Melatonin for prevention of erythema and oxidative stress in response to ultraviolet radiation

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THE THREE ORIGINAL PAPERS ARE

1. Scheuer C, Pommergaard HC, Rosenberg J, Gögenur I. Melatonin's protective effect against UV radiation: a systematic review of clinical and experimental studies. Photodermatol Photoimmunol Photomed 2014; 30: 180-8.
2. Scheuer C, Pommergaard HC, Rosenberg J, Gögenur I. Dose dependent sun protective effect of topical melatonin: a randomized, placebo-controlled, double-blind study. Journal of Dermatological Science 84 (2016) pp. 178-185.
3. Scheuer C, Pommergaard HC, Rosenberg J, Gögenur I. Effect on cognitive parameters when using melatonin cream 12.5%: a randomized, placebo-controlled, double-blind crossover study in healthy volunteers. J Dermatolog Treat. 2016 Nov;27(6):488-494. Epub 2016 Apr 7.

INTRODUCTION:

Skin cancer is an escalating problem in dermatology, and worldwide an increasing incidence of skin cancer, especially non-melanoma skin cancer, has been reported (1-3). Ultraviolet radiation (UVR) from sun exposure is known as a pivotal factor in the initiation and development of skin cancer (4-7). Thus, it seems to be the cumulative sun exposure that leads to the development of non-melanoma skin cancer (8). It is well known that regular use of sunscreen can prevent the development of non-melanoma skin cancer (9). However, despite the growing knowledge about the pathogenesis and development of skin cancer, and the extensive public information regarding use of sun protection, it has not been accomplished to oppose the ongoing increase in incidence (2, 10). It has been suggested that the depletion of the ozone layer and the aging population will only make skin cancer, particularly non-melanoma skin cancer, a progressing problem in the future (10). Furthermore, with the widely use of immunosuppressive medicine in a broad range of diseases, an emerging population of patients with elevated risk of developing non-melanoma skin cancer has evolved (11-17). This calls for development of new and more effective sun protecting agents with other qualities than simple chemical reflection of UVR.

Melatonin, the main hormone of the pineal gland, was first regarded as having great influence on circadian rhythm and reproduction (18). Since then, research has revealed many other actions of melatonin including being the most potent endogenous antioxidant in our organism (19-22). Melatonin exerts its antioxidant effects acting directly as a radical scavenger (22), and indirectly by inducing up-regulation of gene expression and activity of several antioxidant enzymes (23-25). Furthermore, melatonin has shown to have anti-inflammatory effects by regulation of a number of pharmacological targets including inducible nitric oxide synthase (iNOS), cyclo oxygenase 2 (COX2), cytokines such as tumour necrosis factor α (TNF-α), and adhesion molecules (26).

Generation of reactive oxygen species (ROS) and the resulting oxidative stress is known to be a key factor in UVR-induced skin damage and eventually skin cancer (27, 28). Thus, substances with antioxidative activity, such as melatonin, could be effective in the prevention of short- and long-term UVR-induced skin damage. Furthermore, several laboratory and clinical studies have investigated melatonin's protective effect against UVR-induced skin damage (29-46); however, no clear conclusion on melatonin's sun protective effect has been found in the literature.

Previous clinical studies investigating melatonin's protective effect against UVR-induced erythema have all used artificial UVR-sources. In order to give the most clinically relevant evaluation of melatonin's sun protective qualities, studies assessing erythema induced by natural sunlight are warranted, and evaluation of the possible adverse effects has to be explored.

The aim of this PhD thesis was to evaluate the literature and clarify any protective effect of melatonin against UVR-induced skin damage. Furthermore, we wanted to investigate the protective effect of topical melatonin against erythema induced by natural sunlight, and to investigate the extent of side effects.

BACKGROUND:

The skin is our largest organ, and acts as a barrier towards harmful exogenous agents, thereby preserving the homeostasis of the body. The human skin is exposed to stressors, such as UVR and exogenous chemicals, which necessitates an elaborate system of antioxidant substances and enzymes as protection against deleterious agents (47).

Pathophysiology of UVR-induced skin cancer:

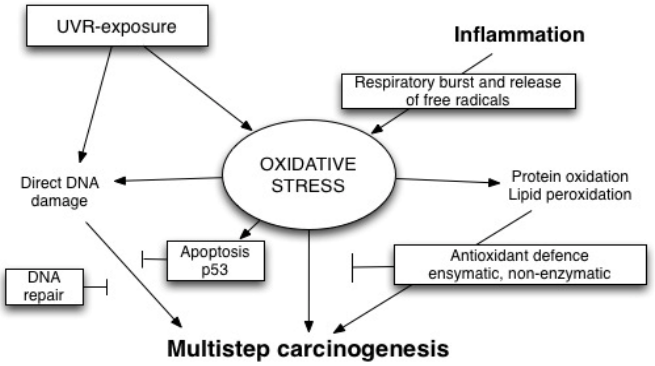
The human skin is continuously exposed to UVR, and the damaging effects can be divided into acute damages represented by erythema, pain and oedema, and more serious chronic damages represented by premature skin aging (4-7), premalignant skin lesions e.g. actinic keratosis (48) and skin cancer (49).

Generation of ROS induced by UVR-exposure leads to depletion of the enzymatic and non-enzymatic antioxidants in the skin. If the generation is further increased and exceeds the antioxidant defence mechanism, a condition of oxidative stress is created. ROS rapidly reacts with nuclear macromolecules and induces damage to several intracellular structures by mechanisms such as lipid peroxidation, formation of 8-OHdG (8-hydroxy-2'-deoxyguanosine) (oxidative damaged DNA) with the ability to induce G:C to T:A transversions, DNA single- and double-strand breaks and DNA protein cross-linking (50, 51). High levels of 8-OHdG has been observed in various kind of human and animal cancers, indicating that oxidative damage of DNA may be part of the aetiology in cancer (52). Mitochondria are known as the main source of free radical generation in human cells, and excess free radical generation is considered to be a consequence of impairment of the electron transport chain (ETC) (53).

Mutations in specific genes and alterations in cell proliferation and apoptosis are important in development of skin cancer. The p53 tumour suppressor gene is the most frequently mutated gene in cancer. P53 is essential in maintaining genomic integrity through its ability to block DNA replication in response to DNA damage, and mutations result in loss of this tumour inhibiting function (27, 49, 54, 55).

Sunburn erythema is a well-known acute cutaneous response to UVR, caused by an inflammatory reaction in the skin. An association between epithelial cancer and inflammation has been established (56, 57), and this association is supported by the findings that mice deficient in TNF-α are resistant to skin carcinogenesis, indicating that an intact TNF-signalling pathway may be required for induction of skin tumours (58, 59). TNF-α is a pro-inflammatory cytokine and a major inducer of NFκB (nuclear factor kappa B), a key regulator of carcinogenesis. Activation of NFκB promotes cellular proliferation and inhibits apoptosis thereby counteracting p53 and promotes cancer development (60).

