The microRNAs as potential biomarkers of neonatal brain ischemia and their alterations following the melatonin treatment

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This thesis has been registered at the Pharmacology course and submitted to the University of Zagreb, Faculty of Pharmacy and Biochemistry. The research was conducted at the Department of Biomolecular Sciences, University of Urbino Carlo Bo, under the expert guidance of Silvia Carloni, PhD, and prof. Walter Balduini, PhD, and the thesis formation co-supervised by prof. Lidija Bach-Rojecky, PhD. Data reported in the thesis are property of the University of Urbino Carlo Bo.

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CONTENTS

1. INTRODUCTION

1.1 Fetal respiration and acid-base balance

Every fetus relies on maternal and placental circulation for the delivery of oxygen (Fig. 1) and the removal of the waste products of metabolism, including carbon dioxide. In the absence of oxygen, cells can continue to produce energy, but this process, called anaerobic metabolism, cannot be maintained over the long term. When oxygen is not present to accept hydrogen ions, they form organic acids such as lactic acid, whose buildup consequently changes the pH within the cells, and if this process continues for too long, the pH will decrease to levels that result in cellular death. When carbon dioxide is not removed from circulation, there is a drop in blood pH as well. A drop in blood values of pH resulting from an increase in the pCO_2 is classified as a respiratory acidemia. The blood pH level is usually kept in balance via the interaction of nonvolatile acid with bicarbonate (HCO_3^-), which is produced by the kidneys. If the production of lactic acid and other metabolic acids outstrips the body's ability to produce enough bicarbonate as a buffer, a decrease in blood pH may result, creating a metabolic acidemia, which, if unimpeded, will progress to a drop in tissue pH (metabolic acidosis) (Fahey and King, 2005). The consequences of impaired gas exchange between maternal and fetal blood and following metabolic acidosis, will be discussed in the next chapters.

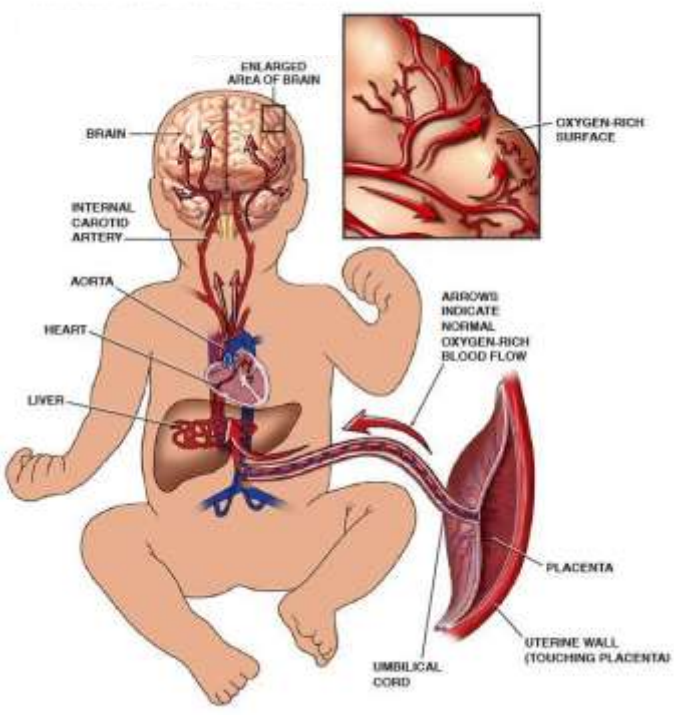

Figure 1. Normal placenta-to-brain blood flow (www.abclawcenters.com)

1.2 Perinatal hypoxia-ischemia: causes and consequences

1.2.1 Anoxia, hypoxia and asphyxia

Damage to the human brain that occurs around the time of birth is attributable to a variety of proximal causes. These include hemorrhage, infection, metabolic difficulties and hypoxia/anoxia. Of these, hypoxia and related conditions constitute the majority of perinatal injuries. Hypoxia can result from conditions related to the mother or the fetus. In the perinatal period the predominant cause is 'birth asphyxia' which is technically referred to as critically impaired intrapartum gas exchange. This may result from many factors, such as umbilical cord prolapse, abruption placentae or immaturity of the lungs. In the prenatal period the most commonly encountered problem of oxygen exchange is prolonged hypoxia due to placental inadequacies; this form of hypoxia often results in babies who are abnormally small for gestational age (Martin and Dombrowski, 2008). Many of these infants sustain significant brain injury and develop long-term sequelae, most commonly cerebral palsy, epilepsy and sensory deficits (Glass and Ferriero, 2007).

Several terms are used to denote a condition in which the mother or the fetus experience inadequate oxygen. Hypoxia is the term used to indicate a deficiency of oxygen in the tissue, while hypoxemia refers to decreased oxygen content in the blood (Fahey and King, 2005). A related term that is often used in relation to perinatal brain injury is anoxia, meaning the complete lack of oxygen. Asphyxia refers to the physiological results of hypoxia or anoxia (Martin and Dombrowski, 2008).

