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Melatonin therapy in preterm infants

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TABLE OF CONTENTS

SUMMARY

Melatonin is an endogenous indoleamine with well known antioxidant properties demonstrated *in vitro*, in animal and in human studies. Therefore, melatonin is an attractive agent in the treatment of "Oxidative stress related diseases" of the newborn. In newborn humans, melatonin has been used at doses ranging from 0.1 to 100 mg/Kg, however, due to the lack of pharmacokinetic data in neonates, it is difficult to establish a therapeutic effective dose. Recent investigations on the pharmacokinetic on newborns, reported that after administration of melatonin, in a single bolus of 0.5 mg/Kg, by intragastric administration, resulted in higher serum melatonin level than adults. Such findings suggest that is possible to obtain and mantain therapeutic concentrations with this dose. However, no data were available on the therapeutic efficacy of these specific doses. The aim of the present study is to investigate the effects of oral administration of melatonin on oxidative stress biomarkers in premature neonates. Therefore 0.5 mg/Kg/die of melatonin has been administrated orally to 35 preterms infants with gestational age <34 weeks, for the first week of life in comparison to placebo. Serum levels of oxidative stress (OS) biomarkers (F2 Isoprostanes, Advanced Oxidative Protein Products, Non-Protein Bound Iron) and serum melatonin were assessed at baseline T0 (0-2h of life), T1 (24h), T2 (72h). We found no significant reduction in serum levels of OS biomarkers except for F2 Isoprostanes at T1 which remains of unclear relevance. Analyzing serum melatonin levels, as expected, we found a significant increase from baseline T0 to T1 and T2 in the group receiving melatonin. However, endogenous melatonin levels increased also in the placebo group, and this result may be considered a response to counteract the elevated oxidative stress associated with the preterm birth. We also found that, despite the fact that melatonin was

administered based on the weight of the newborn, serum melatonin levels were dependent on the total amount of melatonin administered rather than related to the ratio melatonin/weight. Clinical data concerning mechanical ventilation, occurrance of sepsis, Broncho Pulmonary Dysplasia, Necrotizing Enterocolitis, Retinopathy of Premature, Periventricular Leukomalacia and Intraventricular Hemorrage, were also recorded and, although the sample size was too small to make a proper statistical analysis, no difference were found between the two groups. The main limitation of this study is the small number of patients enrolled. The data obtained indicate that studies with more preterm infants are needed to verify the efficacy of the antioxidant effect of melatonin and a higher, fixed dose, instead of a weight based dosage, is required to have a clearer therapeutic efficacy.

INTRODUCTION

Melatonin is an endogenous indoleamine with pleiotropic functions on circadian rhythms, immune system, reproductive physiology, free radicals scavenge. In the last few years, melatonin as antioxidant has been integrated as an adjuvant in the treatment of oxidative stress related diseases, showing a decrease in oxidative stress biomarkers serum levels after supplementation.

Such findings suggested that melatonin supplementation can be considered in the management of the critical preterm newborns. Preterm babies, in fact, are particularly susceptible to oxidative stress: their immature lungs have to face a hyperoxic environment compared to the low intrauterine O2 levels; they often require mechanical ventilation support which represents itself a hyperoxic insult which the preterm newborns is unable to counteract. Moreover, preterm infants are deprived of melatonin protection which is provided by the mother through the placenta, until they start producing circadian levels of melatonin at 52-60 weeks post menstrual age.

In the last few years, melatonin supplementation at high doses in human newborns improved the clinical and biochemical outcomes of OS related diseases of the newborns such as Hypoxic Ischaemic Encephalopathy, Sepsis, Broncho Pulmonary Dysplasia.

Although pharmacokinetic profile of melatonin administration both via intragastric and intravenous route has been recently reported, the therapeutic threshold leading to a clinical and biochemical improvement remains unknown.

Therefore, in the present study we aim to investigate with a starting dose of 0.5 mg/Kg via oral administration, which resulted in higher

levels than adults in previous pharmacokinetic data, results in a therapeutic effect on the preterm newborns.

CHAPTER 1

MELATONIN

1.1 Generalities

Synthesis and secretion

First identified in 1950s, melatonin is the main hormone produced by the pineal gland. Melatonin secretion, starting between the sixth and eighth week of life with very low plasma levels, responds to a circadian rhythm which is established once the baby acquires a proper sleep/wake cycle, enhanced by darkness and inhibited by light. The suprachiasmatic nucleus (SCN) sends an electrical signal to the pineal gland via complex autonomic neural circuitry, which culminates in the release of norepinephrine from post-ganglionic neurons onto pinealocytes, activating melatonin production via adrenergic receptors. Melatonin is also produced in the retina, thymus, bone marrow, respiratory epithelium, skin, lens, intestine, reproductive organs and lymphocytes, from which it may influence other physiological functions through paracrine signalling. Due to its lipophilic nature, melatonin easily crosses the blood-brain barrier and cell membranes, and, as a consequence of that, it is not stored in the pineal gland and its plasma levels faithfully reflects the pineal activity. It is synthesized starting from circulating tryptophan, which is hydroxylated by TPOH to 5-hydroxy-Trp (serotonin), then acetylated on its free amine by serotonin-N-acetyl-transferase (NAT), which is the limiting enzyme for the synthesis of melatonin, and finally O-methylated on the hydroxyl group by hydroxyindole-Omethyltransferase (HIOMT).

