Oxidative and inflammatory biomarkers of ischemia and reperfusion injuries

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PAPERS INCLUDED IN THIS PHD THESIS

PAPER 1:
Halladin N.L, Zahle F.V, Rosenberg J, Gögenur I. Interventions to reduce tourniquet-related ischaemic damage in orthopaedic surgery: a qualitative systematic review of randomised trials. Anaesthesia 2014;69:1033-50.

PAPER 2:
Halladin N.L, Ekeløf S, Alamili M, Bendtzen K, Lykkesfeldt J, Rosenberg J, Gögenur I. Lower limb ischemia and reperfusion injury in healthy volunteers measured by oxidative and inflammatory biomarkers. Perfusion 2015;30:64-70.

PAPER 3:
Halladin N. L, Hansen L.S, Rosenkilde M.M, Rosenberg J, Gögenur I. High potency on MT_1 and MT_2 receptors by melatonin dissolved in isotonic sodium chloride, isotonic glucose or Krebs Ringer-lactate buffers, but not in ethanol.

PAPER 4:
Halladin N.L, Ekeløf S, Jensen S.E, Aarøe J, Kjærgaard B, Heegaard P.M.H, Lykkesfeldt J, Rosenberg J, Gögenur I. Melatonin does not affect oxidative and inflammatory biomarkers in a closed-chest porcine model of acute myocardial infarction. In Vivo 2014;28:483-88.

PAPER 5:
Halladin N.L, Busch S.E, Jensen S.E, Hansen H.S, Zaremba T, Aarøe J, Rosenberg J, Gögenur I. Intracoronary and systemic melatonin to patients with acute myocardial infarction: protocol for the IMPACT-trial. Dan Med J 2014;61(2):A4773.

INTRODUCTION

When an organ or an area of tissue is deprived of its blood supply, the restoration of blood flow to the ischemic area is essential to prevent irreversible tissue necrosis and secure organ function [1]. Perhaps surprisingly, restoration of oxygenated blood flow may augment the injury in excess of that produced by ischemia alone [2], thus producing an injury known as ischemia-reperfusion injury. This injury is defined as cellular damage after reperfusion of previously viable tissues [3]. Ischemia and reperfusion occurs in a variety of clinical settings where the blood supply is temporarily cut-off and restored, both in acute conditions (e.g. myocardial infarction, cerebral stroke) and in elective surgery (e.g. transplantations, vascular surgery or surgery where a tourniquet is applied)[4]. Thus, it is an instrumental part of the disease process in a large number of clinical conditions affecting millions of patients worldwide every year.

Ischemia induces a variety of cellular metabolic and ultrastructural changes promoting expression of pro-inflammatory gene products e.g. cytokines, while repressing protective gene products[1]. Thus, ischemia induces a pro-inflammatory state that increases tissue vulnerability to further injury on reperfusion.

The mechanism of ischemia-reperfusion is multifactorial and involves divergent biological mechanisms, such as immune activation, ion accumulation, and the formation of toxic reactive oxygen species (ROS), also known as free radicals [5]. ROS are considered key molecules in the reperfusion injury [6] due to their potent oxidizing and reducing effects that directly damage cellular membranes by lipid peroxidation [7]. Oxidative stress is defined as a disturbance between the prooxidant and the antioxidant balance resulting in cell injury by oxidation of proteins, lipids and DNA [8]. The elevated oxidative stress level and the inflammatory reaction in reperfused post-ischemic tissue can be so extensive, that exposure of a single organ to ischemia and reperfusion may subsequently cause inflammatory activation in distant non-ischemic organs, eventually leading to multiple organ failure [9,10]. Many of the elements typically expressed during inflammation (cytokines, leukocytes and hormones) are known to have strong diurnal patterns synchronized to the 24 hour light/dark cycle [11]. Cardiovascular physiology and incidences of serious cardiac events e.g. myocardial infarctions, cardiac arrest and ventricular tachycardia are also known to be influenced by the circadian rhythm [12,13].

The circadian rhythm is maintained by secretion of the endogenously produced hormone, melatonin [14], which is mainly synthesized and released from the pineal gland located in the brain [15]. The synthesis as well as the release of melatonin is

stimulated by darkness and inhibited by light [15]. Besides maintaining the circadian rhythm, melatonin is also the body's most potent antioxidant [16,17]. It works both as a direct free radical scavenger and exerts indirect stimulatory actions on antioxidative enzymes [18]. The production of melatonin is reduced in the elderly [19] and in clinical conditions such as cardiovascular disease [20,21]. The blood melatonin concentrations may correlate with the severity of the disease [16] and infarct size following acute myocardial infarctions (AMI) was found to be significantly larger with ST-segment elevation myocardial infarction (STEMI) onset in the dark to light transition period when the melatonin level was at its lowest (6:00-noon) suggesting a circadian variation of infarct size [22]. Thus, the depletion of melatonin is thought to play a critical role in a reduced antioxidant defence against the free radicals formed in AMI. Furthermore, higher endogenous melatonin levels have shown protective effects against myocardial ischemia-reperfusion injuries in coronary artery bypass grafting surgery (CABG) [23].

Reperfusion is undoubtedly paramount in salvaging ischemic tissue. Thus, the major challenge will be to mitigate the unavoidable reperfusion injuries and optimize existing treatments. The use of melatonin in experimental studies involving ischemia-reperfusion injuries is very promising [24-26]; however, the evidence in randomized, clinical settings is still sparse. It is therefore of great importance to explore the potentially beneficial effect of exogenous melatonin in ischemia-reperfusion injuries in humans.

BACKGROUND

The processes contributing to ischemia-reperfusion associated tissue injury are multifactorial and involve many biological pathways [27] (Figure 1). Ischemia-reperfusion injuries occur when the blood supply to an organ or an area of tissue is temporarily cut off and restored. Restoration of blood flow is crucial to prevent irreversible cellular injury and the tissue would inevitably be damaged without blood flow being restored. However, it is widely accepted that the reperfusion in itself may augment tissue injury in excess of that produced by the ischemia alone [28]. Consequently, reperfusion remains a double-edged sword to the clinician, and there is a great interest in developing strategies and treatments to minimize the reperfusion mediated injuries [29].

Ischemia-reperfusion injury contributes to pathology in a wide range of conditions affecting many different organ systems and is thus a common and important clinical problem [4] (Table 1). During surgery and postoperatively, the body is subjected to a physiological stress response, the so-called surgical stress response, in which inflammatory, endocrine, metabolic and immunological mediators are activated [30-32]. Oxidative stress is believed to be an integrated part of the surgical stress response due to the exaggerated generation of ROS [33,34]. In surgical procedures involving ischemia-reperfusion phases, such as major vascular surgery or tourniquet-related surgery, it is thus not possible to distinguish the oxidative stress response elicited by the surgical procedure from the oxidative stress response caused by ischemia-reperfusion per se. Furthermore, the anesthetic compound propofol, which is often used in surgery, is known to work as an antioxidant and scavenge free radicals, thereby reducing the oxidative stress [35]. Hence, in order to investigate the effect of a possible intervention on the response elicited by ischemia-reperfusion solely, it would require a model where the influencing factors of surgery and anesthesia were eliminated.

Figure 1: The pathological processes contributing to ischemia-reperfusion associated tissue injury.

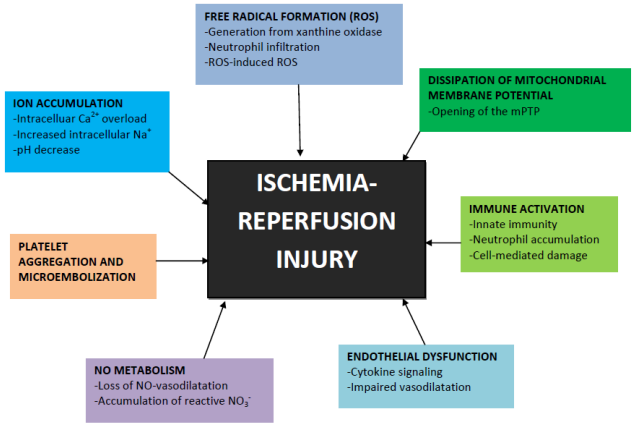


Table 1: Examples of ischemia-reperfusion injuries

		Clinical manifestations
Affected organ	Heart	Acute myocardial infarction
	Brain	Stroke
	Intestine	Intestinal ischemia and reperfusion; multiorgan failure
Ischemia-reperfusion during major surgery	Cardiac surgery	Acute heart failure after cardiopulmonary bypass
	Major vascular surgery	Acute kidney failure
	Organ transplantation	Acute graft failure Early graft rejection
	Tourniquet-related surgery	Deep vein thrombosis; Pulmonary embolism

Modified from Eltzchig HK et al. [4]

Pathophysiological mechanisms of ischemia-reperfusion injuries

Effects of ischemia

Oxygen is critical for cellular existence [36] and oxygen homeostasis is fundamental to human physiology [2]. The reduction of oxygen to water in the mitochondrial transport chain is crucial in maintaining the oxygen homeostasis and supplies the metabolic demands of human life as it enables resynthesis of the energy-rich phosphates adenosine 5'-triphosphate (ATP) and phosphocreatinine, thus maintaining the cellular membrane potentials [1].

During the normal metabolism of oxygen, reactive oxygen species (ROS) are generated as natural by-products. ROS are small molecules or molecular ions, characterized by the presence of unpaired electrons. This molecular structure is associated with a high reactivity with other molecules [7]. ROS can

induce a number of modifications of cellular biological molecules such as DNA, lipids and proteins; a reaction known as oxidative damage [6,27]. The addition of an electron to molecular oxygen (O_2) forms the superoxide anion radical (O_2^-) which is considered to be the primary toxic ROS [6]. The reduction of oxygen mostly occurs in the mitochondria, but specific enzymes, such as xanthine oxidase involved in the ATP metabolism, also generate ROS [6]. If produced in excess of the body's antioxidant capacity the ROS can be lethal to the cells [36]. The essential ATP generation via oxidative phosphorylation is thus balanced by the risk of oxidative damage to cellular lipids, DNA, and proteins [2].