Asphyxia occurs when gas exchange is impaired enough to cause significant metabolic acidosis. As asphyxia progresses, the fetus loses the ability to protect vital organs. This, in turn, leads to marked hypotension and a subsequent further decrease in blood flow to the heart and the brain. If prolonged and unrelieved, asphyxia will lead progressively to cellular death, tissue damage, organ and organ system failure, and, ultimately, fetal death (Fahey and King, 2005). Furthermore, severe intrauterine asphyxia is the main cause of hypoxic–ischemic brain lesions in neonates. This is usually brought about by an acute reduction in the uterine or umbilical circulation (Fig. 2), which in turn can be caused by abruptio placentae, contracture of the uterus, vena cava occlusion syndrome, compression of the umbilical cord etc. (Berger and Garnier, 1999).

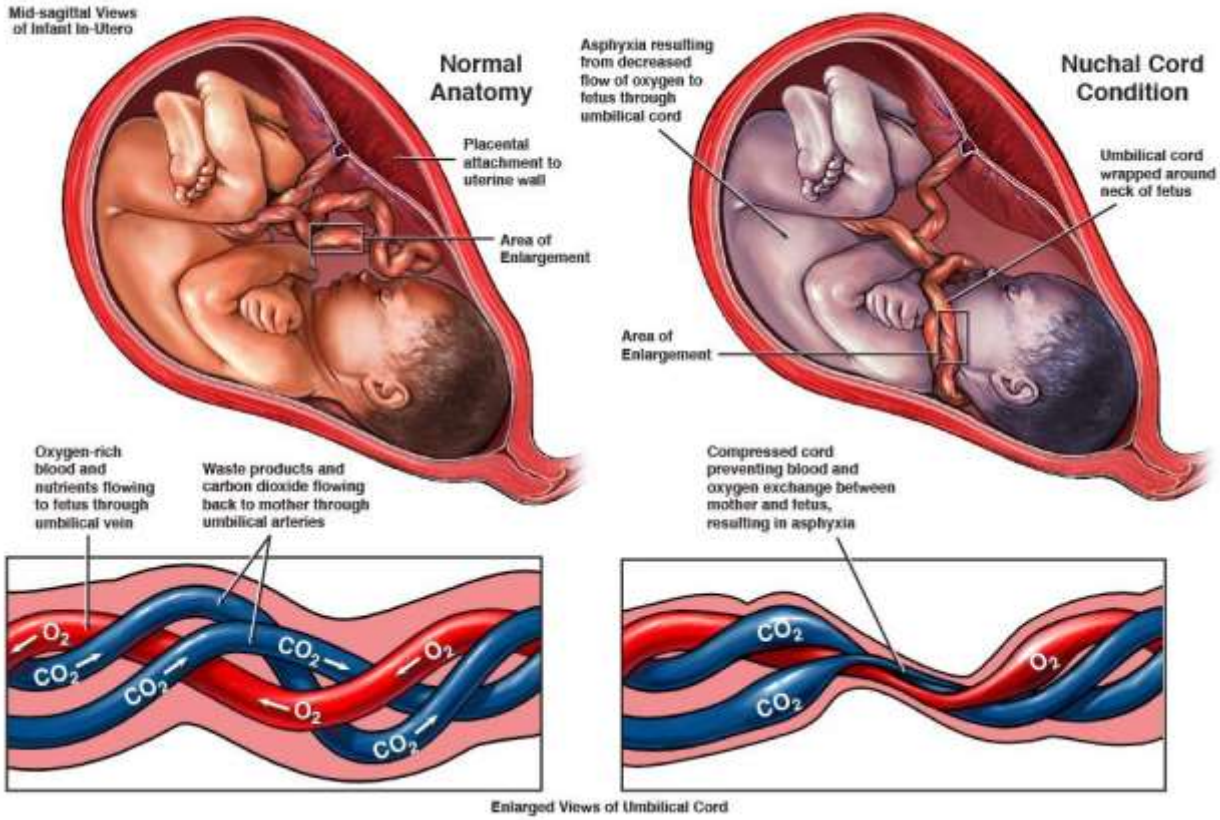

Figure 2. Fetal circulation in the physiological conditions (left) vs. impaired fetal circulation due to an acute reduction in the umbilical circulation, leading to asphyxia (right) [www.abclawcenters.com]

As shown on animal models, the damage occurs after ischemia of only 10 minutes. The pattern of distribution of the cell damage is strongly dependent on perfusion (Berger and Garnier, 1999). Neuronal elements show the most damage, but premyelinating oligodendrocytes (pre-OLs) of subcortical white matter are also involved (Volpe, 2018).

While in term infants brain injury is more likely to occur in the cortex, preterm infants born between 23rd and 32nd week of gestation are at the greatest risk of injury occuring in the crebral white matter. Depending on the severity of insult, the spectrum of white matter injury in the preterm population can differ markedly. On the cellular level, the pathogenetic cascade includes the presence of vulnerable pre-OLs in cerebral white matter, the initiating injurious mechanism of ischemia, with energy deprivation leading to excitotoxicity, microglial activation, calcium influx and free radical attack (Volpe, 2018).