The physiological peak occurs at night, with maximum plasma levels (varying from 80 to 120 pg/ml.) around 03:00/04:00a.m., whereas diurnal levels are nearly undetectable.

Metabolism

Melatonin is mainly metabolized in the liver (90% of circulating hormone has a hepatic metabolism) by CYP1A2. Being this enzyme responsible for the metabolism of most endogenous and exogenous compound, interaction of melatonin with substrate compounds results in alteration of melatonin bioavailability. In the liver melatonin is hydroxylated to 6 hydroxy melatonin which is excreted with urine sulphate, being 6 sulfatoxymelatonin levels in urine very close to plasma melatonin levels. Melatonin is also metabolized in CSF to N-acetyl-N-formyl-5-methoxykynuramine (AFMK) by cleavage of the pyrole ring. AFMK is then converted into N-acetyl-5-methoxykynuramine (AMK). Both AFMK and AMK are proven to be radical scavengers.

Mechanisms of action and Function

Melatonin receptors belong to two distinct classes of proteins: the G-protein coupled receptor superfamily (MT1 and MT2) and the quinone reductase enzyme family (MT3).

Melatonin main function is to regulate circadian rhythms such as core temperature and sleep wake cycling, but besides the well-known effects of melatonin on the regulation of sleep-wake rhythms, melatonin is involved in blood pressure and autonomic cardiovascular regulation, immune system regulation, retinal functions, detoxification of free radicals and antioxidant actions through its action on MT3 receptors protecting the brain from oxidative stress.

The antioxidant actions of melatonin also protect the gastrointestinal tract from ulcerations by reducing secretion of hydrochloric acid and the oxidative effects of bile acids on the intestinal epithelium, and by increasing duodenal mucosal secretion of bicarbonate through its action on MT2 receptors. Concerning the role of melatonin in immune regulation, melatonin stimulates the production of cytokines and more specifically interleukins (IL-2, IL-6, IL-12). Furthermore, melatonin reduces nitric oxide formation which facilitates the decrease of the inflammatory response. [1]

Melatonin in newborns

Melatonin protective role in the newborns takes place from the very early beginning of gestation. In fact, melatonin receptors have been identified in the placenta [2] playing a protective role against oxidative and nitrosative stress by increasing antioxidative enzymes and decreasing lipid peroxidation[3]. Moreover, melatonin is also believed to support umbilical arterial blood flow.[4]

The foetus benefits from maternal circadian secretion of melatonin which is able to cross the placenta barrier [5]. The foetal brain synthesises small amounts of melatonin from mid-gestation, whereas melatonin synthesis in the pineal gland begins in the postnatal period, about 52/60 weeks post menstrual age.

Therefore, preterm and early preterm infants deprivation of the indolamine is consistently longer than it is in term and near-term newborns. Moreover, melatonin production in preterm infants at 9 months is lower when compared with term infants and it correlates with slower mental development. [6,7]

As a consequence of that, this susceptible population may greatly benefit from melatonin supplementation.[8] Furthermore, the pharmacokinetic profile of melatonin in newborns is different from

adults pharmacokinetic, being the half-life of melatonin in preterm and term infants equal to approximately 15h rather than 20 to 50 minutes of the adult population, probably due to the immature liver metabolism and the poor renal excretion of the newborns [9] its slow clearance though (0.045 l/h) makes replacement of a circadian rhythm difficult. [10]

CHAPTER 2

OXIDATIVE STRESS

Oxidative stress was first defined by Sies as *the imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage.*[11]

Free radicals, mainly represented by Reactive oxygen species and Reactive Nitrogen Species (ROS), defined by "any chemical that contains one or more unpaired electrons and can stand alone" [12] are normally produced during oxidative phosphorylation in the mitochondria and they are properly counter balanced by endogenous antioxidant agents both enzymatic such as Superoxide dismutase, Glutathione Peroxidase, Catalase, and non-enzymatic, mainly represented by vitamin E; when antioxidant agents are insufficient, FRs rapidly react with DNA, lipid and proteins producing a cellular damage up to the death of the cell itself. The peroxidation of lipid chains represents a danger for cell membranes function by modifying the membrane structure. ROS, mainly represented by superoxide anion (O2-), hydroperoxyl and hydroxyl (OH) radicals, are highly toxic for the mitochondria although it is the main place for their production; thus, ROS production and mitochondrial dysfunction enhance each other. [13]

Oxidative stress in newborns

Why are newborns more vulnerable to oxidative stress, and, as direct consequence of that, to oxidative stress damage?

In the transition from intrauterine to extrauterine life, the foetus is transferred from an intrauterine 20–25 mmHg oxygen tension (PO2) to 100 mmHg PO2. [15]

On the other hand, due to the low intrauterine PO2 levels, endogenous antioxidant systems in newborns are not properly calibrated to face this sudden change in O2 levels.

As a result of that, a perfectly normal delivery leads to a significant increase of OS. Therefore, pregnancy and perinatal complication such as preeclampsia, PROM, preterm birth, produce an additional charge of ROS, requiring a more consistent antioxidant action which the newborn body it's unable to provide.

