Donzelli, Bilirubin photoisomerization products in serum and urine from a Crigler-Najjar type I patient treated by phototherapy, J. Photochem. Photobiol. 47 (1998) 181–189.
[23] W. Gorczyca, H. Gong, Z. Darzynkiewicz, Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays, Cancer Res. 53 (1993) 1945–1951.
[24] B.B. Noodt, K. Berg, T. Stokke, Q. Peng, J.M. Nesland, Apoptosis and necrosis induced with light and 5-aminolaevulinic acid-derived protoporphyrin IX, Br. J. Cancer 74 (1996) 22–29.
[25] D. Kessel, Y. Luo, Y. Deng, C.K. Chang, The role of subcellular localization in initiation of apoptosis by photodynamic therapy, Photochem. Photobiol. 65 (1997) 422–426.
[26] J.W. McDonald, S.M. Shapiro, F.S. Silverstein, M.V. Johnston, Role of glutamate receptor-mediated excitotoxicity in bilirubin-induced brain injury in the Gunn rat model, Exp. Neurol. 150 (1998) 21–29.
[27] R. Brodersen, Physical chemistry of bilirubin binding to macromolecules and membranes, in: K.P.M. Heirwegh, S.B. Brown (Eds.), Chemistry, Bilirubin, Vol. I, CRC Press, Boca Raton, 1982, pp. 75–124.

Paper 6

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Bilirubin induces apoptosis in the dark, and irrespective of irradiation regimen, in mouse lymphoma cells in culture.

Ellen Bruzell Roll

Norwegian Radiation Protection Authority, POB 55, NO-1332 ØSTERÅS, Norway
and
Department of Physics, Norwegian University of Science and Technology,
NO-7491 TRONDHEIM, Norway

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Paper 7

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Formation of photoproducts and cytotoxicity from bilirubin irradiated with turquoise and blue phototherapy light.

Ellen Bruzell Roll[1,2] and Terje Christensen[1]

[1]Norwegian Radiation Protection Authority
NO-1332 ØSTERÅS, Norway
[2]Department of Physics, Norwegian University of Science and Technology, NO-7491
TRONDHEIM, Norway

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Paper 8

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Effects of bilirubin and blue light on the osmotic fragility and the haematoporphyrin-induced delayed photohaemolysis of erythrocytes and spherocytes.

Ellen Bruzell Roll[1,2], Terje Christensen[1] and Odrun A. Gederaas[3]

[1]Norwegian Radiation Protection Authority, POB 55, NO-1332 ØSTERÅS, Norway
[2]Department of Physics, Norwegian University of Science and Technology, NO-7491 TRONDHEIM, Norway
[3]Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, NO-7489 TRONDHEIM, Norway.

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