1.2.2 The changes in cellular signaling triggered by ischemia and reperfusion

Fetus reacts to an oxygen deficit of this severity by activating the sympathetic–adrenergic system and redistributing the cardiac output in favour of the central organs (brain, heart and adrenals). If the asphyxic insult persists, the fetus is unable to maintain circulatory centralisation, and the cardiac output and extent of cerebral perfusion fall (Berger and Garnier, 1999). Owing to the acute reduction in oxygen supply, glycolysis and oxidative phosphorylation in the brain come to a standstill, which results in decreased production of ATP. The Na^+/K^+ pump at the cell membrane has no more energy to maintain the ionic gradients. The absence of a membrane potential causes anoxic depolarization and opening of voltage-gated Ca^{2+} channels (VGCC) on post-synaptic neurons (Arumugam et al., 2018), allowing for large amounts of Ca^{2+} ions to flow down an extreme extra-intracellular concentration gradient into the cell – the so-called „calcium overload". This leads to cell damage through the activation of degradative enzymes, including calpains, proteases, lipases and endonucleases (Arumugam et al., 2018; Berger and Garnier, 1999), as shown in Fig. 3. During ischemia, besides the influx of Ca^{2+} ions into the cells via VGCC, more Ca^{2+} enters the cells through glutamate-regulated ion channels on post-synaptic neurons due to the impaired function of ATP-dependent glutamate uptake transporters, resulting in excitotoxicity (Arumugam et al., 2018; Berger and Garnier, 1999). The acute lack of cellular energy arising during ischemia induces almost complete inhibition of cerebral protein biosynthesis. Once the ischemic period is over, protein biosynthesis returns to pre-ischemic levels in non-vulnerable regions of the brain, while in more vulnerable areas it remains inhibited. The inhibition of protein synthesis, therefore, appears to be an early indicator of subsequent neuronal cell death (Berger and Garnier, 1999).

A second wave of neuronal cell damage occurs during the reperfusion phase. Restoration of blood flow and reoxygenation is frequently associated with an exacerbation of tissue injury and a profound inflammatory response (called „reperfusion injury") [(Eltzschig and Eckle, 2011)]. Reperfusion injury contributes to the pathology of perinatal brain damage as much as ischemia. The post-ischemic release of oxygen radicals, synthesis of nitric oxide (NO), inflammatory reactions and an imbalance between the excitatory and inhibitory neurotransmitter systems (Berger and Garnier, 1999) are thought to be the main causes of the neuronal damage in this phase.

In addition to the above-mentioned neuronal cell death as a result of ischemia, reperfusion leads to the activation of cell death programs as well.

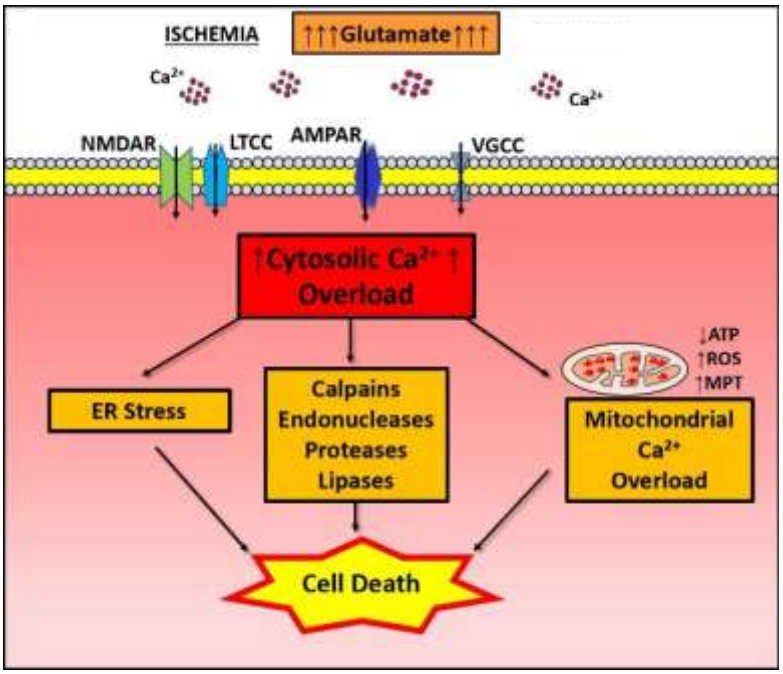

Figure 3. Pathogenic mechanisms of excitotoxicity caused by ischemia. Besides degradative enzymes, cytosolic calcium overload activates endoplasmic reticulum stress and mitochondrial permeability transition. Legend: AMPAR, α-amino-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; ER, endoplasmic reticulum; LTCC, L-type calcium channel; MPT, mitochondrial permeability transition; NMDAR, N-methyl-D-aspartate receptor (Verma et al., 2018)

1.2.3 Cell death programs

Cell death programs that are active in ischemia and reperfusion include necrosis, apoptosis and autophagy-associated cell death (Fig. 4).

1.2.3.1 Necrosis and apoptosis

Necrosis, as a result of an acute increase in intracellular Ca^{2+}, acidotic toxicity and increased Na^{+} influx which leads to the osmotic movement of water, is characterized by progressive cell and organelle swelling, plasma membrane rupture and leakage of proteases and lysosomes into the extracellular compartment (Arumugam et al., 2018). Necrotic cells are highly immunostimulatory and lead to inflammatory-cell infiltration and cytokine production. In contrast, apoptosis involves an orchestrated caspase signaling cascade that induces a self-contained program of cell death characterized by nuclear fragmentation, plasma membrane blebbing, cell shrinkage and loss of mitochondrial membrane potential and integrity. Although

this process has traditionally been viewed as less immunostimulatory with respect to necrosis, studies have shown that extracellular release of ATP from apoptotic cells through pannexin semi-channels acts as a 'find me' signal that attracts phagocytes (Eltzschig and Eckle, 2011).