Figure 1:



Schematic figure illustrating the possible involvement of oxidative stress and antioxidants in UVR-induced multistep carcinogenesis.

MELATONIN:

The synthesis of melatonin is controlled by a light/dark cycle. The regulation is mediated through a multi-synaptic pathway starting with the ganglion cells in the retina. The information is transported via nerve fibres through the retinohypothalamic tract to the suprachiasmatic nucleus, and arrives in the pineal gland via postganglionic sympathetic fibres (61). In humans, the regulation follows an endogenous circadian rhythm with increasing levels of melatonin right after onset of darkness, reaching peak concentrations around 2-4 a.m. and return to undetectable levels in daytime (18, 62). Melatonin serum concentrations vary considerably according to age, with the highest concentrations found in children from one to three years of age, after which serum melatonin declines gradually throughout life (63). The profile of the serum melatonin concentration shows substantial inter-individual differences, but great individual reproducibility (62). Due to its highly hydro- and lipophilic nature, melatonin can easily cross cell membranes and is excreted in saliva, bile, urine, and cerebrospinal fluid (64, 65). The physiological level of melatonin varies greatly between different tissues, body liquids and organs, and in several body compartments concentrations 10-100 times higher than serum have been found. Research has revealed several extra-pineal sites of melatonin synthesis e.g. gut, liver, bone marrow, ovary, eye, lymphocytes, and skin, which may explain the differing concentrations (66-71). Melatonin is mainly metabolized in the liver by hydroxylation to 6-hydroxymelatonin and after conjugation with sulfuric and glucuronic acid is excreted in the urine (18). The bioavailability of exogenous melatonin when given intravenously is 100%, and after a bolus administration, melatonin displays a bi-exponential decay with a first distribution half-life of two minutes and a second metabolic half-life of 20 minutes (62). Oral bioavailability of melatonin is low and is reported to differ up to 37-fold (72) and varies greatly due to a significant first-pass metabolism (73, 74). A dose of 5 mg creates serum melatonin concentrations 10-100 times higher than the natural night-time peak, one hour after ingestion, and declines to baseline values within four to eight hours (18). Transdermal delivery of melatonin shows delayed plasma profiles compared with transmucosal and oral administration (75), suggesting deposition of melatonin in the skin.

It is well established that melatonin is regarded as the hormone responsible for maintaining a circadian rhythm in humans. Furthermore, research has found that melatonin induces a faster onset of sleep and a less disturbed sleep than placebo (76), and due to its hypnotic and chronobiotic properties, the agent is well recognized in the treatment of age-related insomnia (77) and circadian rhythm sleep disorders such as delayed sleep phase syndrome, non-24-hour sleep disorder and jetlag (78, 79). Thus, when using melatonin for purposes not involving sleep or circadian rhythm, the sleep inducing qualities may become an unwelcome side effect.

MELATONIN AS SUN PROTECTING AGENT – RATIONALE:

Melatonin conducts its antioxidant effect acting directly as a radical scavenger (22) and indirectly by inducing up-regulation of gene expression and activity of several antioxidant enzymes (23-25). Furthermore, the metabolites produced during melatonin's metabolism, N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) and N^1-acetyl-5- methoxykynuramine (AMK), have shown to contribute with antioxidant effects (80), which enables melatonin and its metabolites to exert continuous protection referred to as the free radical scavenger cascade (81-83).

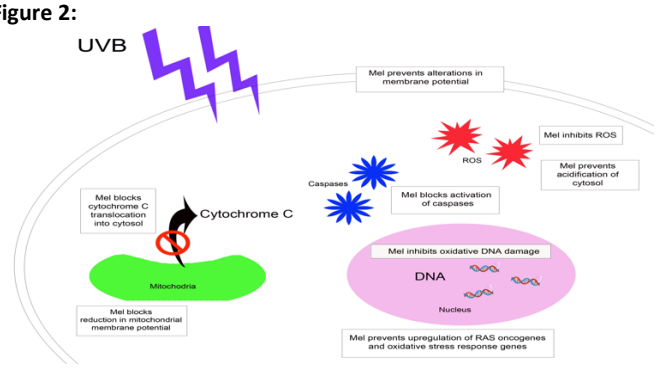
Due to its lipohilic structure, melatonin is able to penetrate cell membranes and can affect both extra- and intracellular structures (84), and an accumulation is seen in the mitochondria. UVR-

induced production of ROS results in mitochondrial membrane damage and thereby impairment of ETC (50, 85). Melatonin reduces electron leakage from mitochondria by stimulating the activities of the respiratory chain complexes (85-87).

Melatonin has shown to have anti-inflammatory effects by regulating a number of pharmacological targets, including iNOS, COX-2, cytokines such as TNF-α, and adhesion molecules (26), and by down-regulating signal transduction pathways including the NFκB pathway (26). As inflammation has been associated to development of cancer (56, 57), melatonin may have a protective effect through its anti-inflammatory effects. Erythema is another result of acute inflammation induced by UVR (88). However, the molecules responsible for light absorption (chromophores) that initiate sunburn inflammation have not been precisely identified (89).

Transdermal applied melatonin is also deposited in the skin for a longer period, leading to a prolonged antioxidant action (75), compared to other routes of administration.

Figure 2:



Schematic figure illustrating the cellular structures, which are affected by UVR and melatonin's possible mechanisms of effect against the UVR-induced cellular damages (reproduced with permission from Photodermatology, Photoimmunology and Photomedicine (90)).

OBJECTIVES:

This PhD thesis was formed on basis of three papers and the objectives were:

- To review and evaluate whether a protective effect of topical melatonin treatment against UVR-induced skin damage exists by evaluating the literature on the topic.
- To clinically assess the protective effect of topical treatment with melatonin against natural sun exposure and determine the optimal concentration.
- To clinically evaluate the degree of cognitive dysfunction with full body application of topical melatonin.

METHODS AND MATERIALS:

Chromatography, Image J:

Erythema is produced as an acute response to UVR, and the intensity of the erythema is correlated to the degree of UVR-induced DNA damage (91, 92). We evaluated erythema with a validated method using software analysis (Image J, version 1.45S, National Institute of health, USA) of digital photos (93). In the software analysis, the "colour space converter" function was used to convert the pictures into red/white colour scale. Erythema was quantified by pixel colour analyses, where all white coloured pixels represent erythema. Erythema degree was represented by an a*-value, where a high a*-value represented high degree of erythema. The pixel analysis was performed on a standardized area measuring 126*126 pixels on digital photos taken under standardized light conditions with the Image J software (Image J, version 1.45S, National Institute of Health, USA).