Despite differences in hypoxic tolerance depending on metabolic rate and intrinsic adaptive mechanisms, cellular necrosis inevitably follows extended periods of ischemia [2]. The time that tissue changes remain reversible (critical ischemic times) depends on the tissue, temperatures and the presence or absence of collateral flow and ranges from 4 hours (muscle) to 4 days (bone) [37]. Multiple cellular metabolic and ultrastructural changes are the result of prolonged ischemia (Table 2). The consequence of ischemia is decreased oxidative phosphorylation and hereby a reduction in the resynthesis of ATP and phosphocreatine. This alters the function of the membrane ATP-dependent ionic pump, favouring the entry of calcium, sodium, and water into the cell causing cellular acidosis, edema and swelling [1,2].

Ischemia and the limited oxygen availability also have deleterious consequences on the endothelial cells lining the microscopic blood vessels and are associated with impaired endothelial cell barrier function[38] resulting in an increase in vascular permeability and leakage [39].

Table 2: Cellular effects of ischemia

Altered membrane potential
Altered ion distribution (↑ intracellular Ca^{2+}/Na^{2+})
Cellular swelling
Cytoskeletal disorganization
Increased hypoxanthine
Decreased adenosine 5'-triphosphate (ATP)
Decreased phosphocreatine
Decreased glutathione
Cellular acidosis

Modified from Collard CD et al. [1] and Eltzschig HK et al. [2]

Effects of reperfusion

Ischemia has detrimental effects on the cells and if not stopped in time, it will inevitably lead to cell death. However, it has long been known, that the histological changes of injury after three hours of ischemia followed by one hour of reperfusion are far worse than the changes observed after four hours of ischemia alone [28]. Thus, reperfusion seems to aggravate the injury caused by ischemia [2].

Ischemia is mainly a local event, but after revascularization, the mediators from the ischemic tissue can enter the systemic circulation and affect other organ systems. For instance, the exposure of plasma to xanthine oxidase-derived toxic ROS, can generate chemotactic factors both in vitro and in vivo and these chemotactic factors may cause the sequestration of inflammatory leukocytes in organs other than the site of the primary ischemic injury [40]. The systemic effects of ischemia-reperfusion injuries are partially caused by ROS and activated neutrophils, promoting the generation of cytokines and vasoactive mediators such as nitric oxide [41,42]. Additionally, ischemia induces the accumulation of intracellular sodium, hydrogen, and calcium ions resulting in a decrease in pH. The acidotic pH, though generally protective in ischemia, is normalized upon reperfusion and this rapid change to normal intracellular pH level, is paradoxically thought to enhance cytotoxicity [43].

The primary function of mitochondria is the generation of ATP through oxidative phosphorylation. Inhibition of oxidative phosphorylation, as it occurs during ischemia, leads to impairment of the normal function. This impairment is largely mediated by a nonspecific pore in the inner membrane of the mitochondria, known as the mitochondrial permeability transition pore (mPTP) [44]. The pore is closed during normal physiological conditions and also during ischemia, but opens upon reperfusion due to mitochondrial Ca^{2+} overload, oxidative stress and rapid normalization of pH [45]. Opening of the mPTP allows free passage of any molecule smaller than 1.5 kDA [46]. Since small molecules move freely across the membrane but proteins do not, this transport results in a colloidal osmotic pressure that causes the mitochondria to swell [44]. If the outer membrane of the mitochondria breaks due to swelling, this leads to the release of the enzyme cytochrome *c*, a potent activator of the apoptotic pathways, into the cytoplasm [47,48]. Furthermore, the inner membrane becomes freely permeable to protons which uncouples the oxidative phosphorylation and leads to the hydrolysis of ATP rather than synthesis. This causes a rapid decline in the intracellular ATP concentrations, which leads to disruption of ionic and metabolic homeostasis and activation of degradative enzymes and ultimately results in irreversible cell damage and necrotic death [49]. There is increasing evidence that opening of the mPTP is critical in the transition from reversible to irreversible reperfusion injury [50,51].

Myocardial infarction and ischemia-reperfusion injury

Although the incidence of AMI is decreasing in Denmark [52], it is becoming an increasing problem globally [53]. Primary percutaneous coronary intervention (pPCI) with restoration of coronary blood flow is considered the treatment of choice for AMI [54], but despite quick and effective treatment the 90-day mortality rate following AMI remains to be 5% [55].

Restoration of blood flow is absolutely necessary in order to salvage the myocardium, however, paradoxically, the re-establishment of blood flow during reperfusion causes cardiac dysfunction: the "no-reflow" phenomenon, myocardial stunning, reperfusion arrhythmias and lethal reperfusion injury [56,57]. The "no-reflow" phenomenon refers to an impairment of coronary flow caused by structural disruption or obstruction of the microvasculature in spite of the opening of the infarct-related artery. The occurrence of no-reflow is associated with a poor clinical prognosis [58]. Myocardial stunning is "the mechanical dysfunction that persists after reperfusion despite the absence of irreversible damage and despite restoration of normal or near-normal coronary flow" [59]. The myocardium normally recovers from this injury within days or weeks [56]. Reperfusion arrhythmias can be potentially harmful but they are easily treated [60]. Lethal myocardial reperfusion injury is characterized by the death of cardiac myocytes that were viable at the onset of reperfusion [6].

The myocyte cell death occurs in a wavefront progression from the inner to the outer regions of the ventricular wall as the duration of occlusion increases [61]. As with reperfu-

sion injuries in general, injuries to the myocardium are caused by increased oxidative stress, intracellular and mitochondrial Ca^{2+} overload, a rapid restoration of physiologic pH, and inflammation involving neutrophil migration into the myocardial tissue with a subsequent release of ROS and degradative enzymes [62].

A subsequent inflammatory response is caused by ROS [6,63] and though ample evidence implicates the involvement of ROS in myocardial stunning [64], there is, however, no general consensus on the harmful effect of ROS upon lethal reperfusion injury [57]. Furthermore, there has been much debate as to whether lethal reperfusion injury is an independent mediator of cardiomyocyte death distinct from that produced by ischemia alone. Some researchers suggest that reperfusion only exacerbates the cellular injury sustained during ischemia [65] while others hypothesize that the survival of the intact but fragile myocytes at the end of the ischemic period will be determined by the conditions of the reperfusion [48]. If the detrimental effects of rapid restoration of oxygenated blood flow could be alleviated, these fragile myocytes might survive [48]. If the size of a myocardial infarct could be reduced by an intervention applied at the beginning of the myocardial reperfusion, it would thus prove the existence of lethal reperfusion injury as a distinct mediator of cardiomyocyte death [57] (see Figure 2).

Another important reason for trying to mitigate the effects of reperfusion is that reperfusion injuries are unfortunately not always confined to the ischemic organ or area of tissue alone and inflammatory mediators released as a consequence of reperfusion appear to activate endothelial cells in remote organs that are not exposed to the initial ischemic event [66]. This distant response to ischemia-reperfusion can result in leukocyte-dependent microvascular injury that is characteristic of multiple organ dysfunction syndrome (MODS) [3]. This devastating complication has been reported after ischemia-reperfusion of organs [67] and after the use of a tourniquet in both humans and animals [68-70]. Several mediators such as ROS, xanthine oxidase and activated neutrophils have been proposed to be implicated in the mechanisms responsible for the remote organ injury induced by ischemia-reperfusion [3].

Mounting evidence exists to support the possibility that ischemia-reperfusion causes the systemic release of inflammatory mediators that can activate and/or attract circulating neutrophils and thereby promote neutrophil activation. This might induce generalized leukocyte and endothelial adhesion molecule expression, and enhance the possibilities for leukocyte-endothelial cell interaction [66,71]. Although the response to ischemia-reperfusion varies greatly among individuals, the presence of risk factors such as hyper-cholesterolemia, hypotension or diabetes further enhances the vulnerability of the microvasculature to the deleterious effects of ischemia-reperfusion [3]. The aforementioned potentially devastating and disabling consequences of ischemia-reperfusion injuries make the investigation of possible interventions urgently needed.

Figure 2: The effect of interventions on ischemia-reperfusion injury

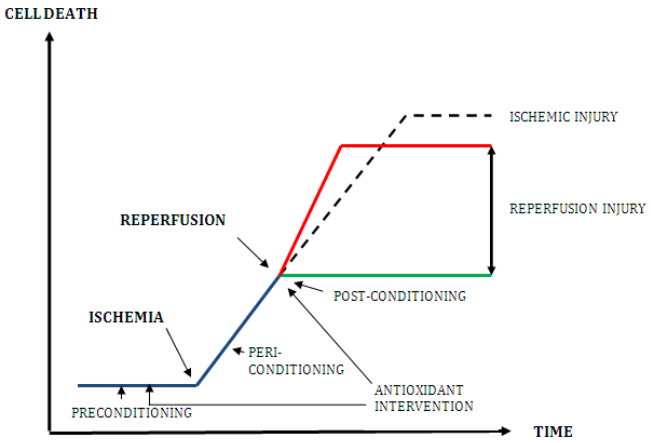


Figure 2: Injury in ischemia– reperfusion develops in two phases. Reperfusion injury adds to the injury developed during initial ischemia (resulting in the red curve). The extent of reperfusion injury can be influenced by protective procedures, such as pre-, peri- or postconditioning or protective agents such as antioxidants (melatonin), applied either before the ischemic event or during the first minutes of reperfusion (resulting in the green curve). When the ischemic tissue is not reperfused, it becomes entirely subject to ischemic cell death (broken black curve).

Melatonin

Melatonin is a hormone mainly secreted by the pineal gland located in the brain [15]. The indole structure of melatonin (N-acetyl-5-methoxytryptamin) is synthesized from L-tryptophan taken up by pinealocytes from the circulation and transformed to serotonin by hydroxylation. Serotonin is then by acetylation and methylation converted to melatonin [14,72]. The synthesis and the secretion of melatonin are inhibited by light and stimulated by darkness by photic information transmitted from the retina via nerve fibres through the hypothalamic suprachiasmatic nucleus to the pineal gland [15]. In humans, the endogenous level of melatonin follows a circadian rhythm with an increase in secretion soon after the onset of darkness and a peak between 2 and 4 AM. Hereafter, the plasma levels gradually falls to undetectable levels in daytime [15,72]. The amplitude of the diurnal serum melatonin peaks at the age of one to three years and is then gradually attenuated with age [19]. The amplitude shows intra-subject reproducibility but it varies greatly between subjects [72].