1.2.3.2 Autophagy

There is strong evidence supporting the idea that autophagy, although classified as a cell death program, acts as an adaptive response to sublethal stress, such as nutrient deprivation. In neonatal tissues, autophagy, manifested as cytoplasmic vacuolization, loss of organelles and accumulation of vacuoles with membrane whorls (Eltzschig and Eckle, 2011), is activated soon after birth and is essential for development and survival. Aminoacids produced from autophagy recycling peroxidized proteins can be used as an energy source or, alternatively, for the synthesis of new proteins for an appropriate response to starvation - a condition that neonates face at birth. Therefore, defective autophagy is likely to represent a lack of adaptation to events, such as birth, that may require a fully efficient autophagy machinery. Furthermore, there is strong evidence showing that maternal inflammation significantly reduces autophagy in the neonatal brain, indicating that the reduced autophagy is likely to contribute to the functional consequences of the inflammatory process. Conversely, autophagy activation protects cells in different types of metabolic, infectious, or inflammatory stresses (Carloni et al., 2016).

However, while it is essential for the well-being of cells, especially neurons, in some situations autophagy can promote cell death. The present evidence shows that autophagy is in most cases involved in cell death by interacting with apoptotic or necrotic cell death programs (Fig. 4). This *modus operandi* is referred to as autophagy-mediated cell death (Descloux et al., 2015). As concerned neonatal HI, autophagy has been found to support apoptosis and delay necrosis (Carloni et al., 2016).

All of the above leads to the conclusion that autophagy is in a dynamic equilibrium in the cell and its impairment can lead to metabolic dysfunctions and cell death.

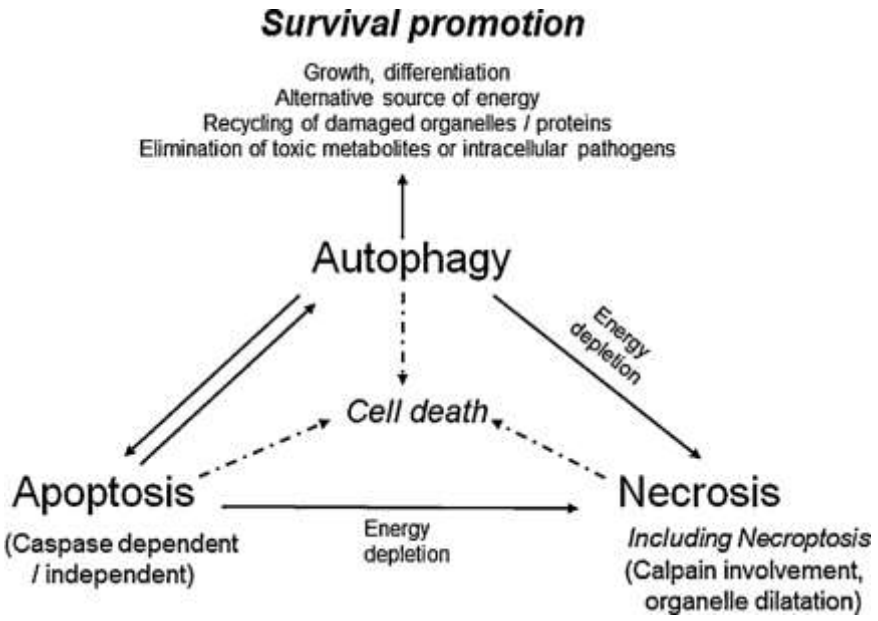

Figure 4. Interconnections between cell death mechanisms (Descloux et al., 2015)

1.3 Hypoxic-ischemic encephalopathy (HIE)

The clinical cerebral entity observed after a severe global asphyxia is termed hypoxic-ischemic encephalopathy (HIE) (Descloux et al., 2015). There are very few intervention strategies at this stage to limit damage from the primary energy failure that occurs within minutes to hours of the initial insult. Primary energy failure occurring from the intrapartum acute hypoxic ischemic (HI) insult is characterized by fetal hypoxemia followed by decreased ATP production and systemic acidosis from increased lactate (Fig. 5) (Berger and Garnier, 1999). Energy failure at the cellular level leads to loss of integrity of the neuronal cell membrane, with calcium entry into the cell (Berger and Garnier, 1999; Nair and Kumar, 2018). However, a period of latency follows primary energy failure, opening up a window of opportunity for interventions to limit further neuronal damage. In the absence of any intervention, secondary energy failure ensues in moderate to severe HIE. However, the degree of resuscitation following HI injury and the severity of primary energy failure mechanisms may impact not only the latency period, but also the subsequent changes in secondary energy failure in the brain. Secondary energy failure typically occurs 6 to 48 h following HI insult and appears to be related to oxidative stress, inflammation, excitotoxicity, and ultimately to cell death. In a subset of infants, persistent active mechanisms may prevent regeneration of neurons or exacerbate brain damage resulting in tertiary brain injury. Tertiary damage to the neurons, such

as myelin deficits, reduced plasticity, and altered cell number, could persist for months to years after the initial insult. Potential mechanisms that oversee these effects include persistent inflammation and epigenetic changes resulting from blockade of oligodendrocyte maturation, impaired neurogenesis, impaired axonal growth, or altered synaptogenesis.