Frosch-Klingman visual scale:

Visual scoring of the erythema was performed using the Frosch-Klingman scale. This scale was developed as a tool for evaluation of skin redness in response to external irritants (94). We used the modified scale: 0: no erythema, 1: slight redness with a blurred boundary, 2: moderate redness with sharp boundary, 3: intense redness, 4: fiery redness with oedema (94).

Hyperalgesia:

Primary hyperalgesia is defined as a decrease in pain threshold and an increase in pain to sub-threshold stimuli (95) due to release of inflammatory mediators into peripheral tissue (96). UVR-exposure induces an acute inflammatory reaction in the skin resulting in a condition of primary hyperalgesia. Testing of primary hyperalgesia was performed using von Frey monofilaments (97), as this is a simple and validated test instrument (95). The skin areas exposed to UVR were tested starting with the thinnest von Frey monofilament, and increase in monofilament size was tried until a pain reaction was sensed.

Figure 3.

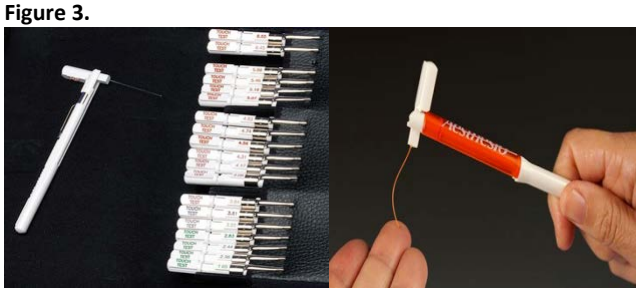


Illustration of von Frey monofilaments, which were used to test primary hyperalgesia.

Karolinska sleepiness scale (KSS):

KSS is as valid scale for measurement of subjective sleepiness in individuals (98, 99) and has been used in several previous studies (100, 101). The scale includes the following nine steps: "extremely alert" (score=1), "alert" (score=3), "neither alert or sleepy" (score=5), "sleepy but no difficulty remaining awake" (score=7), "extremely sleepy fighting sleep" (score 9) (98).

Continuous reaction time (CRT):

CRT is a neurophysiologic test designed to measure vigilance and attention by testing the subjects' ability to rapidly respond to external stimuli over a period of time (102). Previous investigations have shown a correlation between increased reaction time and cognitive dysfunction (103, 104). The test was performed using EKHO software version 1.3 (Bitmatic, Århus, Denmark). Fifty auditory signals (90 db) were presented to the subject through headphones at random intervals (2 to 5 sec) over a period of three minutes. Subjects were instructed to press a button as fast as possible in order to interrupt the signal using the first finger on

the dominant hand, after which the sound was interrupted. The reaction time between the sound signal and activation of the button was registered by a computer and measured in 1/100 seconds. If time to activation exceeded two seconds, this was recorded as no response. Furthermore, reaction time less than 12/100 seconds was not registered as a response, indicating activation of the button without proceeding stimuli (102).

Finger tapping test (FTT):
The FTT is accepted as a simple measure of psychomotor speed and motor control (105). Psychomotor slowing is generally considered as a non-specific consequence of cerebral dysfunction (106-108). In the test, the participant had to tap a key as many times as possible in 10 seconds, and a measuring instrument registered the number of taps. The participants were instructed to use the second finger on the dominant hand, and each test consisted of five rounds of 10 seconds tapping time interrupted by 10 seconds pause between each round. Final score was the mean value for all five rounds (109).

Serum melatonin by RIA technique:
To prove that absorption of melatonin from skin to blood did take place, blood samples were taken 12 hours after cream was applied. Serum melatonin concentrations were analyzed using radio immuno assay (RIA) kits (110).

Natural sun exposure:
Natural sunlight has a different composition compared to artificial UVR-sources. Natural sunlight is composed of approximately 6% UVB and 94% UVA (111), and in artificial UVR-sources the share of UVB is typically much higher (29-32, 112). Since UVB is much more effective in causing biological damage, UVB contributes to approximately 80% of the most harmful damages induced by sun exposure (111). UVB is known to be primarily responsible for induction of erythema (113), and the minimal erythema dose (MED) for UVB is 1000-fold less than for UVA (114, 115). UVR-exposure from natural sunlight is the main UVR-source for most people, and thereby natural sunlight is a more clinically relevant measure in evaluation of potential sun-protecting agents. Natural sun exposure is measured in means of UV-index (UVI). The UVI was launched by WHO and several other partner organizations in 1995, as a method to measure the amount of UVR reaching the Earth's surface (116). The UVI ranges from 0-11, and the higher values imply higher risk of UVR-induced skin damage.

Fitzpatrick skin type scale:
The Fitzpatrick skin typing system was created in 1975 to predict skin reactivity in PUVA (psoralen ultraviolet A) photochemotherapy (117). Since then, the Fitzpatrick classification of skin type has been used worldwide to estimate the risk of skin cancer and malignant melanoma (118-121). There are four categories for white skinned persons: I: Always burn, never tan. II: Usually burn, tan less than average (with difficulty). III: Sometimes mild burn, tan about average. IV: Rarely burn, tan more than average (with ease). Brown skin is classified as skin type V and black skin as skin type VI (117).

Evaluation of sedative side effects of topical melatonin on cognitive parameters:
Synthesis and secretion of melatonin is stimulated by darkness, and melatonin is known to decrease time to fall asleep (122, 123). Due to these qualities, many studies have evaluated potential sedative side effects with a wide range of cognitive test (124-127). The best combination of tests has to cover a variety of different cognitive parameters. We chose three validated tests concerning diverse qualities of cognition e.g. KSS testing subjective sleepiness, FTT testing psychomotor and speed and CRT evaluating attention and vigilance. This combination of tests has been used in several previous studies investigating effect on cognition of sedative drugs (128-130).

ETHICAL CONSIDERATIONS:
Skin cancer is an increasingly serious problem in dermatology, and rising incidence has been reported worldwide (1-3). Furthermore, experts have expressed their concern that incidence will continue upwards due to ozone depletion and increasing age in the population (10). High-risk patient groups are emerging with the wide use of immunosuppressive agents (13-15, 17). There is need for new and more effective sun-protecting agents with other qualities than simple chemical reflection of UVR.

The study evaluating the effect of topical melatonin against natural sunlight (Paper 2) had permissions obtained from the Danish Health and Medicines Authority (2013025193), the local ethics committee (H-2-2013-037) and The Danish Data Protection Agency (HEH-2013-023). The regional GCP unit in Copenhagen monitored the study. The study was registered on clinicaltrials.gov with the registration identifier: NCT01873430.