Due to melatonin being both hydro- and lipophilic, it can easily cross all cell membranes and is excreted in saliva, urine, bile, cerebrospinal fluid etc. [73;74]. Melatonin is mainly metabolized in the liver by hydroxylation to 6-hydroxymelatonin and then conjugated with sulphuric or glucuronic acid and excreted in the urine as 6-sulfatoxymelatonin [15]. The bioavailability of exogenous melatonin when given intravenously is 100% and after intravenous (i.v.) bolus administration, plasma melatonin displays a biexponential decay with a first distribution half-life of two minutes and a second metabolic half-life of 20 minutes [72]. Orally administered melatonin of 80 mg has been reported to result in serum melatonin concentrations up to 10000 times higher than the usual endogenous level at night-time [75]. Industrially produced melatonin is highly soluble in ethanol [76], but the solubility of melatonin in non-ethanol based buffers has not been extensively studied [77-80] and studies without the use of surfactants to enhance the solubility have

not yet been conducted. This limits the applicability of melatonin in direct intracoronary (i.c.) administration due to ethanol's effect on the cardiomyocytes where it can induce myocardial necrosis [81,82].

In humans, two distinct classes of G-protein coupled, seven transmembrane melatonin receptors, MT_1 and MT_2, have been reported [83,84]. Both receptors are widely expressed in a variety of tissues including the cardiovascular system [85,86] and the cardiomyocytes [83]. In the coronary arteries, activation of the MT_1 receptor is thought to initiate vasoconstriction [85] while activation of the MT_2 receptor initiates vasodilatation [86]. During experimental ischemia-reperfusion, melatonin has proven to reduce vasoconstriction and the incidence of ventricular arrhythmias [87] as well as increase coronary blood flow and cardiac function through the MT_1 and MT_2 receptors [88]. Preliminary evidence suggests a circadian variation of the MT_1 receptor in the coronary arteries, but further studies are warranted to explore on the functional relevance of these receptors [89].

Melatonin is a relatively non-toxic molecule and no serious adverse effects related to the use of oral or intravenous routes of melatonin in a broad range of concentrations have been reported [90]. Several safety studies have been conducted, but none of these studies have reported serious side effects [91-93]. In fact melatonin has been proven to reduce the toxicity of many drugs known to produce serious side effects [94].

Melatonin and ischemia reperfusion injuries – the rationale

Melatonin and oxidative stress

As previously discussed, the pathogenesis of ischemia-reperfusion injury is a multifactorial process involving many different biological mechanisms. The generation of ROS, in particular O_2^- [6,27], is one of the known mechanisms which causes cell damage and can initiate local inflammatory responses causing further oxidant mediated tissue injury [95]. Melatonin and the metabolites of melatonin have long been known to act as direct free radical scavengers [96-99] and melatonin's stimulatory effects on antioxidative enzymes such as glutathione peroxidase, superoxide dismutase and catalase enables it to function as an indirect antioxidant as well [100,101]. These properties enable melatonin to attenuate the tissue damages inflicted by ROS.

Due to its non-polar structure, melatonin can penetrate all cell membranes and the highest intracellular concentration of melatonin appears to be in the mitochondria [98]. Since a vast amount of the ROS produced during ischemia-reperfusion is generated in the mitochondria [102], it further increases the interest in melatonin as a possible protective agent. Furthermore, it has been shown that one of the devastating mechanisms during reperfusion is the opening of the mPTP [44]. Evidence suggests that melatonin can protect against myocardial ischemia-reperfusion injury by inhibiting the mPTP opening [24,103].

The anti-oxidative properties listed above are all receptor independent, however, it has recently been demonstrated that melatonin-induced cardioprotection against myocardial ischemia-reperfusion may also be receptor dependent [104] and induce short-term as well as long-term protection [105].

Melatonin and inflammation

When invading organisms are being engulfed by phagocytes, the destruction of the organisms by free radicals is necessary in the inflammatory reaction. However, if the inflammatory response becomes inappropriate, as in sepsis or MODS, the generated free radicals can lead to extensive tissue injury [106]. By its ability to directly scavenge toxic free radicals, melatonin can attenuate this damage in all organs [107]. Furthermore, melatonin is capable of reducing the up-regulation of a variety of pro-inflammatory cytokines such as interleukins and tumor necrosis factor-alpha (TNF-α) by preventing the translocation of the transcription factor, nuclear factor-kappa B (NF-κB), to the nucleus and its binding to DNA [108]. In addition, melatonin has been shown to reduce recruitment of polymorphnuclear leukocytes to the inflammatory sites [109] and to increase natural killer (NK) cell activity [110].

The antioxidant, anti-inflammatory and immunomodulatory actions of melatonin make it a promising, appropriate add-on pharmacological tool in sepsis and multiorgan failure, although the understanding of melatonin's action in the pathogenesis is not fully achieved [106].

Circadian variations

The suprachiasmatic nucleus, located in the hypothalamus, is the main regulator of a variety of the body's cyclic functions including body temperature and secretions of hormones such as melatonin [111]. The cardiovascular system exhibits diurnal rhythms in heart rate, blood pressure and endothelial function, and this rhythm is possibly modulated by the melatoninergic system [112,113].

A retrospective study has shown a circadian variation of the infarct size in patients with AMIs with the largest infarctions occurring between 6.00 AM and noon (dark-to-light period) [22]. It has been proposed that this time-of-day-dependent tolerance to ischemia-reperfusion injury is mediated by the cardiomyocyte circadian clock [114]. Patients with coronary artery disease [21,115] and patients with AMI [20] have been shown to have a significant decrease in their nocturnal synthesis of melatonin. Thus, a possible association between cardiovascular events and the circadian variation might in part be related to the circadian rhythm in cardiovascular disease [20]. It is, however, uncertain whether the low levels of melatonin in patients with cardiovascular diseases are a result of melatonin consumption due to melatonin scavenging free radicals, or if it truly represents a lower melatonin production and consequently a deficient protection against oxidative stress [116]. Beta-blockers, which are prescribed to many patients with coronary artery disease, are known to reduce the production of melatonin via specific inhibition of adrenergic beta1-receptors [117] and this might offer another explanation of the low melatonin levels.

In summary, melatonin displays antioxidant, anti-inflammatory and chronobiotic regulatory functions. All of these actions make melatonin interesting as a promising agent for attenuation of ischemia-reperfusion injuries in humans.

Hypotheses

In view of the above we aimed, with this PhD thesis, to test the following hypotheses:

- It is possible to measure markers of oxidative stress in animals, patients and in healthy volunteers after a period of ischemia and reperfusion.
- Melatonin may have a modulatory effect in experimental and clinical ischemia and reperfusion.

METHODS AND MATERIALS

Oxidative biomarkers

Malondialdehyde

Ischemia and reperfusion of the extremities cause lipid peroxidation. Lipid peroxidation is a chain reaction leading to the oxidation of polyunsaturated fatty acids that, in turn, disrupts the structure of biological membranes and produces toxic metabolites such as malondialdehyde (MDA) [118]. Lipid peroxidation as a free radical generating system may be closely related to ischemia-reperfusion induced tissue damage with an increase at reperfusion, and MDA is a good indicator of the degree of lipid peroxidation [119-121]. Furthermore, there seems to be a close relationship between MDA and cardiac necrosis markers [122]. In the studies measuring MDA in this PhD thesis, we have determined the MDA concentrations in both muscle and plasma using high-performance liquid chromatography (HPLC) with fluorescence detection. When MDA reacts with thiobarbituric acid, a pink fluorescence is formed which then can be assessed by fluorimetry with excitation at 515 nm and emission at 553 nm. This determination of MDA levels is found to be the most reliable method [123].

Ascorbic acid and dehydroascorbic acid

Vitamin C (ascorbic acid, AA) and the oxidized form of AA, dehydroascorbic acid (DHA) are hydrophilic antioxidants, which remove the aqueous phase oxygen free radicals by a rapid electron transfer [124]. The determination of AA and DHA levels is challenging because of the unstable nature of the compounds [125]. In our study (paper 2) we used HPLC with coulometric detection for the determination of AA. This method also measures the levels of total ascorbic acid (TAA), and since no method exists for determination of DHA, the levels of DHA in plasma were calculated by subtracting AA from DHA. This has proven to be the most reliable method [126].

Inflammatory biomarkers

Cytokines are small, nonstructural proteins that are primarily involved in host responses to ischemia, trauma, disease or infection [127]. There are presently 18 cytokines with the name interleukin (IL) and other cytokines have retained their original biological description e.g. tumor necrosis factor (TNF) [127]. While some cytokines clearly promote inflammation and are named pro-inflammatory cytokines, others suppress the activity of the pro-inflammatory cytokines and are named anti-inflammatory cytokines. However, depending on the biological process, any cytokine may function differentially [127].

Pro-inflammatory markers

In our studies (paper 2 and 4) we have used TNF-α, IL-1β, IL-6 and YKL-40 as plasma biomarkers of inflammation. TNF-α and IL-1β are inducers of endothelial adhesion molecules which are essential for the adhesion of leukocytes to the endothelial surface prior to emigration into the tissue. The synergism of TNF-α and IL-1 β is a known phenomenon and both cytokines are produced at sites of local inflammation [127]. IL-6 has both pro-inflammatory and anti-inflammatory properties, but is a potent inducer of the acute–phase protein response [128]. TNF-α, IL-1β and IL-6 have been found in the plasma of patients during myocardial infarction [129]. YKL-40 is a heparin- and chitin-binding lectin which is secreted by macrophages and neutrophils and is used as a plasma marker of inflammation. YKL-40 was determined by a sandwich enzyme-linked immunosorbent assay (ELISA) method (Quidel, Santa Clara, CA, USA) [130].

Anti-inflammatory markers

We have used IL-10, IL-1 receptor antagonist (IL-1Ra) and soluble TNF receptors (sTNF-R) I and –II as anti-inflammatory biomarkers in our studies (paper 2 and 4). IL-10 is considered the most important anti-inflammatory cytokine found within the human immune response [128]. Clinical studies in patients with AMI are, however, inconsistent on the prognostic value of circulating plasma levels of IL-10[131]. IL-1Ra functions as a specific inhibitor of the pro-inflammatory IL-1β while the sTNF-RI and –II function as specific inhibitors of TNF activity on target tissue [128]. Both the pro-inflammatory cytokines and the anti-inflammatory mediators obtained from plasma in the healthy volunteers (paper 2) were measured in a Luminex 100 IS analyzer (Luminex Corporation, Austin, Texas, USA) using appropriate multiplex antibody bead kits (Invitrogen Corporation, Carlsbad, CA, USA) [132]. The pro-inflammatory (IL-1 β, IL-6) and anti-inflammatory (IL-10) cytokines in the porcine-model of AMI were analyzed by sandwich ELISAs from R&D Systems (Duoset DY686, Duoset DY681 and Duoset DY693B, respectively).