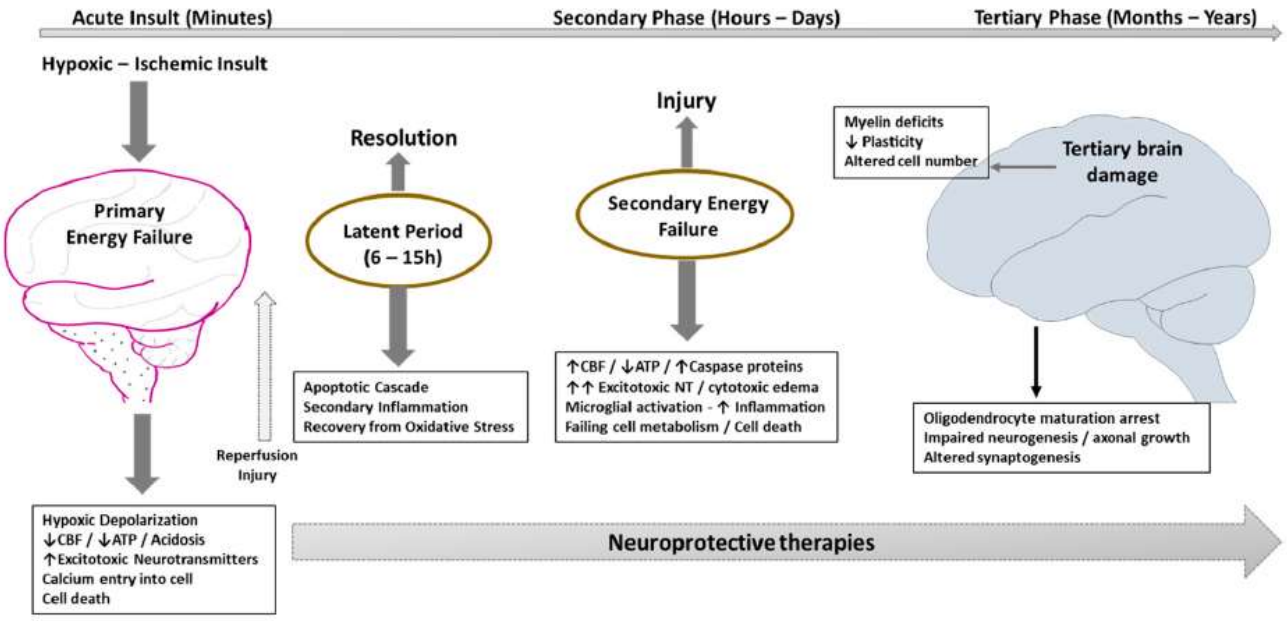

Figure 5. Schematic illustration of pathophysiology of HIE in relation to hypoxic ischemic (HI) insult. Latent period following resuscitation is ideal for interventions to decrease the impact of secondary energy failure. Legend: CBF—cerebral blood flow; ATP—Adenosine tri phosphate; NT—neurotransmitters (Nair and Kumar, 2018)

1.3.1 Evaluation of HIE

To date, the diagnosis and prognosis of an ischemic insult rely on clinical manifestations in combination with neuroimaging, such as cerebral ultrasound (CUS) and magnetic resonance imaging (MRI) (Bersani et al., 2020; Graham et al., 2018). Additionally, even though magnetic resonance spectroscopy (MRS) has significantly improved our understanding of the changes in brain metabolism and evolution of brain injury after a hypoxic-ischemic insult, it is difficult to predict HIE in infants, as in some cases it can occur in spite of a normal neurological exam. Furthermore, due to the varied clinical presentation of HIE, it is difficult to predict outcomes early during the course, when the intervention with targeted therapies would have maximal effect (Nair and Kumar, 2018). Hence, finding quantitative biomarkers that could help detect

subclinical lesions at a stage when routine brain monitoring or imaging is still silent are of high priority in perinatal medicine (Cho et al., 2019; Graham et al., 2018).

1.3.1.1 Biomarkers

In general, biomarkers are defined as chemicals which are measurable in body fluids, such as blood, urine, cerebrospinal fluid (CSF), saliva etc. (Jing-Jie et al., 2014; Nair and Kumar, 2018) or tissue, but mostly researchers focus on peripheral blood biomarkers due to their cost-effectiveness, specificity, sensitivity and ability to give results in a short period of time (Jing-Jie et al., 2014). The optimal biomarker should be reliable, easy and harmless to collect, reproducible and able to guide caregivers in daily practice (Bersani et al., 2020). Biochemical evaluation of the severity of birth asphyxia has traditionally been performed using umbilical arterial blood gases at birth, but this test is poorly predictive of injury (Graham et al., 2018). In the context of HIE, the optimal biomarker should monitor disease progression by longitudinal assessments and possibly correlate with standard procedures such as CUS and MRI to assess the entity of brain injury. The ranges of normality of the ideal biomarker should be available for both healthy term and preterm neonates, and possibly identifiable in different biologic fluids (amniotic, CSF, blood, urine, saliva, milk).