Moreover, both OS and nitrosative stress are highly dangerous for the newborns brain, being the immature cerebral white matter, mainly composed by promyelinating oligodendrocytes (O4 and O1), highly vulnerable to FRs which, indeed, induce their loss, leading to a decreased population of mature oligodendrocytes and to a hypomyelination.[16] Because of this high susceptibility to OS, the term Free Radicals Disease (FRD) was coined for a series of typical prematurity-related diseases in which oxidative damage plays a determining role.

High levels of OS biomarkers have been, indeed, demonstrated in the biological fluids of these patients. Thus, elevated levels of Total Hydroperoxides (TH), Advanced Oxidative Protein Products (AOPP) and Non-protein Bound Iron (NPBI) in cord blood have been found to correlate with an increased risk for FRD.

High TH plasma levels were found in preterm hypoxic newborns providing indirect evidence of an increase in FRs generation during hypoxic conditions. The correlation between TH and hypoxanthine in plasma of preterm newborns strongly suggests that the deeper the hypoxia, the greater is the reactive oxygen metabolite production. The degree of hypoxia correlated with AOPP levels, indicating that plasma proteins are impacted in FRs damage in preterm hypoxic newborns. [17]

Over the last decades, emerging data have suggested that OS is involved in the lung development and in the development of bronchopulmonary dysplasia (BPD) in the preterm newborns. Human studies have shown a quantitative increase in oxidative damage to proteins and lipids in lung tissue and decrease in levels of antioxidants in biological fluids of ventilated preterm infants. [18]

Several studies suggested a role of OS in the pathogenesis of Necrotizing Enterocolitis (NEC).[19] Aydemir et al. found that preterm infants with NEC had significantly higher total oxidant status (TOS), and oxidative stress index (OSI) levels compared with controls without NEC, with higher levels of TOS and OSI being associated with the severity of NEC.[20] Furthermore, a strong association between the concentration of OS markers in cord blood and the occurrence of NEC in preterm infants was found; in particular, AOPP, TH and NPBI cord blood levels were significantly higher in babies with NEC than in babies without. [21]

NPBI has been found increased in cerebrospinal fluid of preterm infants with post-haemorrhagic ventricular dilatation- PHVD, suggesting an association between IVH and subsequent white matter damage.[22]

The predictive role of a default panel of OS biomarkers for the early identification of infants at high risk of hypoxic-ischemic encephalopathy (HIE) and their validation through the correlation with MRI findings was recently reported. The presence of an association between biomarkers of oxidative stress measured in the first hours of life and brain damage (successfully evaluated through neuroimaging), emphasizes the possibility of early identification of newborns at greater risk of brain damage. Knowing also that after a hypoxic-ischemic insult cellular damage on energy substrates continues to evolve over the first 12–48 h, it suggests that the introduction of new neuroprotective strategies and antioxidants in such an early stage of life could change the long-term outcome of these infants. [23]

Oxidative stress related diseases

Oxidative stress damage plays a crucial role in the onset and the maintenance of many chronic diseases such as atherosclerosis, ischaemia, diabetes, cancer, neurodegenerative diseases. OS plays a key role in the endothelial disfunction which is the common denominator among hypertension, diabetes, atherosclerosis, dyslipidaemia, being NADPH oxidase (NOX) the main source of ROS production in the vascular wall. The figure below shows that for each disease a specific antioxidant agent is particularly overloaded, leading to an increased ROS production which, in turn, contributes to maintain the tissue damage.

Postulated mechanisms by which cardiovascular risk factors affect ROS generation and endothelial function along the interplay of oxidant and antioxidant systems, in generation of ROS. [24]

As above mentioned, ROS exceeding production is able to produce DNA damage with consequent genetic instability eventually leading to **tumorigenesis**. Moreover, ROS production is also involved in the induction of angiogenesis, epithelial-mesenchymal transition (EMT) and "cross-talking" with surrounding cells that support tumour development as well.[25]

Concerning **neurodegeneration**, due to its high content in PUFA and consuming about 20% of total body oxygen, the brain is highly sensitive to OS damage. Furthermore, redox/active metals such as copper and iron, highly represented in the brain, actively participate in ROS generation. As the brain cell membranes are rich in PUFA, they are more prone to lipid peroxidation. [26]

Oxidative stress Biomarkers

"A biomarker is any substance, structure, or process that can be measured in the body or its products and influences or predict the incidence of outcome or disease." (WHO, 2001) To acquire clinical relevance, a biomarker should be (a) specific (b) have a prognostic value (c) correlate with disease activity (d) stable.

Oxidative stress biomarkers are usually ROS-induced modifications on proteins (AAOP), lipids (Isoprostanes, MDA), and others and they reflects pharmacologic response to antioxidant interventions. Lipid peroxidation provides a number of possibilities for assays. It is a radical process whereby polyunsaturated fatty acid (PUFA) contained

in the phospholipids of cellular membranes undergo a reaction with oxygen, yielding lipid hydroperoxides. The reaction occurs through a free radical chain mechanism initiated by the abstraction of a hydrogen atom from PUFA by a reactive free radical, followed by a complex sequence of propagative reactions. Hydroperoxides are the major initial molecular products of lipid peroxidation and can be measured in plasma by a variety of techniques. Total hydroperoxide (TH) represents a measure of overall OS, because it is indicative of intermediate oxidative products of lipids and peptides. Lipid and protein damage from FRs exposure leads to lipid hydroperoxide generation from lipids and to carbonyl formation and protein hydroperoxide generation from proteins.