Permissions for the MELATOX study (Paper 3) were obtained from the Danish Health and Medicines Authority (2013-081100), the local ethics committee (H-1-2013-085) as well as the Danish Data Protection Agency (HEH-2013-072). The regional GCP unit in Copenhagen monitored the study. The study was registered on clinicaltrials.gov with the registration identifier: NCT02224937.

Both studies were conducted in accordance with the Helsinki declaration.

PRESENTATION OF INCLUDED PAPERS:

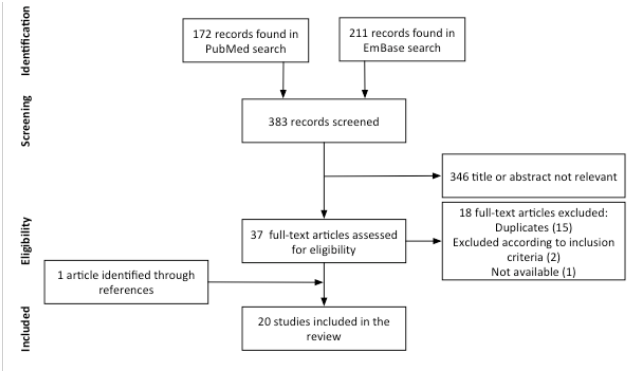
Paper 1: Melatonin's protective effect against UV radiation: a systematic review of clinical and experimental studies.

Aim:
The aim of this study was to evaluate evidence in the literature of a protective effect of topical melatonin against UVR-induced skin damage and clarify the cellular mechanisms behind.

Method:
This study was a systematic review conducted in accordance to PRISMA guidelines (131). A literature search was performed in January 2013 on Medline (1946 to 2013), EMBASE (1918 to 2013), and CINAHL (1981 to 2013). The literature search aimed to identify both laboratory and clinical studies investigating melatonin's effect against UVR-induced skin damage. The search was supplemented by manual reference list searches of the included studies to identify additional studies. Only studies using human skin or human cells were included. For the study selection process, see

Figure 3. Eligibility assessment of abstracts was performed in an unblinded standardized manner by the principal author (CS).

Figure 4:



Study selection process in accordance with PRISMA guidelines (reproduced with permission from Photodermatology, Photoimmunology and Photomedicine (90)).

Results:
Twenty studies, 16 experimental and four human, were included. The human studies were equal in study design as they were all randomized controlled trials. However, they differed in use of UVR-source and time of melatonin application. Even though the studies differed in choice of UVR-source, they all used artificial UVR-sources, and they all had a larger share of UVB than a natural UVR-source.
There is convincing evidence of a protective effect with topical treatment with melatonin when applied before UVR-exposure, and the protective effect is present with different doses. Furthermore, evaluation of experimental studies indicated a possible protective effect of melatonin against UVR-induced damage to cellular structures and DNA. However, the studies varied in the composition of UVR-source and concentration of melatonin.

Conclusion:
Melatonin has sun protective effects in both human and cellular studies. Nevertheless, to answer the question if melatonin can be used as a sun protective agent in a clinical setting, further studies are needed to evaluate melatonin's sun protective effect with use of natural sunlight. It is also of great importance to identify the optimal concentration of melatonin, which would be most effective in reducing UVR-damage to the skin.

Strengths:
The systematic review was conducted in accordance to PRISMA guidelines (131). Reporting of PICO (population, intervention, comparison and outcome) was used. Furthermore, the literature search was made with use of three different databases.

Limitations:
An obvious limitation to a systematic review may be the quality of included studies. In this review, we did not assess the quality of the studies. However, at least for the experimental studies no suited bias tool was available. Moreover, we did not publish a review protocol although this is recommended in the guidelines.

Only the principal author performed the literature search, screening of possible titles and abstracts and assessment of relevant studies. However, any doubt in the inclusion process was discussed with the other authors.

We only included studies published in English or Scandinavian languages, however, the language bias arising from this has shown to be very limited (132). As we did not search any clinical trial databases in order to identify any unpublished or ongoing clinical trials, this review may be subject to an unknown extent of publication bias. This could imply a potential risk of overestimating the effects of melatonin against UVR-induced skin damage.

All the included studies used artificial UVR-sources to induce skin damage, whether it was erythema or cellular damages, they investigated. However, the studies were very diverse in choice of UVR-composition and exposure time, which made it difficult to compare them. As a consequence, we were unable to make any conclusions on maximal exposure time in which melatonin would still have a protective effect. Thus, no conclusion could be made regarding if the protective effects found with one composition of UVR could be replicated with use of another composition.

Paper 2: Dose dependent sun protective effect of topical melatonin: a randomized, placebo-controlled, double-blind study.

Aim:
The aim of this study was to investigate the sun protective effect of topical treatment with different doses of melatonin against UVR-induced erythema from natural sunlight.

Methods:
This study was a randomized, double-blind, placebo-controlled study. The included participants had a specific randomization sequence generated, and three different concentrations of melatonin cream (0.5%, 2.5%, 12.5%) were applied to their backs before sun exposure, and compared to placebo cream and no treatment. Blinding and randomization of the study was carried out on several levels. The five different creams were concealed in five identical boxes only marked by a number, and there was no visual difference between the creams. Furthermore, the participants were blinded to in what order the creams were applied. Three helpers without any connection to the study did all the practical work regarding cream application etc. The investigators had no contact with the creams, and randomization was kept sealed until data analyses were executed. The participants were exposed to the sun for 40 minutes with an UV-index of 9.

Erythema was evaluated with chromatography by a validated method using software analysis (Image J, version 1.45S, National Institute of health, USA) of digital photos (93). A special "colour space converter" function was used, and increasing degree of erythema was expressed as rising a*-values. Participants were divided into two groups according to their reaction to sun exposure. High responders were defined as participants with a change in a*-value of five or more, and low responders were defined as participants with change in a*-value below five. Eleven participants fulfilled the criteria and were defined as high responders. Furthermore, erythema was quantified by visual scoring using the Frosch-Klingman scale (94). Testing of primary hyperalgesia was conducted on the sun-exposed areas using von Frey monofilaments (97). Tests were performed at baseline, one, four, eight and 24 hours after UVR-exposure.

Results:
Twenty-three volunteers were enrolled. Of the 23 participants, one did not meet the inclusion criteria due to sun exposure prior to the study. One participant was lost for follow-up before the last measurement 24 hours after exposure, but the prior four measurements were included in data analyses. Thus, 21 participants completed the study.