However, to date only a few biomarkers meet these criteria for routine use. Furthermore, despite the fact that several biomarkers, such as calcium binding protein S100B, cytoskeletal monomeric filament protein GFAP and neuron-specific cytoplasmatic enzyme UCH-L1, have recently been approved by European Medicine Agency (EMA) and US Food and Drug Administration (FDA) (Table 1.) for use with adults and children, they are still not included among standard monitoring procedures in the perinatal period (Bersani et al., 2020). Instead, a number of biomarkers normally used to identify other conditions are currently being used to identify neonatal brain injury. For instance, markers of cardiac injury are promising in this respect. In addition, the time-dependent measurement of various metabolic biomarkers that are significantly increased during tissue hypoxia-ischemia (e.g. lactate dehydrogenase, choline, betaine, cytidine, uridine), could improve the prediction of injury. Some inflammatory biomarkers are being used as well, considering that microglial activation and subsequent release of proinflammatory mediators, including IL-6, TNF-α, IL-1α, IL-1β, IFN-γ, is a well-studied process that undoubtedly occurs during the neonatal HIE. Another process following the microglial activation is the production of ROS, indicating oxidative stress. Thus, levels of lipid

peroxidation products correlate, and selenium levels negatively correlate with the severity of HIE (Graham et al., 2018).

Table 1. Studied biomarkers of HIE and their optimality according to the FDA and the EMA criteria ("+" indicates that the biomarker fulfills certain criterion) (according to Bersani et al., 2020)

Biomarker	Brain damage	Degree of injury	Lesion extension	Longitudinal monitoring	Pediatric population	Available kit	Reference curve	Biological fluid
S100B	+	+	+	+	+	+	+	CSF, AF, CB, PB, U, S, M
AM	+	-	-	+	-	+	-	CSF, A, C, P
AcA	+	-	-	+	-	+	-	CSF, A, C, P, U, M
NSE	+	+	-	+	-	+	-	CSF, C, P
OSM	+	+	-	-	-	+	-	CSF, C, P
GFAP	+	-	-	+	-	+	-	CSF, C, P
UCH-L1	+	+	+	+	-	+	-	C, P

Legend: AM, adrenomedullin; AcA, activin A; NSE, neuron specific enolase; OSM, oxidative stress markers; G-FAP, glial fibrillary acid protein; UCH-L1, ubiquitin carboxyl-terminal hydrolase L1; +, yes; -, no; CSF, cerebrospinal fluid; AF, amniotic fluid; CB, cord blood; PB, peripheral blood; U, urine; S, saliva; M, milk. A, waiting for studies in wider healthy populations

Nevertheless, it is necessary to highlight the fact that none of these biomarkers are exclusive to the perinatal HIE.

1.3.2 Assessment and treatment of HIE

Any pregnancy that is identified as being at high risk for neonatal complications should ideally be delivered at a tertiary care center, where trained and experienced resuscitators would handle the management of an infant who is depressed at birth, following accepted guidelines (Nair and Kumar, 2018). The infant is evaluated for therapeutic hypothermia (TH), which is currently the standard of care in the management of moderate to severe HIE in infants (Bersani et al., 2020; Bustelo et al., 2020; Graham et al., 2018; Nair and Kumar, 2018). The mechanism by which hypothermia exposes its therapeutic effects seems to be connected to general cell metabolism retardation (Bustelo et al., 2020). However, TH is expensive, limited by narrow

therapeutic window for initiation (Bustelo et al., 2020; Nair and Kumar, 2018), needs a multidisciplinary team (Nair and Kumar, 2018) and, even if all of these conditions are met, about 45% of neonates have abnormal outcomes despite the treatment (Graham et al., 2018). In addition to TH, supportive management of seizures, fluid balance, hematological and cardiovascular abnormities is essential in ensuring optimal outcomes (Nair and Kumar, 2018).

1.4 Non-coding RNAs

In humans, only 1-2% of the genome encodes proteins, while the rest is composed of non-coding DNA. Therefore, RNAs that do not translate into proteins account for the great majority of the human transcript (Bustelo et al., 2020). Noncoding RNAs can be divided into two categories according to nucleotide (nt) length: long non-coding RNAs (lncRNAs; >200 nt); and short noncoding RNAs (<200 nt) (Zhang et al., 2019).

1.4.1 microRNAs

Recent studies indicate that protein expression is tightly controlled by a group of short (~22 nucleotides), single-stranded, endogenous, non-coding RNAs, called microRNAs (miRNAs) (Cho et al., 2019; Jing-Jie et al., 2014; Ma and Zhang, 2015). They have been detected in all animal model systems and some were shown to be highly conserved across species (O'Brien et al., 2018). To our knowledge, human genome encodes 2693 miRNAs (Valihrach et al., 2020), but there are still new ones being discovered (O'Brien et al., 2018). The roles of miRNAs in modulating protein expression are well recognized on both transcriptional and posttranscriptional levels (Ma and Zhang, 2015; O'Brien et al., 2018). Thus, miRNAs are a part of a complex net of genetic regulation system that can modulate many fundamental biological processes at different levels, in physiological as well as pathological conditions (Cho et al., 2019).