Lipid and protein hydroperoxide, in the presence of traces of free iron, produces several secondary reactive radical species, which can be measured collectively as organic hydroperoxide. Because of the rapid degradation in vitro, an accurate measurement of hydroperoxides is very difficult. The fact that the origin of lipid peroxidation products cannot be directly demonstrated represents a significant problem with the lipoperoxidation tests. This limitation can be overcome by measuring a series of prostaglandin-like compounds, called Isoprostanes (IsoPs) and isofurans (IsoFs). Isoprostanes and isofurans are produced independently of the cyclooxygenase pathway, and their formation results respectively from oxidation of arachidonic acid (AA) and docosahexaenoic acid (DHA). The F2-isoprostanes are prostaglandin-like products, which originate from in vivo and in vitro peroxidation of arachidonic acid and phospholipids. They are not produced by cyclooxygenase but just by free radicals' reactions.

F2-isoprostanes are protein G-like molecules generated by the peroxidation of arachidonic acid and phospholipids. They are more stable compared with other peroxidation products, such as aldehydes

or peroxyl radicals; they are mainly measured in plasma and urine using methods such as the gas chromatography coupled to mass spectrometry (MS) technique, liquid chromatography coupled to MS (LC-MS) and immunological assays.[17]

Normal adult humans are found to have stable plasma levels of F2-Isoprostanes. When compared with adults, the plasma F2-IsoP levels of newborns are significantly higher and an inverse relationship between IsoPs levels and gestational age was reported [27] suggesting that lipid peroxidation is already active in the antenatal period and that it goes to fade during the last gestational weeks and throughout postnatal life. A recent study made it possible to determine the F2-IsoPs reference levels in newborns. [28]

Non-protein bound Iron (NPBI)

Iron, a highly reactive element, is one critical component for the generation of free radicals being a strong biologic oxidant and a reducing agent. In particular, iron catalyses the formation of the highly reactive °OH from hydrogen peroxide by the Fenton reaction:

$$Fe2+ + H2O2 > Fe3+ + °OH + OH-$$

In moderate quantities and bound to protein, it is an essential element for growth and for all aerobic metabolic processes, but it is toxic when unbound. Physiologically, iron is safely sequestered in transport proteins such as transferrin and lactoferrin and stored in proteins like ferritin and hemosiderin. Iron ions cannot exist in plasma, so the term non protein-bound iron (NPBI) was introduced to indicate a low molecular mass iron form, free from the high-affinity binding to transferrin. In this case, iron is available to react with reduced intermediates of oxygen and generate reactive oxygen species (ROS). These FRs are capable of releasing even more iron by mobilizing it from ferritin. Therefore, the toxicity of iron is inversely proportional

to the availability of ferritin necessary for sequestering and detoxifying ferrous ion, and directly proportional to the quantity of hydrogen peroxide available for producing hydroxyl radicals by the Fenton reaction. Asphyxia and acidosis supply redox-cycling iron, predisposing the increase of the free iron content of erythrocytes. In newborns, the release of NBPI in erythrocytes correlates with plasma NBPI: the released iron has a tendency to diffuse from erythrocytes into the surrounding medium, suggesting the appearance of plasma NBPI.[29] In hypoxic newborns, increased concentrations of plasma NBPI significantly correlate with the severity of the brain injury and alteration of neurodevelopmental outcome until the second year of age.[30] Plasmatic free iron seems to be a reliable index of brain damage, reaching 100% sensitivity and specificity at high concentration. Moreover, a supposed interrelation between NPBI and white matter injury in preterm hypoxic newborns has been advanced. Supportive of a relationship between iron and periventricular leukomalacia (PVL) is the observation that, many weeks after human intraventricular haemorrhage and post-haemorrhagic ventricular dilatation (disorders that sharply increases the risk of PVL), levels of non-protein-bound iron in the cerebrospinal fluid are markedly increased [22] this increase could not be explained by haemolysis alone.

Advanced Oxidation Protein Products

As plasma proteins are the first target of free radicals, the detection of advanced oxidation protein products (AOPP) in biologic fluids can be an optimal strategy to detect and to estimate the degree of oxidative stress mediated protein damage. Indeed, AOPP are terminal products of protein exposure to FRs without oxidant properties.

AOPP levels are elevated in hypoxic newborns, especially if preterm [31] [17].

Oxidative stress related diseases of the newborn: the role of melatonin

OS related diseases of the newborns

As first suggested by Saugstad,[32] oxygen radical disease of neonatology is nowadays a serious issue to deal with.

A brief overview on the main OS related diseases of newborns is provided below.

- Bronchopulmonary dysplasia

  Defined by the need for supplemental oxygen and/or ventilator support for 28 days (mild) or beyond 36 weeks PMA (moderate and severe),[33] BPD is a chronic lung disease of the premature which usually occurs in RDS affected preterm newborns.; pulmonary remodelling and vascular remodelling induced by hyperoxia are the basis of BPD onset.