Visual Analogue Scale

The visual analogue scale (VAS) has been used for many years for the assessment of subjective phenomena [133]. We used the VAS in our study (paper 2) for assessment of pain during tourniquet-induced ischemia and in the following reperfusion period. The pain severity was evaluated by a total of eight VAS measurements. We used a 100 mm long horizontal line without end-lines or numbers. The left side was stating “no pain” (= 0 mm) and the right side was stating “worst possible pain” (= 100 mm). The healthy volunteers drew a vertical line on the horizontal line representing the pain intensity they perceived when asked. To ensure uniformity all the VAS were identical and had the same direction of increasing severity in pain. There has been much discussion as to whether the VAS represents ordinal or continuous data [134]. In our study we interpreted VAS scores as continuous data and reported data in median and interquartile range (IQR) and used non-parametric statistics.

Muscle biopsies

In the healthy volunteer study (paper 2) we took a baseline and a post-reperfusion muscle biopsy to examine our primary outcome, MDA. For this procedure we used a 5 mm Bergström muscle biopsy needle (Figure 5) to extract muscle tissue from the m. vastus lateralis. This is a safe and widely used method of obtaining a muscle biopsy [135].

Serum Element Response (SRE) assay

In the MT_1 and MT_2 receptor activation study (paper 3) we tested the receptor activation mediated by melatonin dissolved in three classical non-ethanol based buffers and compared it with the receptor activation of melatonin dissolved in ethanol. For this we used an SRE-assay in which the receptor activation is facilitated by intracellular mechanisms. The MT_1 and MT_2 receptors are G protein-coupled and signal through G proteins within the cell when stimulated by melatonin. Although the receptors are known to couple $G\alpha_i$, in our study they recruited a chimeric G protein which facilitates the signal through the $G\alpha_q$ pathway. When the intracellular signaling terminates in the nucleus, the SRE is activated to initiate DNA transcription. In this assay the SRE-gene was fused with a luciferase-gene. When the response element was

activated, it synthesized this SRE/luciferase-peptide. On the last day of the experiment, the cells, when added a substrate which was cleaved by luciferase, created luminescence in proportional amounts reported as relative light units.

ETHICAL CONSIDERATIONS

For the study on healthy volunteers (paper 2) approval was obtained from the Danish National Committee on Biomedical Research Ethics (H-4-2011-110), and the Danish Data Protection Agency (2007-58-0015/HEH.750.89-15). Written informed consent was obtained from all study subjects and the trial was registered on Clinicaltrials.gov (NCT01486212).

For the porcine closed-chest model of acute myocardial infarction (paper 4) the study was performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication no. 85-23, revised 1996) and approval was obtained from the Danish Animal Experiments Inspectorate, license no. 2012-15-2934-00583.

For the IMPACT-protocol (paper 5) approval was obtained from the Danish National Committee on Biomedical Research Ethics (H-3-2010-117), the Danish Medicines Agency (EudraCT nr. 2010-022400-53) and the Danish Data Protection Agency (HEH.afd.D.750.89-9). The study was also registered on Clinicaltrials.gov (NCT01172171).

STATISTICAL CONSIDERATIONS

In the studies included in this PhD thesis, we have used non-parametrical statistics after testing for normality using the Kolmogorov-Smirnov test. For paired data we have used Wilcoxon signed rank test if there were two groups and Friedman analysis of variance if there were more than two groups. For unpaired data we have used Mann-Whitney U test if there were two groups and Kruskal-Wallis if there were more than two groups. Data were presented as median (IQR) unless specified otherwise. Results with p-values ≤ 0.05 were considered statistically significant. Data were analyzed using SPSS version 20.0 software (IBM Corp., Armonk, NY, USA).

Biological markers, such as MDA and cytokines, typically exhibit a positive skew (skewed to the right). Hence, for the oxidative and inflammatory markers obtained in the porcine closed-chest model (paper 4), we log transformed the data in order to normalize the distribution. However, some of the log-transformed markers had p-values ≤ 0.05 when testing with the Kolmogorov-Smirnov test, thus we decided to perform non-parametric testing.

OBJECTIVES

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

- To review the literature on tourniquet-related oxidative damage in orthopaedic surgery (paper 1).
- To clinically test an ischemia-reperfusion model in healthy volunteers measuring biochemical oxidative and inflammatory markers in order to produce a potential model for future intervention studies (paper 2).
- To test if melatonin could be dissolved in aqueous buffers and still activate the melatonin receptors, MT_1 and MT_2 (paper 3).
- To explore the effect of intracoronary and systemic melatonin administration in a porcine closed-chest model of myocardial infarction measured by oxidative and inflammatory biomarkers released in the reperfusion phase (paper 4).
- To design a trial and publish the protocol on a RCT aimed at testing if intracoronary and systemic melatonin administration in patients suffering from acute myocardial infarction and undergoing primary percutaneous coronary intervention can reduce infarct size measured by cardiac MRI (paper 5).

PRESENTATION OF THE INCLUDED PAPERS

PAPER 1: INTERVENTIONS TO REDUCE TOURNIQUET-RELATED ISCHAEMIC DAMAGE IN ORTHOPAEDIC SURGERY: A QUALITATIVE SYSTEMATIC REVIEW OF RANDOMISED TRIALS

Objective: The objective of this paper was to review the biochemical oxidative biomarkers that are released in the reperfusion phase following tourniquet-related surgery, explore which interventions that might reduce these markers, and finally investigate whether a potential biochemical reduction was reflected in a better postoperative clinical outcome.

Methods: The review was conducted according to the PRISMA guidelines [136]. A literature search was performed in September 2013 in the following electronic databases: PubMed, Embase and Cochrane Central Register of Controlled Trials. The search was supplemented by manual reference list searches of the included studies to identify additional studies. The whole search strategy is shown in Figure 3. We only included randomized, clinical trials which aimed at reducing oxidative stress measured by biochemical markers in adult patients undergoing tourniquet-assisted surgery on the extremities. Only articles reported in full-text and in English were included. From each included study we extracted information regarding study design, population, intervention, primary outcome measures and postoperative clinical outcomes or complications. Data were extracted without any data transformation. For assessment of the methodological quality of the trials we used the Jadad scale [137]. This assessment was supplemented with a table covering inclusion- and exclusion criteria, sample size calculation, and intention-to-treat analysis.

Results: The literature search, the screening and the assessment of the included studies were performed independently by the first and the second author. From a total of 66 records screened, we included 17 studies with a total of 565 patients (Figure 4). The interventions used in the included studies were divided in to three groups. Nine of the studies used anesthetic interventions (propofol, dexmedetomidine, ketamine and spinal anesthesia), four studies used antioxidants (N-acetyl-cysteine, vitamin C and mannitol) and four studies used ischemic preconditioning. All but one study were of poor quality assessed by the Jadad scale. Only two studies reported postoperative clinical outcomes or complications. Fifteen studies reported that the applied interventions significantly reduced the levels of biochemical oxidative stress markers, while two studies, using dexmedetomidine and mannitol, did not find any reduction compared to the control groups.

Conclusion: Tourniquet-related surgery elicits an expression of biochemical oxidative stress markers in the reperfusion period. This expression can be reduced by intervention of primarily propofol and ischemic preconditioning. A correlation between the reduction in oxidative stress and postoperative clinical outcomes/complications remains to be further investigated.

Strengths and limitations: An obvious limitation in any systematic review is the quality of the included studies. In this review the quality was very low as assessed by the Jadad scale. This scale has rather fallen out of use e.g. within the Cochrane Collaboration, where a non-numerical risk of bias tool is now preferred. One of the reasons for this is that the Jadad scale has more focus on the quality of reporting than on the methodological quality [138]. Furthermore, for randomization, the scale addresses explicitly the sequence generation but not concealment of allocation and the scale does not address blinding of caregivers or intention-to-treat analysis. Yet, we find that with our additional reporting of inclusion- and exclusion criteria, sample size and intention-to-treat, we were able to make a fair assessment of the individual studies. We also found that the included studies were too heterogeneous with regard to interventions, study population and outcomes and we have therefore not performed a quantitative meta-analysis.

Another potential limitation in systematic reviews in general, is the search strategy. This also applies in this review. Even though we searched several of the largest most relevant databases and supplied with reference list searches, the risk of not including relevant studies cannot be eliminated. Furthermore, we limited the literature search to only including oxidative stress or oxidative stress markers, and the search strategy did not include clinical outcomes or complications. This was deliberately done because we wanted to review the literature on the different biochemical stress markers following tourniquet-related ischemia-reperfusion as a primary outcome and secondly look at the correlation between a reduction in oxidative stress markers and the clinical outcomes or complications. Our search strategy, however, did not include these terms in the search. Therefore, there are probably relevant randomized trials which have looked at clinical outcome following tourniquet-related surgery but have not measured oxidative stress and these studies, though relevant, would not have been identified through our search. To fully explore this very interesting topic it would require a different literature search.

The literature search, screening of possible titles and abstracts and assessment of relevant studies were performed by two independent authors. Discrepancies between the assessors were rare and were all settled in consensus and we did not perform comparative statistics on the degree of disagreement.