About a half of currently known miRNAs are intragenic (O'Brien et al., 2018), i.e. transcribed from the DNA sequence located within protein-coding genes (Liu et al., 2019), and processed mostly from introns, with relatively fewer exons of these genes. The other half of them is intergenic, meaning that they are transcribed independently of a host gene and regulated by their own promoters (O'Brien et al., 2018).

Following its formation, a mature miRNA is loaded onto the Argonaute (AGO) family of proteins in an ATP-dependent manner, to form a miRNA-induced silencing complex (miRISC), which then target-specifically participates in gene silencing. Once incorporated in

the miRISC, miRNA, via its seed region (an 8-nucleotide sequence located on the 5' end of a mature miRNA (Kehl et al., 2017)), interacts with complementary sequences on the target mRNA. These sequences are called miRNA response elements (MREs) (O'Brien et al., 2018). The interaction, however, does not have to be perfect (Kehl et al., 2017); the degree of miRNA:MRE complementarity determines whether the translational inhibition or degradation of the target mRNA will occur (O'Brien et al., 2018). If the 3' UTR of the target mRNA is fully complementary to the miRNA seed site, the mRNA is targeted for degradation. If it is only partially complementary, the mRNA is targeted for translational inhibition. In this way, an individual miRNA can post-transcriptionally regulate the expression of hundreds of mRNAs (Cho et al., 2019; Liu et al., 2019; Valihrach et al., 2020) and, at the same time, one mRNA can be regulated by several miRNAs. Moreover, besides their repressive role, there is considerable evidence to support posttranscriptional stimulation of gene expression by miRNAs in specific situations (Cho et al., 2019).

Most miRNAs are expressed in a developmental or tissue-specific manner. Furthermore, central nervous system (CNS) is confirmedly the prominent site for their expression. It is estimated that approximately 70% of currently detectable miRNAs are expressed in the brain, and that half of them are either brain specific or brain enriched (Ma and Zhang, 2015). This is consistent with the growing evidence, which has elucidated that miRNAs play the important role in the regulation of CNS development and homeostatic function (Cho et al., 2019; Ma and Zhang, 2015), as well as under pathological conditions of HI, as mediators of neuroinflammation and neurodegeneration.

In addition to the role of miRNAs in the patophysiology of perinatal brain injury, evidence now suggests that cells in the CNS secrete stable miRNAs into the plasma, which are either bound to protein, HDL, or packaged within exosomes/microvesicles following stroke. As their release is closely related to genomic changes in the brain, they have immense potential as biomarkers of perinatal brain injury, leading to early diagnosis and well-timed treatment (Cho et al., 2019).

1.5 Melatonin

Melatonin (Fig. 6), an endogenous indoleamine mainly produced by the pineal gland, is well known for regulating the circadian rhythm. However, increasing evidence indicates that it also plays a significant role in visual, reproductive, cerebrovascular, neuroendocrine and neuroimmunological systems (Carloni et al., 2017a). Today it is well known that melatonin has the ability to remove singlet oxygen, superoxide anion radical, hydroperoxide, hydroxyl radical and the lipid peroxide radical by acting as a direct scavenger. Considering that the brain is especially sensitive to free radical injury due to its high utilization of oxygen, inadequate antioxidant defense and high amount of easily oxidizable PUFAs (Paprocka et al., 2019; Zhao et al., 2018), melatonin is emerging as a potential therapeutic for the treatment of several neurological disorders, including amyotrophic lateral sclerosis (ALS), Alzheimer's, Parkinson's and Huntington's disease, multiple sclerosis and adult ischemic stroke (Carloni et al., 2017a; Paprocka et al., 2019). Another proposed use of melatonin is in the treatment of perinatal ischemic and inflammatory brain injuries. During the perinatal period, children are more exposed to oxidative stress as a consequence of the transition from the hypoxic intrauterine environment to extrauterine life. The peculiar perinatal susceptibility to oxidative stress indicates that prophylactic use of antioxidants, such as melatonin, could help to prevent or at least reduce oxidative stress-related diseases in newborns (Paprocka et al., 2019).

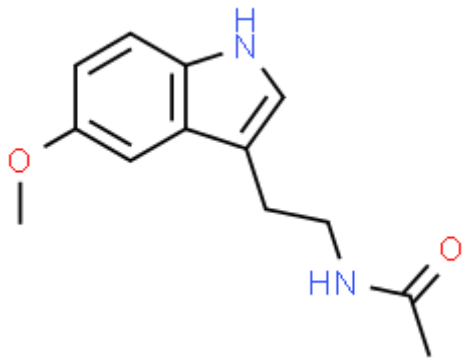

Figure 6. Chemical structure of melatonin (www.chemspider.com)

1.5.1 Melatonin supply in fetal and neonatal period

Melatonin is coming to fetal circulation exclusively from the mother, through the placenta. Therefore, it is clear that impaired blood flow between the mother and the fetus influences melatonin levels in the fetal circulation. In the absence of maternal melatonin, the appearance of fetal circadian rhythms depends principally on neurological maturation and very little on the environment.