  The high inspiratory concentrations of oxygen required to achieve adequate arterial oxygenation leads to a massive ROS production which is responsible of the lung damage, whom specific targets are the vascular endothelial cells and the alveolar epithelial cells. At the state of art, guaranteed volume ventilation and -in selected patients- the administration of exogenous surfactant are the main strategies for BPD. [34]

- Sepsis

  Clinical signs of sepsis in newborns are often non-specific such as tachy or bradycardia, respiratory distress (grunting, nasal flaring, cyanosis), mottled skin as a result of a poor perfusion, either

irritability or lethargy; the clinical suspect is usually confirmed by isolating the responsible agent of the infection from a normally sterile body site (bloodstream, urine, CSF). [35]

The massive inflammatory response which is activated to face the noxa on one hand, is on the other hand responsible for the overwhelming production of ROS compared to the elevation of antioxidant enzymes, suggesting that antioxidant therapy might be useful in the management of neonates with sepsis.[36]

- Hypoxic Ischaemic Encephalopathy

  HIE is the result of a hypoxic–ischemic insult -*asphyxia*- during the delivery, where two phases of injury take place: the initial damage caused by the interruption of brain perfusion, during which inflammatory cascade and apoptosis of neurons begins, is followed by the reperfusion damage, mainly operated by ROS production because of the shift to anaerobic metabolism.

  Pathophysiology of hypoxic ischemic encephalopathy.[37]

  Therapeutic hypothermia is currently the only recognized treatment for HIE. [38]

- Necrotizing Enterocolitis

  Necrotizing enterocolitis (NEC) is a gastrointestinal surgical emergency in premature low BW neonates[38] characterised by the necrosis of the intestinal mucosae. It responds to a multifactorial aetiology including immature gut barrier, enteral/parenteral feeding, inadequate perfusion of the gut. Though has not yet been demonstrated a direct cause-effect relationship between OS and NEC in humans, high levels of OS markers such as non-protein bound iron (NPBI), and markers of FRs damage: advanced oxidation protein products (AOPP) and total hydroperoxides

(TH), were measured in the cord blood of NEC affected babies. [40]

- Retinopathy of prematurity

  ROP is a proliferative disease of the retinal vasculature of the premature, remaining one of the major cause of blindness in newborns. Its pathogenesis mainly responds to hyperoxia exposure and light exposure when the retina vessels development is not complete. In fact, 21% oxygen environment, compared to the hypoxic intrauterine environment, determines the suppression of VEGF, which is essential for normal retinal vascular development; [41] if the abnormal neovascularization progresses through the retina into the vitreous, blood and fluid leakage will spread into the different parts of the eye. this leads to scar formation (cicatrix) and traction on the retina which, in turn, leads to complete retinal detachment and ultimately permanent blindness. [42]

2.4.2 the role of melatonin

In the light of the above mentioned antioxidative properties of melatonin and, on the other hand, the vulnerability of the newborns to oxidative stress, it is reasonable that a supplementation of melatonin could play a therapeutic role in OS related diseases of the newborns and in selected at-risk patients (preterm and early preterm patients).

In fact, melatonin supplementation has already been shown to prevent the increase of myeloperoxidase, malondialdehyde, nitrite/nitrate levels in hyperoxia damaged lung tissue after mechanical ventilation in rats,[43] to reduce pro inflammatory cytokines [interleukin (IL)-6, IL-8 and tumour necrosis factor (TNF)-alpha] in tracheobronchial aspirate in mechanically ventilated human newborns,[44] to increase plasma superoxide dismutase (SOD) and serum nitric oxide in

asphyxial newborns, [7][45] to reduce the levels of lipid peroxidation products, such as serum MDA and4-hydroxylalkanal (4-HDA) in septic newborns,[46] to increase retinal ganglion cell (RGC) survival in ischemic mouse retina by the inhibition of HIF-1α. [47]

CHAPTER 3

OPEN QUESTIONS ON THE USE OF MELATONIN

Safety profile of melatonin has been largely demonstrated in preclinical and clinical data. No adverse effects have been reported. Melatonin has been proved to improve overall survival in septic newborns when used at high doses, [1] and can reduce ventilator associated lung injury in preterm infants,[46] without any adverse event. Finally, while melatonin supplementation aids the establishment of appropriate circadian rhythms, it does not suppress endogenous secretion of the pineal hormone [48].

However, the translation from adults data to newborns leads to different outcomes because of the different pharmacokinetic of melatonin in infants; indeed, melatonin is extremely lipophilic and the body fat content of newborns is very low, resulting in a different volume of distribution and higher plasma concentration. Following this assumption, Merchant et al. reported the pharmacokinetic profile of melatonin iv after infusion of 0.1 mg/Kg melatonin for two hours in preterm infants and they confirmed that, compared to adults and older children, the half-life and clearance rate of melatonin are longer and its volume of distribution decreased. [10]

Gitto et al. reported the pharmacokinetic profile of pharmacological doses of melatonin in preterm neonates after intragastric

administration using three different doses of melatonin: 0.1, 0.5 and 5 mg/Kg. After administration of a single 0.5 mg/Kg intragastric bolus, blood melatonin resulted in the high nM range (higher compared to adults physiological peak), and reached the μM range after administration of both 1 mg/Kg and 5 mg/Kg. [49]

CHAPTER 4

THE STUDY

Materials and Methods

Study Population

The study was designed and conducted in accordance with the Declaration of Helsinki, and was approved by the Ethics Committee of the University Hospital of Messina (approval number E41/13). Prior to the study, written informed consent was obtained from the parents. Thirty-five premature newborns admitted to the Neonatal Intensive Care Units of the University Hospital of Messina and Catania, Italy, were enrolled in the study within 2 h after birth.