Maximal erythema reaction was seen eight hours post exposure, and a significant decrease in erythema was produced by melatonin cream 12.5% compared with placebo or no treatment (Figure 4). However, no effect on primary hyperalgesia was found.

Figure 5:

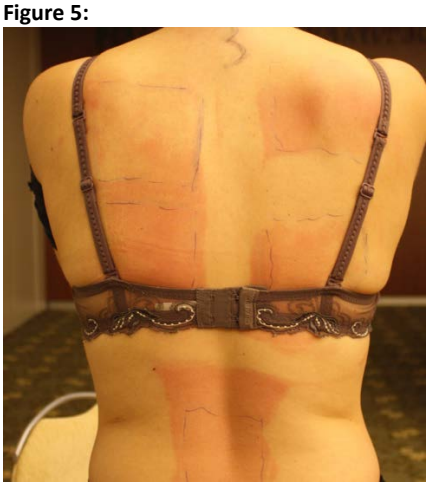
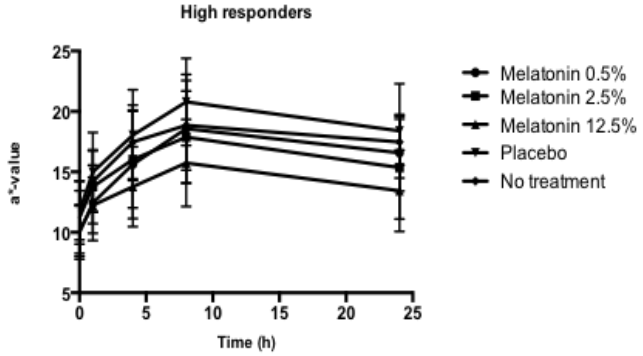


Photo of one of the participants' back 8 hours after sun exposure. There is a distinct difference in erythema formation in the five test areas.

Figure 6:



Effect of topical treatment with melatonin on UVR-induced erythema in participants with high reaction to the sun (change in a*-value of more than five). Graph visualizes mean +/- standard deviation (SD) values over time.

Conclusion:
In conclusion, we have demonstrated a protective effect of topical melatonin against erythema induced by UVR from natural sunlight. However, the exact mechanisms remain to be clarified, and the results obtained need to be supported by studies evaluating the possible side effects when using melatonin as sunscreen on larger areas of the body.

Strengths:
Participants were their own control, which gives the optimal settings for comparison of the five different treatments.

For most people, natural sunlight is the main source of UVR, and thereby also the main source contributing to development of skin cancer. Due to this using natural sunlight in our study gives a more clinical relevant evaluation regarding if melatonin cream 12.5% is suitable as a sun-protecting agent, than studies conducted using artificial UVR-sources.

We used two different parameters to assess erythema (chromatography and visual evaluation using Frosch-Klingman scale). Assessing the same outcome from two different perspectives gives more reliable results.

The randomization and blinding of the study was carried out on three levels. From the pharmacy, the different creams had been concealed in identical boxes only marked with a number, corresponding to a number in the randomization sequences. The participants were blinded to the order in which they had the different creams applied, and there was no visual difference between the creams. The investigators conducting the data analysis had no contact with the creams and were blinded to the random allocation sequences until data processing was completed.

Limitations:
As we did only apply cream to very little skin areas, we were not able to conclude on the presence of any possible adverse effects.

Exposure time may have been too short, as many did not have a reaction to the sun exposure (low responders). We chose the exposure time based on knowledge about the UV-index at the location of the study, and earlier studies (133) showing that an exposure time of 40 minutes induced erythema in most people with the current UV-index. Extending the exposure time to more than 40 minutes would be with risk of producing serious burns in some participants, and this could not be defended from an ethical perspective. Despite this short exposure time, there was a significant effect of topical treatment with melatonin. However, this was only seen in participants with an erythema in response to the sun exposure (high responders). We anticipate that the effect would be even greater if the exposure time was longer and the erythema was more severe. Furthermore, due to this relatively short exposure time we cannot make any conclusions about the duration of the protective effect of topical treatment with melatonin cream. Thus, no guidelines can be made on time between each application in order to obtain continuous protection.

The dose of melatonin may not have been high enough to conduct the maximum reduction of erythema, as we only found a significant reduction of erythema with the highest dose. Therefore, we have no knowledge of the protective effects of higher doses, and we cannot conclude that the maximal protective effect is found with the 12.5% dose. However, due to the chemical properties of the melatonin content and the basis cream, it was estimated that the concentration of 12.5% would be close to the maximum dose, which could be dissolved.

Participants had, as expected, very diverse reactions to the 40 minutes of sun exposure, and were therefore divided into subgroups in accordance to the strength of their erythema. This subgroup division may have weakened the strength of the results, which could have been avoided with inclusion of more participants.

Conflicting results regarding if melatonin's protective effect is due to sun-screening qualities have been found. Previous studies have revealed sun-screening properties by melatonin, as they found that formulations containing melatonin showed significant UVB/UVC absorption (30, 45). However, another research group found that melatonin's UVR absorbance characteristics did only concern UVR at wavelengths of 225-275 nm, which was below the

UVR range used in their study (32). In our study, we did not make any measurements to characterize the UVR-screening qualities of the creams used. However, a sun-screening effect conducted by the basis cream can be eliminated, as there was no difference in erythema formation between fields treated with placebo/basis cream and fields given no treatment. Nevertheless, we cannot reject that some of the protective effect against erythema induced by natural sunlight is in fact on behalf of a sun-screening effect conducted by melatonin.

Paper 3: Effect on cognitive parameters when using melatonin cream 12.5%: a randomized, placebo-controlled, double-blinded crossover study in healthy volunteers.

Aim:

The aim of this study was to assess the degree of cognitive dysfunction when cream containing 12.5% melatonin was used as full body application.

Methods:

This study was a randomized, placebo-controlled, double-blind crossover design with healthy volunteers. Participants were their own controls, as they were randomized to receive either melatonin cream 12.5% or placebo on the first or second day of investigation, in accordance to a randomization sequence generated by Glostrup Pharmacy. Placebo and melatonin cream 12.5% were concealed in 20 tubes marked with X or Y, representing the first and second day of investigation, and a number from 1-10 representing the ten participants. There was no visual difference between the two creams, and the order in which the participants received either melatonin or placebo was unknown to both the participants and the investigator. The study was conducted over two days with a gap of 14 days in-between. The test battery was identical on the two days of investigation, and settings were kept as constant as possible.