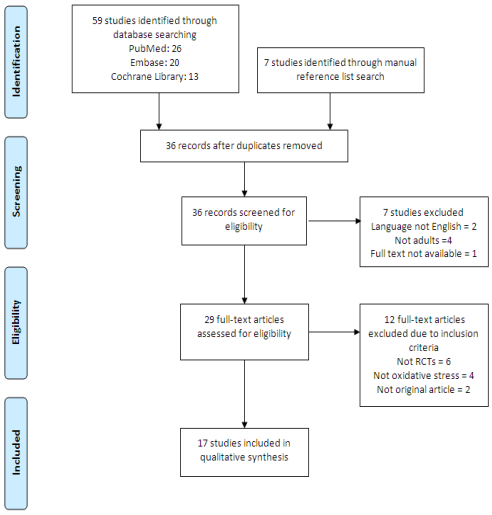
We only included studies published in English, however the potential language bias arising from this has been shown to be very limited [139] and the only relevant identified study in a non-English language (Italian) was not an RCT. Moreover, we did not search clinical trial databases in order to identify any unpublished or ongoing studies which might be of relevance. This could imply a potential risk of overestimating the effect of the interventions if several RCTs on this topic were not published due to negative findings. However, due to heterogeneity and clinical diversity, this review resulted in a qualitative analysis as opposed to a meta-analysis, and it would thus not have had the same influencing impact on the summarizing conclusion.

The conducting of the review using the PRISMA guidelines has added strength to the study due to the systematic and thorough approach it provides in conducting and reporting. It is a possibility to register a systematic review prospectively at e.g. the PROSPERO register. As with the registration of clinical trials, this is thought to minimize the risk of publication bias, enhance the transparency and avoid duplication of effort [140,141]. No formal protocol for our review was registered.

Figure 3: The comprehensive literature search as performed in Pubmed. The literature search in Embase and Cochrane databases were similar to this search. Limitations were (Humans [Mesh].

((((((((((((((ischaemia reperfusion injur*) OR ischaemia-reperfusion injur*) OR ir injur*) OR ir-injur*) OR ischemia reperfusion injur*) OR ischemia-reperfusion injur*) OR reperfusion damage) OR ischaemia reperfusion damage) OR ischaemia-reperfusion damage) OR ir damage) OR ischemia reperfusion damage) OR ischemia-reperfusion damage)) AND ((oxidative stress) OR oxidative stress marker*)) AND (((tourniquet*) OR tourniquet operations) OR bloodless surgery).

Figure 4: PRISMA flow diagram of the study selection process.



Reprinted with permission from Paper 1, Halladin et al. [142].

PAPER 2: LOWER LIMB ISCHEMIA AND REPERFUSION INJURY IN HEALTHY VOLUNTEERS MEASURED BY OXIDATIVE AND INFLAMMATORY BIOMARKERS

Objective: The objective of this study was to characterize a human ischemia-reperfusion model measuring biochemical markers locally in muscle biopsies and systemically in the circulation. We wanted to characterize an ischemia-reperfusion model without the influencing factors of surgery and anesthesia.

Methods: Ten healthy male volunteers had a pneumatic tourniquet applied to their lower limb (Figure 5). It was inflated to a pressure of 300 mmHg and sustained for 20 minutes before deflation. Blood samples were collected from an intravenous catheter

in the cubital vein at baseline, 5, 15, 30, 60, and 90 minutes after start of reperfusion. For a complete overview of the study see Figure 6. Before inflation a muscle biopsy was taken from the vastus lateralis muscle of the opposite limb. Thirty minutes after start of reperfusion a muscle biopsy was taken from the vastus lateralis muscle just below the placing of the tourniquet. The muscle biopsies were taken with a 5 mm Bergström muscle biopsy needle [135] (Figure 7).

The volunteers scored their pain at baseline, every 5th minute during inflation and until 15 minutes after deflation using a visual analogue scale (VAS).

Our primary outcome was malondialdehyde (MDA) in the muscle. Secondary outcomes were circulating plasma markers of oxidative stress (MDA, vitamin C (ascorbic acid, AA) and dehydroscorbic acid (DHA)) and markers of inflammation (tumor necrosis factor (TNF)-α, interleukin (IL)- 1β, IL-1 receptor antagonist (IL-1Ra), IL-6, IL-10, TNF-receptor (TNF-R)I, TNF-RII and YKL-40).

Results:
MDA in the muscle biopsies revealed no differences when comparing baseline 46.9 (38.8-50.8) nmol/g tissue with 30 minutes of reperfusion 40.1 (31.4-48.0) ($p = 0.39$). Data were analyzed using the Wilcoxon signed rank test.

There were no significant changes in any of the oxidative markers, MDA, AA or DHA from baseline to after reperfusion ($p = 0.71$, $p = 0.94$, $p = 0.88$). Data were analyzed using Friedman analysis of variance test.

There were no significant changes in any of the inflammatory markers (TNF- α, IL-1β, IL-1Ra, IL-6, IL-10, TNF-RI, TNF-RII and YKL-40) from baseline to 90 minutes after start of reperfusion. ($p = 1$, $p = 0.68$, $p = 1$, $p = 1$, $p = 0.12$, $p = 0.1$, $p = 0.56$, and $p = 0.54$, respectively. Data were analyzed using Wilcoxon signed rank test and Friedman analysis of variance for YKL-40.

VAS scores peaked at 20 minutes of ischemia with a median of 22 (10-56) mm. Ten minutes after reperfusion the VAS scores were not significantly different from baseline values ($p = 0.14$). Data are presented as median (IQR) and were analyzed using the Wilcoxon signed rank test.

Conclusion: This study showed that 20 minutes of ischemia of the lower limb was not enough time to produce a response that could be measured by a broad range of local or circulating oxidative and inflammatory markers up to 90 minutes after start of the reperfusion.

Strengths and limitations: The characterization of an ischemia-reperfusion model without the influencing factors of surgery or anesthesia are warranted for future intervention studies aimed at reducing the reperfusion injury. Therefore, we found this model interesting. However, the study holds certain limitations. We knew of no previous studies which had explored this simple healthy volunteer model and to calculate sample size we therefore used a study examining MDA in patients undergoing reconstruction of the ligaments in the knee. This, however, might have underestimated the sample size as the patients are also exposed to surgical stress and this might in itself have increased their oxidative stress level. We might therefore have made a Type II error.
However, we did not find any difference from baseline to after the reperfusion, which could suggest an error in design rather than too small a sample size. The primary outcome, MDA [118,125] has been shown to be reliable biomarkers of oxidative stress and the method of measurement is validated [123].

It has previously been shown that 60 minutes of ischemia and 60 minutes of reperfusion are enough to elicit hepatic myeloperoxidase (MPO) production and subsequent neutrophil recruitment, whereas 120 minutes of ischemia and 60 minutes of reperfusion, increased the thiobarbituric acids (TBARS) level. This confirms the aggravating effects of prolonged ischemia on oxidative stress [67]. Thus, it could implicate that the length of the ischemic event in this healthy volunteer study was too short for the expression of oxidative stress and inflammation. However, if we had measured MPO as a marker for neutrophil sequestration we might have seen a response.

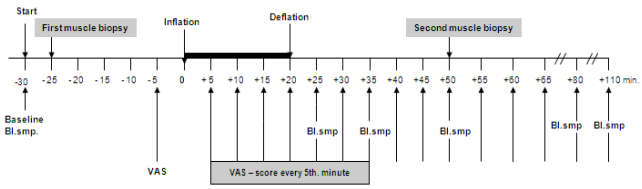
The lack of a sufficient ischemia-reperfusion response in this model was further stressed by the fact that we did not detect any differences in the levels of AA or DHA. If the ischemic period and the subsequent reperfusion had caused injuries that were too subtle to detect using MDA as a marker, it might have been because the body was using its antioxidant capacity (AA) to protect against this injury. However, since the levels of AA or DHA were not decreased from baseline to the late reperfusion phase, this indicates that the ischemic event was not severe enough.

Hence, the most obvious reasons for not obtaining an expression of reperfusion injury were that the ischemic period as well as the reperfusion period was too short. It might also have improved the design had we used additional biomarkers to measure the reperfusion response.

Figure 5: Setup of the tourniquet study.

A baseline muscle biopsy was taken from the right thigh and the tourniquet was placed and inflated around the left thigh. A catheter for blood sampling was placed in the right cubital vein.

Figure 6: Overview of the time course in the healthy volunteer study.



Bl.smp = blood sample, VAS = visual analog scale

Figure 7: The 5 mm Bergström muscle biopsy needle.

PAPER 3: HIGH POTENCY ON MT_1 AND MT_2 RECEPTORS BY MELATONIN DISSOLVED IN ISOTONIC SODIUM CHLORIDE, ISOTONIC GLUCOSE OR KREBS RINGER-LACTATE BUFFERS, BUT NOT IN ETHANOL

Objective: The objective of this study was to examine whether melatonin could be dissolved in non-ethanol based buffers and still be able to activate the melatonin receptors, MT_1 and MT_2 and compare this to melatonin dissolved in ethanol. Furthermore, we wanted to test whether light exposure to melatonin dissolved in the non-ethanol based buffers would suppress melatonin's effect on receptor activation.

Methods: We used three classical, non-ethanol based buffers; isotonic sodium chloride, isotonic glucose and Krebs Ringer-lactate to test the aqueous solubility of melatonin. We used ten-fold dilution rows starting with a concentration of 2.5 mg/ml, and the solutions were then diluted until the melatonin was dissolved. Solubility was defined as no visible precipitate determined by visual inspection. The receptor activating abilities of melatonin dissolved in the three classical buffers and in ethanol (96%) were assessed at 37 ºC by the functional readout measuring the activity of the Serum Response Element (SRE) transcription factor.

Furthermore, solutions of melatonin dissolved in isotonic sodium and in Krebs Ringer-lactate, were placed at room temperature and exposed to five hours of lamp-light before testing the receptor activation using the SRE-assay.

Results: Using dilution rows we found melatonin to be soluble in all three non-ethanol based buffers at a concentration of 0.1 mg/ml (0.43×10^{-3} M) and melatonin dissolved in all three solutions was still able to activate both the MT_1 and MT_2 receptors (EC_{50} at 2.8-114 pM). Melatonin was easily dissolved in ethanol; however, it required a 250-26000 times higher concentration (EC_{50} at 28-73 nM) than melatonin dissolved in the non-ethanol based buffers in order to activate the MT_1 and MT_2 receptors. We found no significant reduction in the potency and efficacy of melatonin solutions exposed to five hours of lamp-light.

Conclusion: Melatonin can be dissolved in non-ethanol based buffers without losing the potency of the MT_1 and MT_2 receptor activation. In fact, when dissolved in ethanol, melatonin acts with lower potency than when dissolved in non-ethanol based buffers. Receptor activation is not reduced in melatonin solutions exposed to light.