Inclusion and exclusion criteria of the enrolled newborns were as follows:

a. inclusion criteria: gestational age <34 weeks; normal liver function test (i.e., serum bilirubin, alkaline phosphatase, serum glutamic-oxaloacetic transaminase, serum glutamic pyruvic transaminase, etc.), normal kidney function test (i.e., serum creatinine levels, blood urea nitrogen);

b. exclusion criteria: obvious congenital malformation.

Newborns were fed with formula milk when mother's milk was not available. The following data have been recorded: gestational age, birth weight, type of delivery, Apgar score, pH, mechanical ventilation, non invasive ventilation, oxygen requirement (FiO2), PCR as marker of sepsis. Moreover, GMH, IVH and PVL were assessed by cerebral ultrasound scan. The occurrence of NEC, ROP and BPD were also recorded.

Demographic and clinical data are reported in Table 1.

	Melatonin Group		**Placebo Group**		**p-value**
	Median	**Interquartile range**	**Median**	**Interquartile range**	
Age (weeks)	31	28-34	32	29-34	0.112
Sex (M:F)	13:6		8:8		0.268
Weight (gr)	1550	1100-1850	1790	1150-2200	0.09
Apgar 1′	7	4-9	8	6-9	0.155
Apgar 5′	8	7-9	9	8-10	0.121

Dose and Medication

Using a computer-generated randomization schedule, newborns were randomly assigned to two different groups. Group 1 received a daily intragastric bolus of 0.5 mg·kg−1 melatonin for 7 days, whereas group 2 received placebo.

Melatonin (Pisolino® Gocce, Pediatrica, Italy) was administered by a nurse through a nasogastric tube. After administration, the tube was

flushed with 0.5 mL of sterile water to ensure the full delivery of melatonin.

To determine the OS biomarkers (F2IsoPs, AOPP, NPBI) and melatonin levels, blood samples (1.5 mL) were collected through an indwelling arterial or venous catheter immediately before (time 0), 24h and 72 h after melatonin/placebo administration. Samples were collected in plastic tubes without anticoagulant agents.

The serum was immediately separated by centrifugation, and stored at −20 ◦C until assayed.

Statistical Analysis

In order to describe the variables of interest, descriptive statistical measurements have been calculated (i.e. mean, sd, median, percentiles).

Moreover, absolute and relative frequencies have been reported to describe the behaviour of categorical variables. The non-parametric approach has been used for the statistical analysis, both for the low sample size, either because the Kolmogorov Smirnov test confirmed the existence of significant differences with respect to normal distribution, which is why the most appropriate approach for data analysis is the non-parametric one.

The Mann Whitney test has been used to evaluate the differences between continuous variables (i.e. weight, days of life, Apgar, Isoprostanes…) respectively in the melatonin group and in the placebo group.

The categorical variables (i.e. birth type, sex, mechanical ventilation, non invasive ventilation…) has been evaluated using the Chi- Square

test.

The correlation between the melatonin level and some variables of interest as well as birth weight has been estimated using the Spearman correlation coefficient. The Spearman p values have been calculated in order to evaluate the significance of each correlation coefficient.

The Wilcoxon test has been used to evaluate the differences between continuous variables before and after the treatment/placebo. A p value below 0.050 was considered statistically significant. The statistical software with which all statistical analyses were carried out is SPSS For Windows 22.

Results

Thirty-five preterm infants have been enrolled in the study: 19 were randomly assigned to the melatonin group and 16 to the placebo group. Median gestational age was 31 weeks (interquartile range 28-34w) for the melatonin group, versus 32 weeks (29-34) for the placebo group (p= 0.112). The two groups were comparable for sex distribution (p= 0.269), birth weight (p= 0.09), Apgar score at 1' and 5' (respectively p=0.155 and p=0.121) and type of delivery (p=0.82).

No differences were found as well in needing of MV (p=0.077), NIV(p=0.142) and oxygen requirement (p=0.523), occurrence of sepsis (p=0.100), abnormalities at the CUS (p=0.893) between the two groups. No case of NEC occurred in either of the two groups, whereas a single case of BPD has been reported in the melatonin group and two cases of ROP occurred, one in each group.

In the comparison of serum level of IsoPs, AOPP, NPBI and melatonin between the two groups, we found difference only in the IsoPs levels at T1 which were significantly lower in treated group were (p 0.032) than the placebo group (Table 2); as expected, serum

melatonin levels as well were significantly higher in the treated group both at T1 and T2 (respectively p=0.007 and p=0.025) (Fig 1) (Table 2).