Participants had their body surface area calculated using the following formula:

$$A = W^{0,425} * H^{0,725} * 0,007184 \ (134)$$

Where weight (W) was given in kg and height (H) was given in cm. The individual amount of cream was calculated using 80% of surface area (A) multiplied with 0.0027 ml cream per cm^2. This particular dose of cream per cm^2 has been determined in previous studies to produce a relevant protective effect against UVR-induced erythema (29, 30, 32). As dosage of cream was calculated for each individual in accordance to their specific surface area, participants were treated with increasing total doses of melatonin with increasing body surface area. The cream used in this study contained 12.5% melatonin, and as we applied 0.0027 ml/cm^2 this corresponded to a melatonin dose of 0.3375 mg/cm^2. At baseline, participants had cream applied to 80% of their body surface area.

The degree of cognitive dysfunction was assessed using a test battery consisting of three tests: KSS was the primary outcome and secondary outcomes consisted of two neuropsychological tests: FTT and CRT. The test battery was performed at baseline and 1,2,3,4,5,6,7,8,12,24 and 36 hours after cream was applied. The neuropsychological tests were administered with the first test being FTT followed by CRT. Before administration of melatonin cream 12.5%, the test battery was repeated three times to ensure familiarity with the tests and to minimize a learning effect through the study. The baselines for all tests were defined as the scoring in the third repetition of the test battery.

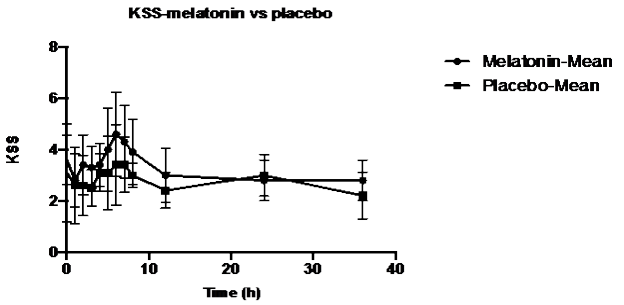
A blood sample was taken 12 hours after cream was applied to demonstrate that absorption of melatonin from skin to blood did take place.

Results:

Three male and seven female with a median age of 26 (range 20-56) were included. Based on height and weight of the participants, the median melatonin dose was 4800 mg (range 4100–5600 mg). All ten participants completed the study.

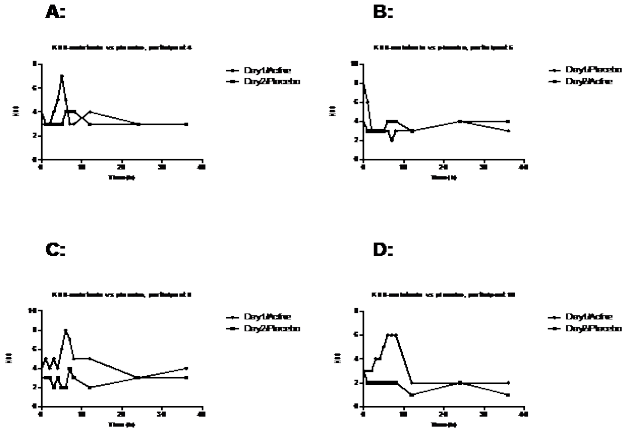
There was no significant differences on any of the cognitive parameters when comparing melatonin cream 12.5% with placebo: KSS (p=0.117) (Figure 5), FTT (p=0.658) and CRT (p =0.248). However, there were few participants on whom melatonin had a great impact, and they reported high values of KSS (Figure 6). Serum melatonin concentration 12 hours after cream application showed supraphysiologic serum concentrations, proving that absorption of melatonin from skin to blood was substantial.

Figure 7:



Effect of topical full body application of melatonin cream 12.5% on subjective sleepiness expressed as KSS. Graph shows mean +/- standard deviation (SD) values over time.

Figure 8:



Individual AUC measures for four participants illustrating the large inter-individual difference in KSS. Graph visualizes KSS values over time.

Conclusion:

In conclusion, we have demonstrated that there was no significant group effect on cognition when using melatonin cream 12.5% on 80% of body surface area.

Strengths:

Strength to this study was that the participants were their own controls, as this ensures comparability of the results from melatonin and placebo. Also, settings were kept as alike as possible on the two days of investigation.

The randomization and blinding of the study was carried out on several levels. Participants were randomized in accordance to a random allocation sequence generated by the pharmacy. Furthermore, creams were sealed in tubes only marked with X or Y corresponding to the first and second day of the investigation and a number from 1-10 representing the number of participants. No visual difference could be made between the two creams, and the order in which the participants received either melatonin or placebo was unknown to both the participants and the investigator. The investigators performing data analyses of the primary and secondary outcomes were blinded to the randomization sequences until data analyses were finished.

The neurophysiologic tests used to assess the degree of cognitive dysfunction were diverse and covered a broad range of cognitive parameters. Furthermore, the same combination of tests has been used in several studies evaluating sedative drugs' effect on cognition (128-130, 135). They are well accepted and valid as tools for assessing cognitive parameters (99-101, 103, 104, 106-108).

Limitations:

The calculation of sample size was based on the conditions, that participants become sedated to a degree, which induces an increased risk of accidents. Therefore, we cannot reject that participants have been affected to a lesser extent, as we cannot detect smaller sedative effects, since this would have required more participants. Furthermore, even though tests were the same on both days of investigations, participants were free to do any activity between each hourly test, and the only restriction was no sleep during daytime. We had not made any standards to ensure same intake of calories on the two days of investigation. Optimal settings would have provided participants with the exact same amount of calories and same activities on the two days of investigation.

Due to its many diverse qualities melatonin is, and has been, subject to a lot of research, and many studies have evaluated melatonin's sedative effects (124-127). However, these studies have used other cognitive tests in their evaluation, and therefore no direct comparison was possible. Furthermore, no previous studies have evaluated the sedative effect of topical melatonin, and comparison could only be made with studies using oral or intravenous administration of melatonin.

We found a surprisingly large inter-individual difference in the impact on KSS. The explanation may in part be found in the huge individual span of serum melatonin concentrations found in our study. However, there was no clear correlation between high serum melatonin concentrations and reported values of KSS.

No studies have evaluated the pharmacokinetics with full body application of topical melatonin, and therefore we cannot draw any conclusions on the strength of a possible deposition effect in the skin. Furthermore, we cannot conclude on the percentage of melatonin that is transported into the blood, and if any local metabolism takes place.

DISCUSSION:

Principal findings:

With this PhD thesis, we aimed at testing the usefulness of topical melatonin as protection against oxidative stress induced by UVR from exposure to natural sunlight. Furthermore, we wanted to investigate potential adverse effects with topical use of melatonin as full body application.

There is convincing evidence of a protective effect of melatonin against UVR-induced skin damage in both human and cellular studies in laboratory settings, and this protective effect is achieved through a broad range of cellular signal pathways.