Strengths and limitations: For the determination of the melatonin concentrations we used a ten-fold dilution row, hence we were not able to determine the exact concentration at which melatonin could be dissolved in non-ethanol based buffers. Ideally, the concentration of melatonin should be tested using high performance liquid chromatography (HPLC). However, the objective of this study was to test whether melatonin could be dissolved in classical buffers without losing the receptor activating properties, and compare it to the standard solvent of melatonin; ethanol. This objective has been met.

Ethanol as a solvent agent might also in itself have influenced the readout measuring the activity of the SRE transcription factor. It has previously been described in a luminescence resonance energy transfer (LRET)-assay that the maximal readout was reduced up to 40 % if the assay buffer was mixed with ethanol prior to the addition of proteins (a 5% mix of ethanol to the total buffer volume) [143]. In our setup, 5 µL of the ethanol/melatonin solution was added to 100 µL cell media corresponding to a 5% mix, however, the difference being that in our setup the melatonin was dissolved in ethanol 96% and then further diluted with ethanol, before adding it to the cell media. Though, whether this possible readout reduction also reflects a reduced potency remains to be determined.

This study is the first to examine the receptor activating properties of melatonin dissolved in non-ethanol based buffers without the use of surfactants. The results from this study increase the applicability of melatonin in clinical settings where dissolving in ethanol is not an option.

PAPER 4: MELATONIN DOES NOT AFFECT OXIDATIVE AND INFLAMMATORY BIOMARKERS IN A CLOSED-CHEST PORCINE MODEL OF ACUTE MYOCARDIAL INFARCTION

Objective: The objective of this study was to examine whether melatonin could reduce oxidative and inflammatory markers elicited in the reperfusion phase in a closed-chest porcine model of acute myocardial infarction.

Methods: This study reports secondary outcomes (oxidative and inflammatory biomarkers) from an RCT that primarily sought to investigate the cardioprotective effects of intracoronary (i.c.) and intravenous (i.v.) melatonin in a closed-chest porcine model of myocardial infarction assessed by cardiac magnetic resonance imaging (CMR).

Twenty Danish Landrace pigs were anesthetized, and under X-ray guidance a PCI guiding catheter was inserted through an introducer sheath in the left coronary ostium through the femoral artery (Figure 8). A baseline coronary angiogram was performed and an over-the-wire balloon catheter was placed in either the left anterior descending artery (LAD) or the circumflex coronary artery (Cx). Ischemia was induced by

inflation of the angioplasty balloon and the vessel was kept occluded for 45 minutes. Occlusion was verified by ECG changes.

The pigs were randomized to receive i.c. and i.v. of either 200 mg melatonin dissolved in isotoninc saline (0.4 mg/ml) or placebo (isotonic saline). Five minutes prior to reperfusion an i.v. infusion of either 495 ml isotonic saline containing 198 mg melatonin or 495 ml isotonic saline (placebo) was started. This infusion lasted 30 minutes in total. One minute prior to reperfusion, a bolus of 5 ml 0.4 mg melatonin/ml isotonic saline or placebo (5 ml isotonic saline) was injected through the over-the-wire catheter directly into the occluded coronary artery. This injection lasted until the first minute of reperfusion (total injection time was 2 minutes). The myocardium was then reperfused for four hours before the hearts were explanted.

From a catheter in the femoral vein, blood samples were collected at baseline, 30 minutes and 1, 2, 3 and 4 hours after the start of reperfusion and analyzed for markers of myocardial injury; high-sensitivity troponin T (hs-TnT), markers of oxidative stress; MDA, and markers of inflammation; IL-1β, IL-6 and IL-10.

Results: Three pigs died due to irreversible ventricular fibrillation, thus eight pigs in the melatonin group and nine pigs in the placebo group completed the experimental protocol and were included in the final analysis. Data were analyzed using non-parametric statistic.

The highest increase in hs-TnT levels was two hours after the start of reperfusion. There was no difference between the two groups at this time point; 5881 (1398-9158) ng/L in the placebo group versus 3413 (2389-6326) ng/L in the melatonin group (p = 0.63).

With regard to development over time of the MDA levels, we found no significant difference between the melatonin group and the placebo group (p = 0.06 and p = 0.23, respectively). Four hours after the start of the reperfusion, there was no significant difference in MDA levels between the two groups (p =0.63).

With regard to development of IL-1β, IL-6 and IL-10 levels over time, there was no significant difference in either the melatonin group (p = 0.25, 0.08, 0.08, respectively) or the placebo group (p = 0.11, 0.13, 0.14, respectively). Four hours after the start of the reperfusion, we found no significant difference between the two groups in any of the inflammatory markers (p = 1.0, 0.39, 0.35, respectively).

Conclusion: The porcine closed-chest model of myocardial infarction with four hours of reperfusion was not successful in demonstrating the cardioprotective effects of melatonin when assessed by circulating biochemical markers of oxidative stress and inflammation.

Strengths and limitations: This study reports secondary outcomes from an RCT designed to evaluate the cardioprotective effect of melatonin assessed by CMR, hence the sample size was originally calculated for this primary outcome. Consequently, the lack of an effect could thus be due to the fact that we did not include enough animals to find an effect (Type II error). However, a post-hoc sample size based on MDA, with a Type I error of 5% and a power of 90% revealed that we would need 18 pigs in total.

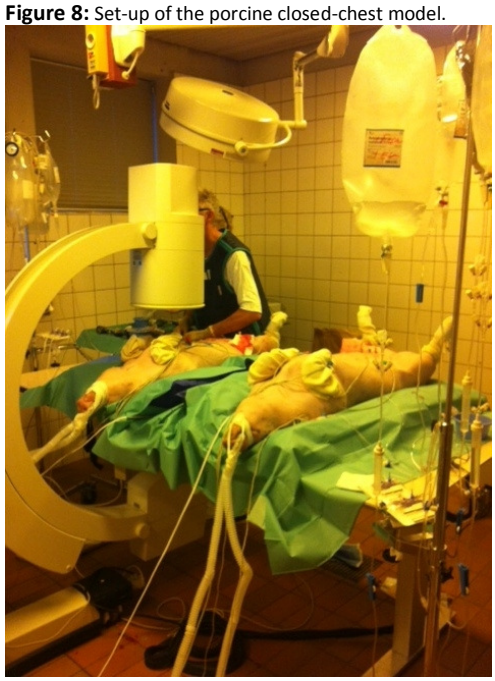
The animals were randomized and both the experimenters and the laboratories carrying out the biochemical analyses were blinded. Hence, we have a low risk of selection, performance and detection bias.

The cytokine response secondary to myocardial ischemia and reperfusion has not previously been examined with the porcine closed-chest model. Following acute myocardial infarctions in humans the peak of inflammatory cytokines varies between 2 hours (IL-1β) and 6 hours (IL-6) [129], thus it might have improved the design of the study if we had extended the reperfusion period with at least 2 hours. However, in the present study this was not an option due to the logistic circumstances.

The level of MDA did not increase in the reperfusion phase as we had anticipated. A previous study with an open-chest porcine model reported of significantly elevated levels of MDA after only 30 minutes of ischemia and three hours of reperfusion [144] and another study also reported of significantly elevated levels of MDA immediately after 60 minutes of ischemia and further increased upon reperfusion [145]. These studies were porcine open-chest studies, thus the response might have been augmented by the surgical stress. However, in humans presenting with AMI, the level of MDA was found to be increased within 12 hours after onset of symptoms compared to controls [146].

Due to the similarities of anatomy, physiology and pathology between the porcine and the human heart [147], the porcine closed-chest model has proven to be superior in interventional studies of ischemia-reperfusion injury, as the surgical stress is minimized compared to the open-chest model [148] and we considered it to be the most optimal experimental model with regard to our set-up. However, the use of experimental models as a model for AMI in humans has built-in limitations. As compared to humans, the animals were young and did not take any additional medicine, they had no co-morbidities, they had been exposed to the same environment and they had not developed atherosclerotic arteries. Furthermore, age is known to augment the ROS formation after AMI [149] and the contribution of this factor would not be recognized in a model using young animals. The degree to which the findings in animal models can be extrapolated to a human suffering from AMI is thus limited.

Figure 8: Set-up of the porcine closed-chest model.



The cardiologist is inserting a catheter in the femoral artery under X-ray guidance. The other pig has already had the catheter inserted.

PAPER 5: INTRACORONARY AND SYSTEMIC MELATONIN TO PATIENTS WITH ACUTE MYOCARDIAL INFARCTION: PROTOCOL FOR THE IMPACT-TRIAL

Objective: The objective of this paper was to publish a protocol on the design, rationale, and statistical analyses of a trial aimed at testing whether intracoronary and systemic melatonin administered to patients with acute myocardial infarctions undergoing primary percutaneous coronary intervention (pPCI) can increase the myocardial salvage index (MSI) assessed by cardiac magnetic resonance imaging (CMR).

Methods: The IMPACT-trial is a multicentre, randomized, double-blinded, placebo-controlled study designed to test the effect of intracoronary and systemic melatonin administration to patients undergoing pPCI following STEMI. The original protocol was written and approved by the relevant agencies in January 2011. Recruitment of patients started in July 2013 and is ongoing. The protocol manuscript was published February 2014, after the trial was commenced.

Primary endpoint in the IMPACT-trial is MSI assessed by CMR on day 4 (±1). MSI is calculated as follows:

MSI = (area at risk (AAR) – infarct size)/AAR

AAR will be assessed by short tau inversion recovery T2-weighted (T2-STIR) imaging. Infarct size will be measured by inversion recovery gradient echo sequence (late gadolinium enhancement (LGE) imaging).

Secondary endpoints are:

- high-sensitivity troponin I (hs-TnI) or high-sensitivity troponin T (hs-TnT) measured in blood samples collected before the reperfusion and 6, 24, 48, 72 and 96 hours post-intervention. hs-TnI or hs-TnT will be calculated as area under the curve.
- Creatinkinase myocardial band (CK-MB) measured before the reperfusion and 6, 24, 48, 72 and 96 hours post-intervention.
- Plasma melatonin, advanced oxidative protein products (AOPP), MDA and MPO collected 24 hours post pPCI.
- Clinical events occurring within the first 90 days post pPCI: Sustained ventricular arrhythmias, resuscitation after cardiac arrest, cardiogenic shock, revascularization of a new coronary artery, CABG, major bleedings, re-infarction, stent thrombosis, cardiac and non-cardiac re-hospitalization, and death. Information will be obtained from the patient's medical journal.