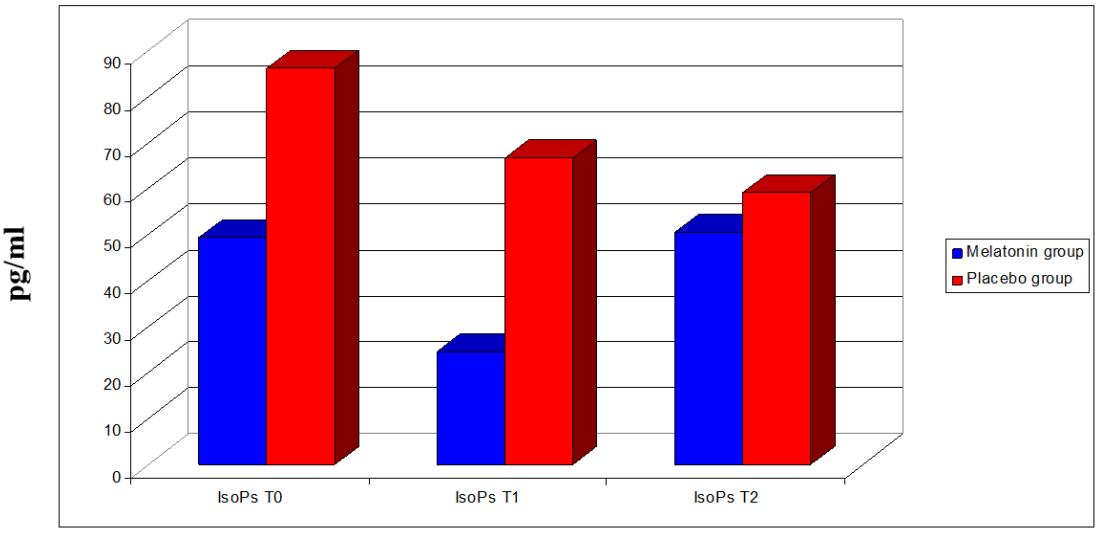
Melatonin Group			Placebo group		P value
Median		Interquartile Range	Median	Interquartile Range	
IsoPs 0 (pg/ml)	49,40	33,00-93,20	86,30	119,75	,380
IsoPs 1 (pg/ml)	24,70	11,03-74,10	66,70	127,00	**,032**
IsoPs 2 (pg/ml)	80,60	32,35-147,00	59,30	120,00	,382
AOPP 0 (µmol/dL)	32,12	24,13-43,48	35,07	45,34	,246
AOPP 1 (µmol/dL)	43,03	37,28-58,16	53,55	74,88	,167
AOPP 2 (µmol/dL)	56,67	37,95-65,25	59,66	81,64	,462
NPBI 0 (µmol/L)	3,64	0,51-5,97	1,37	4,30	,358
NPBI 1 (µmol/L)	0,90	0,65-4,38	,79	5,41	,357
NPBI 2 (µmol/dL)	1,14	0,76-8,31	1,72	7,62	,902
Melatonin 0 (pg/ml)	9653,33	13,52-32128,03	25,28	105,42	**,007**
Melatonin 1 (pg/ml)	110553,18	21545,53-529731,96	9922,50	100020,88	**,025**
Melatonin 2 (pg/ml)	14868,69	5085,27-40334,61	2244,60	89151,64	,310

Table 2. A p value <0.05 has been considered significant.

We evaluated the change in IsoPs, AOPP and serum Melatonin levels at time 0, 1, 2 with Wilcoxon test and we found that in the melatonin group, IsoPs at T1 levels were significantly decreased compared to IsoPs at T0 (p 0.023) (Table 3) (Fig. 7). Melatonin serum levels increased from T0 to T1 and from T1 to T2 both in placebo and treated group (Table 4). The difference between baseline and follow-up melatonin was significantly higher in the melatonin group: median melatonin levels increased from 96 pg/ml to 110553 pg/ml from T0 to T1 ($p<0.001$) followed by a decrease at T2 (14868 pg/ml; $p=0.036$). In the placebo group as well melatonin increased from a basal level of 25 pg/ml to 9922 pg/ml at T1($p=0.033$). Also, AOPP1 increased compared to AOPP0 (Table 3).

	IsoPs1-IsoPs0	IsoPs2-IsoPs1	AOPP1-AOPP0	AOPP2-AOPP1	NPBI1-NPBI0	NPBI2-NPBI1
p value	**0.023**	0.128	0.002	0.889	0.109	0.499

Table 3. A p value of 0.05 has been considered significant.

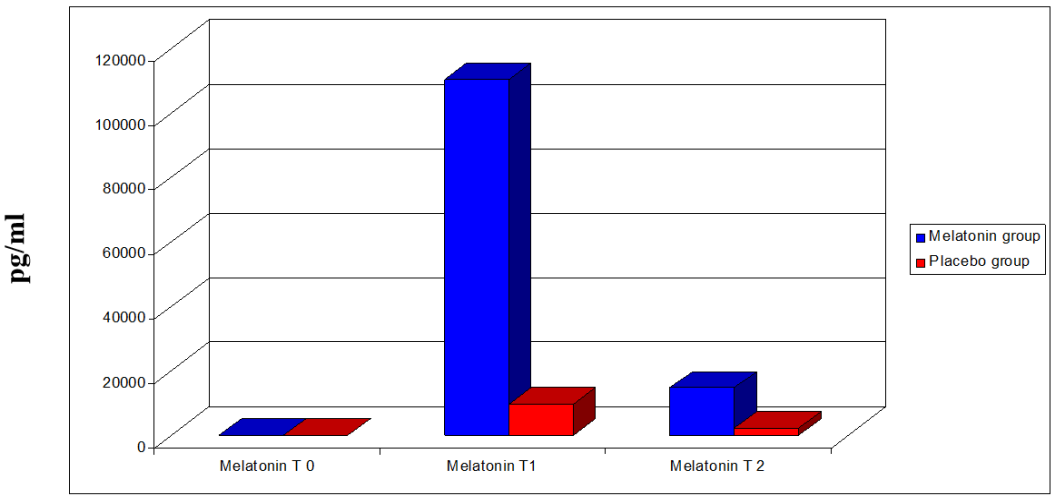


Serum Isoprostanes levels

(Fig 1)

	Melatonin1- melatonin0	Melatonin2-melatonin1
p value	**<0.001**	**0.036**

Table 4.