We tested the protective effect of three different doses of melatonin cream against erythema induced by natural sunlight. A protective effect was found represented as reduced erythema formation with use of melatonin cream 12.5% in high responders. However, no protective effect on primary hyperalgesia was found.

With application of melatonin cream 12.5% on 80% of the body surface area, no significant cognitive dysfunction was found when looking at the whole group of participants.

Comparison with previous findings:

Melatonin as topical sun protection:

Our research supports previous findings suggesting melatonin as a protective agent against UVR-induced erythema (29-32). However, we could only reproduce the protective effect using a much higher dosage of 12.5% melatonin. Furthermore, we used natural sunlight in our study, as we believe this gives a more clinically relevant evaluation of melatonin's quality as a protective agent. The previous studies used artificial UVR-sources with a different composition of UVA and UVB than natural sunlight (29-32). The share of UVB is higher in artificial UVR-sources (111, 112). Due to UVB being mostly responsible for inducing erythema (111, 113), the erythema induced by artificial UVR-sources may have been more intense, and thereby a protective effect has probably been more distinct and easier established with a lower dose of melatonin.

The study presented in paper 3 confirmed previous findings showing that melatonin could be absorbed over the skin (75, 136), as we registered supraphysiologic serum concentrations after 12 hours. Furthermore, we have supported the idea of melatonin being a safe drug for topical treatment, even in previously unknown high dosages.

Due to earlier studies' conflicting results regarding whether melatonin contains substantial sun-screening properties that lead to photoprotection (30, 32, 45), it cannot be rejected that this has had influence on the results found in our studies. However, as presented in paper 1 several previous studies have supported that melatonin has a protective effect against UVR-induced oxidative damage, which is on behalf of strong antioxidant qualities (33-43, 45, 46, 137, 138). Therefore, we must expect some of the protective effect being due to antioxidant mechanisms. Even if the results alone are due to a sun-screening effect, topical treatment with melatonin as protection against UVR-induced skin damage will still have the advantage of producing it's desired effects through both chemical reflection and through interfering with cellular pathways.

Melatonin and pain management:

Several earlier studies in mice (139, 140), hamsters (141), rats (142), and humans (143) showed that there is a circadian rhythm in pain perception, and this lead to the idea that melatonin may possess some analgesic effects. Since then melatonin's anti-noci-

ceptive qualities have been demonstrated in various animal models, and an effect on acute pain (144, 145), inflammatory pain (146, 147) and neuropathic pain (148, 149) has been established. The mechanisms behind melatonin's analgesic effect are still not fully clarified and understood. However, studies have shown that it is possible to block melatonin's analgesic effects by naloxone (an opioid antagonist), suggesting that melatonin conducts its anti-nociceptive effects through the opioid receptor system. Furthermore, melatonin is known to have substantial antioxidant (19-22) and anti-inflammatory qualities (26). As both free radicals and inflammatory agents are known to play a substantial role in pain induction and perception, this may also be a pathway through which melatonin produces its analgesic effects. Thus, melatonin may conduct its analgesic effect through both receptor-dependent and receptor-independent mechanisms.

Melatonin and cancer:

In this PhD thesis, we did a thorough review of the literature regarding melatonin's protective effect against UVR-induced skin damage. This revealed that melatonin has a protective effect, which is induced through a wide range of cellular pathways. It can be proposed, that topical melatonin counteracts or buffers external and internal stressors to preserve the biological integrity of the skin, and to maintain its homeostasis. However, no clinical studies confirming melatonin's eventual preventive effect against UVR-induced skin cancer have been conducted.

Already in the seventies, researchers proposed a correlation between melatonin and breast cancer (150). They suggested that a decrease in pineal function could induce a state of relative hyperestrogenism, and the early and prolonged exposure of the breast tissue to the estrogens could be involved in breast carcinogenesis. Since then, several studies have suggested an association between melatonin levels and cancer progression, as reduced levels of melatonin has been found in patients with certain types of cancer compared with healthy people of same age (151-154). Furthermore, a significant correlation between low serum levels of melatonin and the presence of endometrial cancer has been found (155), and several epidemiological studies have shown an increase in breast- and colorectal cancer risk in women working nightshifts (156-159). The mechanisms behind these effects are not entirely clarified.

Melatonin and cognition:

Timing of the administration of melatonin is now known to have great impact on the degree of sedation, as melatonin administered at noon had less sedating effect compared with melatonin administered in late afternoon (160). Also the settings in which melatonin is given have effect on the extent of sedation, as light, activity and keeping in an upright position can lessen it (161). All these factors with modulatory effects on melatonin's sedating effect may be part of the explanation on the lack of impact on KSS found in our study. Furthermore, melatonin differs from other sedatives, as melatonin does not impair motor skills (125, 162).

Clinical implementation of melatonin as a sun protective agent:

To our knowledge, we are the first to show a protective effect of topical melatonin against UVR-induced erythema induced by natural sunlight in a randomized clinical trial, which gives us reason to hypothesize a protective effect against UVR-induced skin cancer. However, we need more knowledge concerning the pharmacokinetic relations when using melatonin cream 12.5% as full body application. Also, we have with our research shown that there are no significant acute effects of melatonin on cognitive dysfunction. More knowledge regarding the effect of long-term use of topical melatonin on circadian rhythm, sleep quality and risk of inducing circadian disturbances, known as phase-shift, are required. As we found an increased risk of accidents to be a clinically relevant outcome, we based our sample size calculations on this outcome. Studies investigating sedative effects of topical melatonin with less severity are warranted, before any conclusions can be drawn regarding clinical implementation of topical melatonin treatment against UVR-induced skin damage.

Future perspectives and suggestion for future research:

When studying the literature on the subject of skin cancer and UVR, it seems that the pathogenesis of UVR-induced skin cancer may be multifactorial and caused by a combination of several cellular pathways. In addition, the intensified widespread use of immunosuppressive medicine has resulted in a growing patient group with special needs in the battle against the increased risk of UVR-induced skin cancer (12, 14-16).

The bioavailability of melatonin is a major challenge, as the liver metabolism of melatonin can differ as much as 37-fold in healthy individuals (72). This huge first pass metabolism is avoided with topical treatment (75, 163); thus, investigation in topical administration of melatonin for various purposes may be a future topic of interest, also for other indications than skin protection.

Review of the cellular studies investigating melatonin's effect against UVR-induced oxidative stress has given convincing evidence of a possible protective effect obtained through various cellular mechanisms (33-46, 137, 138), of which some may be involved in cancer development (28, 52). These results contribute to the hypothesis that topical treatment may have potential as a preventive agent against development of skin cancer. However, further research is warranted, and an obvious next step would be to investigate this hypothesis in experiments with UVR-induced skin cancer in experimental animals.