If the patients meet the inclusion criteria (Figure 9), they are randomized to a total dose of 50 mg melatonin (0.1 mg/ml) or placebo (isotonic sodium chloride). A bolus of 10 ml melatonin or placebo is given intracoronarily during the first minute of reperfusion, and the remaining 490 ml is given systemically over a period of six hours starting immediately after the pPCI.

pPCI will be performed according to the standard guidelines at the trial centres. The investigator, the operating cardiologist and the patients will be blinded throughout the study and the analysis of the CMR will also be blinded. The allocation code will only be revealed after all the statistical analyses are completed.

In this study we wish to include 2 x 20 patients. The sample size is based on a previous study which tested the cardioprotective effect of exanatide following pPCI and reported the average salvage index measured by CMR to be 0.62 with a standard deviation of 0.16. With a Type I error at 5%, a Type II error at 20% and a minimal relevant difference (MIREDIF) at 25%, it revealed a sample size of 34 patients (17 in each group) in order to detect a difference in MSI between groups. To account for possible drop-outs we chose to include 2 x 20 patients.

Figure 9: Inclusion and exclusion criteria in the IMPACT-trial.

TABLE 1

Inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
Adults > 18 years of age	Known prior myocardial infarction
pPCI initiated within 6 h from symptom onset	Prehospital thrombolysis
Having an electrocardiogram indicative of an acute ST-elevation myocardial infarction showing: ≥ 0.2 mV in V2 or V3 and/or ≥ 0.1 mV in the other leads for men < 40 years: ≥ 0.25 mV in V2-V3	ASA class ≥ 4
	> 1 artery with coronary stenosis that requires treatment
	Known history of renal failure (GFR < 60 ml/min./1.73 m^2 p-cratinin > 200 µmol/l)
Occlusion of a large (> 2 mm) infarct-related coronary artery with TIMI 0-1	Known history of autoimmune diseases (systemic lupus erythematosus, mulitple sclerosis, rheumatoid arthritis, type 1 diabetes mellitus)
Being willing and able to provide informed consent after written and oral information	Servere concurrent illness with reduced short-term prognosis (e.g. terminal cancer, terminal AIDS, severe infection)
	Atrial fibrillation before or after pPCI
	Pregnancy
	Breastfeeding
	Fertile woman < 12 months since menopause or nonsterilised)
	Cardiogenic shock
	BMI > 40 kg/m^2
	Contraindications for CMR (pacemaker, parts of metal in the body and claustrophobia)

ASA class = The American Society of Anesthesiologists Physical Status classification; BMI = body mass index; CMR = cardiovascular magnetic resonance imaging; GFR = glomerular filtration rate; pPCI = primary percutaneous coronary intervention; TIMI = thrombolysis In myocardial infarction.

Reprinted from Paper 5, Halladin et al. [150].

Strengths and limitations: The intention of publishing a protocol for clinical trials serves several purposes. First of all, it promotes transparency and gives a full description of what is planned. Secondly, it minimizes the risk of reporting bias, as the authors cannot perform post hoc changes without a valid explanation. Furthermore, it also increases awareness on the topic. This may benefit several of the involved parties ranging from the trial participants up to the policymakers.

For our primary outcome we have chosen MSI assessed by CMR. Although infarct size is a rough estimate of myocardial salvage, it is dependent on coronary diameter, branching and location of the occluding lesion [151], whereas with MSI we can quantify the area of salvaged myocardium out of the area at risk and this would be a better measure of therapeutic efficacy. Furthermore, it enables the inclusion of patients with occlusion of all coronary arteries (> 2mm) and not only the left anterior descending artery (LAD) which is frequently used in these set-ups. The inclusion of patients with varying occluded arteries increases the external validity of the trial. Although, the use of T2 weighted MR scan as an indirect measure of myocardial edema has been questioned [152], it is the diagnostic tool of choice when estimating area at risk [153] and with respect to our trial design we regard MSI measured with CMR as the optimal outcome.

By administering the melatonin intracoronarily and at the time of reperfusion we thereby allow the melatonin to

work at the site of the myocardial injury at the most vulnerable time. As it is not possible to administer the melatonin prior to the ischemic event, we regard this method as the most optimal. In an experimental model it has been shown that melatonin, given at the time of reperfusion, prevents ventricular arrhythmias [154].

In 2013, a guideline for the minimum content of a clinical trial was published (Standard Protocol Items: Recommendations for Interventional Trials; SPIRIT) including a 33-item checklist [155]. Due to a restriction in number of words allowed from the publishing journal's side, the contents in our published protocol are not in complete accordance with these guidelines. The incomplete information mainly applies to: the data management (item 19), access to data (item 29), ancillary and post-trial care (item 30) and dissemination policy (item 31a-31c). Although, these items are not addressed in the published manuscript, the protocol approved by the relevant agencies fully covers these issues. Retrospectively, although we did not adhere to the SPIRIT guidelines, our main protocol is overall in accordance with the recommended guidelines.

DISCUSSION

Principal findings

With this PhD thesis we aimed at testing if it was possible to measure markers of oxidative stress in animals, patients and in healthy volunteers after a period of ischemia and reperfusion. Furthermore, we wanted to test if melatonin may have a modulatory effect in experimental and clinical ischemia and reperfusion.

Oxidative stress following tourniquet-related orthopedic surgery can be measured by biochemical markers. Moreover, ischemic preconditioning and propofol seem to reduce the oxidative stress markers, however, the correlation with postoperative clinical outcomes and complications still needs to be investigated.

Twenty minutes of lower limb ischemia was not sufficient to produce an oxidative or an inflammatory response that could be measured by a broad range of biochemical markers up till 90 minutes after the start of reperfusion. The porcine closed-chest model of myocardial infarction with 45 minutes of myocardial ischemia and four hours of reperfusion was also not optimal with regard to examining the circulating oxidative and inflammatory stress markers. Both models need to be improved in order to detect ischemia and reperfusion injury. Thus, it still remains to be tested whether melatonin can reduce ischemia and reperfusion induced oxidative stress in a large animal model.

Melatonin was soluble in aqueous solution without losing the ability to activate both the MT_1 and MT_2 receptors, thus making melatonin applicable in a coronary artery administration route. This finding was used in the porcine closed-chest model.

We are still awaiting the results from the IMPACT-trial on intracoronary and systemic administration of melatonin to patients suffering from acute myocardial infarction.

Comparison with previous findings

Many experimental models have reported very convincing results with regard to the ability of melatonin to attenuate ischemia-reperfusion injuries [24-26,156,157]. However, there is still a considerable lack of evidence regarding randomized trials in humans involving melatonin.

In a current trial from a Spanish group (The MARIA-trial, Clinical trial identifier: NCT00640094), the investigators are randomizing patients with AMI who are undergoing pPCI to a total intravenous melatonin dose of 11.61 mg (approximately 166 microgram/kg) or placebo. Their primary outcome is infarct size determined by the cumulative release of alpha-hydroxybutyrate dehydrogenase (area under the curve: 0 to 72 h) [158]. In the IMPACT-trial we use a higher dose of total melatonin (50 mg), and 1 mg of this dose is administered intracoronarily in order to allow the melatonin to function at the site of the injury. Our primary outcome is also infarct size, but measured with CMR. The MARIA-trial, just as ours, is still recruiting.

A recent study investigated how peak and cumulative levels of circulating markers of inflammatory response, and reactive oxygen species, were related to myocardial infarct size (by CMR) in patients with acute coronary occlusion treated with PCI. The authors reported of no significant correlation to any parameter, thereby challenging the effect of inflammatory treatment in reperfusion [159]. Despite the results of this study, there is still ample evidence which points in the direction of the ischemia-reperfusion injury being caused, at least in part, by oxidative stress and inflammatory responses [27,160].

Besides its anti-inflammatory and antioxidative effects, melatonin might still be beneficial due to its effect on the mPTP. In a much cited pilot-study from 2008, the authors reported that administration of cyclosporine at the time of reperfusion was associated with a smaller myocardial infarct measured by CMR [161]. Cyclosporine is known to inhibit opening of the mPTP during reperfusion [162], and it was probably this property which mediated the beneficial effect found in the study. As melatonin is also known to inhibit the opening of the mPTP [24], and since melatonin [116] as opposed to the immunesuppressive agent, cyclosporine [163], has no known serious adverse side effects, it could thus offer an interesting and safe alternative in the treatment of myocardial ischemia-reperfusion injuries.

It is to be noted, that myocardial ischemia-reperfusion injuries are only a part of the whole range of ischemia-reperfusion injuries. The remote organ injuries or multiple organ dysfunction syndrome caused by ischemia and reperfusion of e.g. the extremity during tourniquet-related surgery, fully display the detrimental consequences of ischemia-reperfusion. Optimizing the existing treatments and exploring new suitable interventions for the whole range of ischemia-reperfusion injuries, is therefore of great interest.

One aspect within this range could be the prevention of ischemia-reperfusion injuries during elective surgery with the use of melatonin. When performing e.g. organ transplantations, CABG, tourniquet-related surgery or vascular surgery, the ischemic event is planned and it is thus possible to administer the melatonin in advance. The effect of melatonin might relate to the time of administration, as it has been shown in an experimental study that melatonin administered 30 minutes prior to the ischemic event protected against myocardial injury [164]. The administration of melatonin prior to the ischemic event might therefore increase the likelihood of attenuating the reperfusion injury and it is possible to implement this administration in elective surgery.

The antioxidant effect of melatonin needed to mitigate conditions with excessive oxidative stress probably only occurs at pharmacological concentrations [15,165,166] and the effect of melatonin might be dose-dependent. In the above mentioned study [164] they reported decreasing markers of reperfusion injury with increasing doses of melatonin (5 to 40 mg/kg bodyweight). In patients undergoing liver resections it has been shown that melatonin is well tolerated and safe [92] and in this

safety-study they used a single dose of 50 mg melatonin pr. kg bodyweight, which is much higher than the 200 mg total melatonin dose we used in the porcine closed-chest study (paper 4).