Serum melatonin levels

(Fig. 2)

Finally, through Rho Spearman test we investigate a possible relationship of interdependence between melatonin levels and the total dose of melatonin administered (based on weight) (p=0.475) and between melatonin levels and weight. We found a significant correlation between total amount of melatonin administered and serum melatonin levels at T1 and T2 (p=0.035 and p=0.045) whereas no correlation was found between weight and serum level of melatonin at none of the times examined. (p=0.475, p=0.455, p=0.167 respectively for T0, T1 and T2).

Discussion

Antioxidant properties of melatonin has been demonstrated both in in vitro and in animal studies. In newborns humans, melatonin, administered both via oral and iv route at high doses (ranging from 10

to 100 mg/Kg), resulted in reduction of serum oxidative stress biomarkers (malondialdehyde, nitrite/nitrate levels, superoxide dismutase). In small clinical studies, melatonin improved clinical outcome in babies with septic shock (20 mg orally within 12 h of diagnosis), reduced ventilator-associated lung injury and inflammatory markers in preterm babies (10 doses of 10 mg over 72 h) reduced malondialdehyde (MDA) and nitrate/nitrite in asphyxiated babies (80 mg orally over 16 h) and reduced cytokines and improved clinical outcome in surgical babies (100 mg IV over 72 h). However, these doses have been chosen on the base of results of in vitro and animal studies and the paucity of pharmacokinetic data in neonates led to a difficult establishment of a therapeutic effective dose . Based on adult pharmacokinetic data, Merchant et al. First performed a pharmacokinetic study in preterm infants, starting with a iv dose of 0.1 mg kg-1 h-1 for 6 h. This resulted in plasma melatonin concentrations higher than predicted probably due to several factors: melatonin is extremely lipophilic and the fat body content of the preterm newborns is very low; moreover, preterm infants present immature liver metabolism and poor renal excretion. Reliable pharmacokinetic data and melatonin concentrations closest to physiological adult concentrations were achieved with a 2 h infusion of 0.1 mg kg-1 h-1. Recently, has been reported, for the first time, the pharmacokinetic profile of oral pharmacological dose of melatonin in preterm neonates. After administration of a single 0.5 mg/Kg bolus, blood melatonin resulted in the high nM range, pointing out the possibility to obtain and maintain protective concentrations of melatonin in blood using oral administration of the indoleamine repeated every 24h. These data has been considered helpful to prescribe proper dosage and frequency of administration of melatonin in preterm infants. However, no data were available on the efficacy of

these specific dose. Therefore, in the present study we investigated the effect of treatment with 0.5 mg/Kg every 24h of oral melatonin on the serum levels of F2 Isoprostanes, AOPP and NPBI in comparison with placebo.

We found no significant reduction in serum levels of OS biomarkers except for IsoPs at time 1 which remains of unclear clinical relevance, even because it increases again at T2 almost reaching the placebo group levels. Analysing serum melatonin levels, as expected, we found a significant increase from baseline to T1 and T2 in the group receiving melatonin, but melatonin levels increased also in the placebo group at the same time, and this can be attributed to the attempt to counteract the oxidative stress associated with the preterm birth.

We investigated a possible relationship of interdependence between melatonin levels and the total dose of melatonin administered and between melatonin levels and weight and we found a significant correlation between total amount of melatonin administered and serum melatonin levels at T2 and T3 whereas no correlation was found between weight and serum level of melatonin at none of the times examined.

These results suggest that the serum melatonin level reached after administration is dependent on the total amount rather than on the dosage/Kg. Therefore, in our opinion, in the planning of future studies aiming to investigate the efficacy of melatonin as antioxidant, a higher standard fixed dose of melatonin should be preferred to a weight base dosage.

Although the sample size is too small to make a proper statistical analysis, no difference were found in the incidence of oxidative stress related diseases (BPD, NEC, ROP, PVL) between the two groups.

A limitation of this study is the small number of patients enrolled. Larger studies with extended follow-up are needed to examine long-term clinical effects.

CHAPTER 5

CONCLUSION

Despite melatonin is a promising molecule in providing reduction of OS biomarkers in newborns, further knowledges are needed to identify the proper dosage and frequency of administration. Although the pharmacokinetic studies suggested that a regimen of 0.5mg/Kg/die lead to supraphysiologic levels of melatonin in preterm in newborns, our study demonstrated that this dose is not effective in reducing oxidative stress biomarkers in premature infants. Therefore, larger studied are needed to investigate the efficacy of higher doses of oral melatonin supplementation.

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