The exposure time used in paper 2 was very limited; as this was the duration we could defend from an ethical perspective. However, this sparse time span excludes us from making any suggestions on the duration of the protective effect of melatonin cream 12.5%, and the time interval in which a recurrent application has to be made. Therefore, studies resembling a more realistic setting of a day of sun exposure are warranted.

As shown in paper 2, we only found a significant reduction in UV-induced erythema with the highest dose; thus, we are unable to reject an even greater protective effect with higher doses of melatonin. To determine the optimal dose of melatonin cream, studies using the same setting, but higher doses of melatonin are warranted.

In paper 3, we investigated the cognitive dysfunction with use of full body application of melatonin cream 12.5%. However, our sample size was made on the calculation that participants became sedated in a degree, which increased risk of accidents. Studies evaluating cognitive dysfunction to a minor extent are necessary before any clinical implementation of melatonin cream as a sun protective agent can be done. Furthermore, even though the cognitive test used in our study represents a broad range of cognitive parameters, studies evaluating other cognitive parameters

e.g. memory, overview and mood, will give a more elaborate picture of the impact on cognition, when using melatonin cream.

Melatonin is known to have effect on sleep quality and time of sleep induction (164-166). Therefore, studies investigating the effect of continuous treatment with topical melatonin on sleep with acthigraphy and sleep EEG are required to elaborate any adverse effects on sleep.

In paper 1, we reviewed cellular studies investigation the protective effect of melatonin against UVR-induced skin damage, and found indications of a protective effect (33-46, 137, 138). This protective effect was achieved through direct antioxidant and indirect antioxidant mechanisms, but also through altered gene expression favouring tumour suppressor genes, and thereby enhancing the body's own defence against carcinogenesis (33, 40, 41, 46). Patients treated with immunosuppressive medicine are constantly immunosuppressed and thereby their defence mechanisms against cancer too may be suppressed, resulting in an increased risk of non-melanoma skin cancer, even with proper use of the sun protective agents known today (167, 168). In addition to the increased incidence these tumours often tend to be more aggressive, as evidenced by clinical presentation, severity of histological presentation and increased metastatic potential (169). It can be hypothesized, that these patients can achieve better protection against UVR-induced skin damage and eventually skin cancer, with use of sun protective agents that induce their protective effects through alterations of cellular pathways and mechanisms, rather than chemical reflection of UVR. Melatonin could be such an active protective agent, however, to clarify this, studies with immunosuppressed patients are warranted.

CONCLUSION:

With the objectives of the thesis in mind and on the basis of the conducted studies it can be concluded that:

- Clinical and experimental studies evaluating melatonin's effect against UVR-induced skin damage indicate a protective effect.
- Our studies support previous findings suggesting melatonin to be a sun protective agent (29-32).
- To our knowledge, this is the first study evaluating the protective effect of topical melatonin against natural sun exposure. This adds a more clinically relevant evaluation of melatonin's potential use as a sun protective agent in humans.

Also, to our knowledge, we have conducted the first study investigating the degree of cognitive dysfunction with topical treatment of melatonin. We found no group effect on cognitive dysfunction with topical treatment of melatonin cream 12.5%.

ENGLISH SUMMARY:

Skin damage induced by UVR is an escalating problem in dermatology, and increasing incidence of skin cancer, especially for non-melanoma skin cancer, has been reported worldwide. UVR from sun exposure and the production of reactive oxygen species (ROS) is known to be a pivotal factor in the aetiology of skin cancer. The pineal hormone melatonin is recognized as the most potent endogenous antioxidant. Melatonin conducts its antioxidant effects acting directly as a radical scavenger and indirectly by up regulation of antioxidant enzymes. It has been proposed, that melatonin may have a protective effect against UVR-induced skin damage. The aim of this thesis was to:

- Clarify melatonin's protective effect against UVR-induced skin damage in laboratory and clinical settings trough a systematic review of the literature.
- To clinically assess the protective effect of topical treatment with melatonin against natural sun exposure, and determine the optimal concentration.
- To clinically evaluate the degree of cognitive dysfunction with full body application of topical melatonin.

Study 1 :

This was a systematic review using the databases Pubmed, EMBASE and Cinahl. The databases were searched up to January 2013 to identify studies evaluating melatonin's protective effect against UVR-induced erythema in humans, and damage on a cellular level. Twenty studies were included, four human and 16 experimental. The results indicated that melatonin had a protective effect against UVR-induced erythema if applied before exposure, and this effect was probably obtained by melatonin acting directly as an antioxidant, and indirectly by regulating gene expression and inducing a DNA stabilizing effect. As these results were obtained using artificial UVR-sources and without investigating possible side effects, studies using natural sunlight and evaluating possible side effects of topical melatonin administration were warranted.

Study 2:

This study was a randomized, double-blind, placebo-controlled study. We evaluated the protective effect of three different doses of topical melatonin against erythema induced by natural sunlight. The primary outcome was reduction in erythema, evaluated by chromatography, after sun exposure, when treated with topical melatonin (0.5%, 2.5%, 12.5%) versus placebo and no treatment. A significant difference in erythema formation was found between areas treated with melatonin 12.5% and areas receiving placebo or no treatment. However, this was only seen in participants with an erythema reaction to the sun exposure. Furthermore, the treated skin areas were very small and studies assessing any potential adverse effects were necessary.

Study 3:

This also was a randomized, double-blind, placebo-controlled, cross-over study. We assessed the degree of cognitive dysfunction with full body application of topical melatonin 12.5%. Cognition was evaluated using a neuropsychological test battery consisting of Karolinska sleepiness scale (KSS), finger tapping test (FTT) and continuous reaction time (CRT). The impact on KSS was the primary outcome. We found no significant effect on cognition, however, large inter-individual variation was observed. These results support that melatonin is a safe drug for dermal application.

The studies in this thesis may be valuable in the research field of melatonin's protective potential against UVR-induced oxidative skin damage. Increasing incidence of skin cancer is reported worldwide, and experts have suggested that the problem will only increase further, due to depletion of the ozone layer and the aging population. Furthermore, high-risk patient groups are emerging with the widely use of immunosuppressive medicine in various diseases, and this high-risk is in spite of use of protective measures known today. Therefore, development of new and more effective sun protective agents, with other qualities than simple chemical reflection of the UVR, is more important than ever. We have supported the suggestion of melatonin as a sun protective agent, and added the clinical relevant feature, that melatonin also has a protective effect against natural sunlight.

Furthermore, we have supported the idea of melatonin being a safe drug for topical treatment, even in previous unknown high dosages. However, before any clinical implementation of melatonin as a sun protective agent can take place, further studies evaluating the long-term effects are warranted.

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