In an RCT a total dose of 50 mg melatonin was administered to patients undergoing major vascular surgery without a significant reduction in oxidative stress measured by MDA, AA and DHA [166]. This lack of effect might be due to melatonin being administered at the time of the first incision and not prior to the start of surgery and the dose of melatonin was possibly too low. The authors did, however, find that significantly fewer patients randomized to melatonin had elevation of troponin-I postoperatively, as well as significantly fewer ST-segment deviations (unpublished data). These results were the rationale for our choice of 50 mg melatonin in the IMPACT-trial.

FUTURE PERSPECTIVES

In studying some of the ever-expanding literature on the topic of ischemia and reperfusion, there seems to be little doubt that the pathogenesis of ischemia-reperfusion injuries is multifactorial and caused by a combination of several biologic pathways. Thus, a possible treatment would consequently have to target several pathways.

One of the up-coming and very promising treatments of special interest is conditioning of the tissue or organ – either before the ischemic event (pre-conditioning), during ischemia (as remote ischemic conditioning) or at the time of reperfusion (post-conditioning) [167]. The possible effect of conditioning in myocardial ischemia-reperfusion injury can be seen in Figure 2. Although the concept of conditioning is very promising and interesting, there are still divergent opinions as to its effects. Some studies have shown very promising results, also on long-term cardiac function [168], whereas some studies have not found any effect [169] and even found that it might be potentially harmful [170]. Thus, it remains to be fully explored if and how ischemic conditioning treatment can be applied as to maximize the effect and minimize the ischemia-reperfusion injuries.

Melatonin has proven to reduce ROS, up-regulate antioxidant defense mechanisms and inhibit the mPTP. Conditioning has proven to reduce oxidative stress and up-regulate pro-survival kinases [171]. Thus, a combination of melatonin and conditioning might prove beneficial in reducing ischemia-reperfusion injuries. The optimal dosage and 'time and route' of administration for melatonin will have to be examined further, depending on the clinical setting in which it is to be applied.

Suggestions for future research

Overall, there seems to be a lack of studies examining the effect of a reduction in biochemical oxidative stress markers on the postoperative clinical outcomes in patients undergoing tourniquet-related surgery. In order to elaborate on the correlation between a reduction in oxidative stress and a reflection in clinical outcomes it would be interesting to conduct a systematic review that covers a wider range of postoperative clinical outcomes without the limitation that there should be included measurement of oxidative stress parameters.

The development of an ischemia-reperfusion model without the influencing factors of surgical stress and anesthesia is still warranted. Important data were collected in the healthy volunteer study (paper 2), which could easily be incorporated in a new design. First of all, the ischemic period should be expanded to 30 minutes, as an attempt to increase the tissue damage upon reperfusion. Inflammation markers should be assessed in a period of up to six hours. In addition, it would probably improve the design to use the tourniquet on the upper arm, giving the possibility to collect blood from a local vein and thus increasing the possibility of detecting a change [8,172].

Orthopedic surgery with e.g. a total knee arthroplasty is often performed in otherwise healthy persons, and a potential fatal outcome due to a reperfusion injury would be worth intervening against, especially if the interventions are almost without adverse effects. As before mentioned melatonin and pre- or postconditioning target different biological pathways in the ischemia-reperfusion injury. We know from the systematic review (paper 1) that both ischemic preconditioning and propofol, due to its free radical scavenging effects, work at reducing the oxidative stress level. If we were to conduct a new randomized clinical trial, it would thus be interesting to examine if the effect of melatonin in combination with pre- or postconditioning [173] in patients undergoing tourniquet surgery would increase the effect of the conditioning, due to the simultaneous targeting of multiple mechanisms.

In the porcine closed-chest study (paper 4), we only measured circulating MDA and cytokines in order to examine the cardio-protective effect of melatonin. To improve the design it could also be interesting to include analysis of microRNA (miRNA) in plasma as well as in myocardial tissue. Elevated levels of several circulating miRNAs (miR-1, miR-133a, miR- 208b and miR-499-5p) have been reported in a closed-chest porcine model of myocardial infarction as well as in patients with STEMI [174]. In this study, release of miRNAs was furthermore found to correlate with the ejection fraction, cardiomyocyte necrosis markers and the glomerular filtration rate (GFR), indicating a possible role for miRNAs, both in the diagnosis of STEMI and the prediction of long-term outcomes [174].

With regard to the IMPACT-trial (paper 5), it might also have been interesting to examine the possible role of miRNAs in the patients treated with melatonin as this could add to the knowledge of a possible long-term effect of melatonin on clinical outcomes. If the IMPACT-trial shows promising results with regard to increased myocardial salvage index, a larger scaled randomized trial will have to be performed.

CONCLUSION

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

- The biological mechanisms contributing to the ischemia-reperfusion injuries are many and divergent and the treatment of the injuries may implicate interventions that are directed at more than one mechanism and/or several interventions at the same time.

- The tourniquet-related oxidative damage following orthopedic surgery can be reduced with the intervention of especially propofol and ischemic preconditioning – however, it is not properly explored whether this reduction in oxidative stress also has clinical relevance.

- An ischemia-reperfusion model without the influencing effects of anesthesia and surgery should be optimized with regard to design and choice of outcomes compared to our model (paper 2).

- The porcine closed-chest model works well with respect to simulating an acute myocardial infarction,

however the use of MDA and cytokines as biomarkers of oxidative and inflammatory injury is not optimal in this model.

- Melatonin can be dissolved in non-ethanol based buffers without losing the ability to activate the MT_1 and MT_2 receptors. In fact, when dissolved in ethanol, melatonin acts with lower potency than when dissolved in non-ethanol based buffers. Melatonin is thus applicable in clinical settings where ethanol is not an option.

- The antioxidant, anti-inflammatory and mPTP inhibiting properties of melatonin along with its low toxicity make melatonin an extremely interesting compound with regard to an effective intervention of ischemia-reperfusion injury treatment. Thus, we await the results of the IMPACT-trial with great interest.

SUMMARY

Ischemia-reperfusion injuries occur when the blood supply to an organ or tissue is temporarily cut-off and then restored. Even though the restoration of blood flow is absolutely essential in preventing tissue death, the reperfusion of oxygenated blood to the oxygen-deprived areas may in itself augment the tissue damage in excess of that produced by the ischemia alone.

The process of ischemia-reperfusion is multifactorial and there are several mechanisms involved in the pathogenesis. Ample evidence shows that the injury is in part caused by an excessive generation of reactive oxygen species or free radicals. The free radicals consequently initiate an inflammatory response, which in some cases may affect distant organs, thus causing remote organ injuries.

Ischemia-reperfusion injuries are a common complication in many diseases (acute myocardial infarctions, stroke) or surgical settings (transplantations, tourniquet-related surgery) and they have potential detrimental and disabling consequences. The tolerance of ischemia-reperfusion has proven to be time-of-day-dependent and the size of myocardial infarctions has proven to be significantly higher when occurring in the dark-to-light period. This period is characterized by and coincides with a rapid decrease in the plasma levels of the hormone melatonin.

Melatonin is the body's most potent antioxidant and is capable of both direct free radical scavenging and indirect optimization of other anti-oxidant enzymes. It also possesses anti-inflammatory properties and is known to inhibit the mitochondrial permeability transition pore during reperfusion. This inhibiting property has been shown to be of great importance in reducing ischemia-reperfusion injuries. Furthermore, melatonin is a relatively non-toxic molecule, which has proven to be safe for use in clinical trials. Thus, there is compelling evidence of melatonin's effect in reducing ischemia-reperfusion injuries in many experimental studies, but the number of human clinical trials is very limited.

In this PhD thesis we set out to explore the oxidative and inflammatory biochemical markers of ischemia and reperfusion injuries and the possible effect of melatonin on these markers.

We have reviewed the literature on the tourniquet-related oxidative damage and found that ischemic preconditioning and the use of propofol could significantly reduce the release of such markers. However, the relevance of this reduction in terms of clinical outcomes is still to be investigated (paper 1).

We undertook the characterization of a human ischemia-reperfusion model without the influencing factors of surgery and anesthesia, and subsequently found ways to improve this model (paper 2).

In order to apply an intracoronary melatonin administration, we investigated whether melatonin could be dissolved in non-ethanol based buffers and still activate the melatonin receptors (paper 3). We found this to be possible, and in a porcine closed-chest model of acute myocardial infarction (AMI) we randomized the pigs to intracoronary and systemic melatonin or placebo in order to test whether melatonin could attenuate the oxidative and inflammatory biomarkers following reperfusion (paper 4). The outcomes were not optimal for this model, and the effect of melatonin still remains to be explored in a large animal model.

We are currently still awaiting the results of the IMPACT-trial -a randomized, placebo-controlled, clinical trial exploring the effect of intracoronary and systemic melatonin given to patients suffering from AMI and undergoing primary percutaneous coronary intervention (pPCI) (paper 5). Though pPCI is undisputedly life-saving, it holds a built-in consequence of aggravating the ischemic injury, paradoxically due to the reperfusion. The optimization of existing treatments and the exploring of new suitable interventions, such as melatonin, for minimizing the ischemia-reperfusion injuries is therefore of great interest.

ABBREVIATIONS

AA: ascorbic acid (vitamin C)
AMI: acute myocardial infarction
ATP: adenosine 5'-triphosphate ROS: reactive oxygen species
Ca^{2+}: calcium
CABG: coronary artery bypass graft surgery
CMR: cardiac magnetic resonance imaging
DHA: dehydroascorbic acid
HPLC: high performance liquid chromatography
hs-TnT: high sensitivity troponin T
i.c.: intracoronary
i.v.: intravenous
IL: interleukin
IMPACT-trial: **I**ntracoronary **M**elatonin for **P**atients with **A**cute myo**C**ardial infarc**T**ion –trial
IQR: interquartile range
MDA: malondialdehyde
MODS: multiple organ dysfunction syndrome
MPO: myeloperoxidase
mPTP: mitochondrial permeability transition pore
MT_1 and MT_2: melatonin receptor 1 and melatonin receptor 2
O_2^-: superoxide anion radical
(p)PCI: (primary) percutaneous coronary intervention
PRISMA: Preferred Reporting Items for Systematic reviews and Meta-Analyses
RCT: randomized controlled trial
SPIRIT: Standard Protocol Items: Recommendations for Interventional Trials
SRE: serum response element
STEMI: ST-elevation myocardial infarction
TNF: tumor necrosis factor
VAS: visual analog scale

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