After birth, even though the structures like the suprachiasmatic nucleus and pineal gland are well functioning, the neural network concerning the brain circuitry of melatonin secretion remains immature. Therefore, the newborn's main source of melatonin becomes breast milk. During the first 4-5 days after birth, it contains colostral mononuclear cells capable of synthesizing melatonin (autocrine synthesis) and after that it only contains the melatonin derived from the mother. Generally, nocturnal levels of melatonin in newborns may normalize 48 hours after birth, while the rhythmic secretion of endogenous melatonin appears 2-3 months after birth (Paprocka et al., 2019).

1.5.2 Therapeutic potential - neonatal HIE

At the moment, there are several mainly animal studies showing the usefulness of melatonin application in the oxidative stress-related diseases in newborns (Paprocka et al., 2019). In animal models, melatonin was particularly effective as a neuroprotective agent, as it was found to reduce brain injury and its long-lasting consequences after HI and oxidative damage in immature rat brain. In addition, melatonin showed promising results in improving the LPS-induced neonatal inflammation and related brain injury in rats (Carloni et al., 2016).

Even though the exact dose of melatonin needed for neuroprotection is still unknown, the data collected from the animal and human studies revealed that the neuroprotective doses are much higher than the physiological ones (Carloni et al., 2017a; Paprocka et al., 2019). Of course, not only the adjustment of dose, but also the choice of a proper timing to administer melatonin is of fundamental importance in the case of preterm and term infants (hours or days before or after hypoxia) (Paprocka et al., 2019).

2. RESEARCH OBJECTIVES

This thesis reports preliminary data of an in progress study aimed at determining new diagnostic and/or prognostic biomarkers connected with the potential use of melatonin in the treatment of neonatal hypoxic-ischemic encephalopathy (HIE).

Birth asphyxia, which normally leads to the development of HIE, is the most common contributor to early neonatal mortality. Four million newborn infants experience birth asphyxia each year, accounting for an estimated one million deaths and 42 million disability-adjusted life years (Glass and Ferriero, 2007). Therefore, on a global scale, advances in managing infants with HIE will contribute significantly to achieving the sustainable developmental goals. Even though progress has been made in this area, the management of neonates with HIE is still hindered by the lack of quantifiable biomarkers that could measure the degree of injury, assist in triage to therapy and give prognostic information (Graham et al., 2018; Nair and Kumar, 2018). Additionally, with hypothermia currently being the only therapy for perinatal HIE, there is a strong need for alternative and supplementary neuroprotective agents (Nair and Kumar, 2018; Paprocka et al., 2019). Melatonin has emerged as a promising molecule in that aspect due to its neuroprotective properties, low toxicity and ability to readily cross the blood-brain barrier (Paprocka et al., 2019).

Focus of this thesis is set on the modifications of two different miRNAs induced by HI and melatonin in cerebral cortex, as well as in the serum of pup rats. The miRNAs analyzed were miR-126, mainly involved in angiogenesis, and miR-146a, shown to be involved in the inflammatory response. These processes are activated during HI and seem to play a major role in brain damage and/or neuronal protection.

First, measurements based on the intensity and uniformity of the staining of brain slices were performed to determine in which brain areas neonatal brain HI caused the greatest damage. Then, the RT-qPCR analysis was used with the purpose of determining the levels of target miRNAs (miR-126 and miR-146a) in three different time points: 1h, 6h and 24h after HI and melatonin treatment, in the right cerebral cortex and serum of each experimental group. The hypothesis was that a correlation exists between the levels of these miRNAs in the cerebral cortex and in the serum. If this was the case, it could be clinically relevant to measure the levels of these miRNAs for potential diagnosis of the ongoing brain damage and for evaluating the efficacy of neuroprotective drugs. For this purpose, the alterations between the levels of these miRNAs were also studied following the treatment with melatonin, which has previously shown the efficacy in reducing the consequences of HI insult (Carloni et al., 2017b).

3. MATERIALS AND METHODS

All surgical and experimental procedures were carried out by prof. Silvia Carloni, PhD, in accordance with the Italian regulations for the care and use of laboratory animals (according to the EU Directive 2010/63/EU), and were approved by the Animal Care Committee of the University of Urbino "Carlo Bo" (Carloni et al., 2014).

3.1 Animals

Time-pregnant Sprague-Dawley rats (Charles River, Calco, Lecco, Italy) were housed in individual cages. They had free access to food and water and were bred at 22°C with a normal light cycle (Carloni et al., 2016). The day of delivery was considered day 0 for the pups.

For the brain damage assessment, total of 30 pup rats was used, divided in three experimental groups: (i) non-ischemic group which has not received melatonin, i.e. the control group (CTRL); (ii) ischemic group which has not received melatonin (HI); (iii) ischemic group which has received melatonin (HI+Mel). There were 10 pups in each group (N = 10/group) (Carloni et al., 2014).

For the RT-qPCR analysis, another 30 pup rats were used. The CTRL group included six pups (n = 6), while HI and HI+Mel groups included 12 pups each (n = 12/group).

3.2 Materials

All the materials used for the assessment of brain damage, as well as for RT-qPCR analysis, are listed in Table 2.

Table 2. Chemical compounds, instruments and kits used for the experiment.

CHEMICAL COMPOUND	PROVISIONER
isoflurane (05653/4166)	Virbac
melatonin (M5250-1G)	Sigma-Aldrich
dimethyl sulfoxide (DMSO; D5879-1L)	Sigma-Aldrich
2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris; T1503-1KG)	Sigma-Aldrich
ethylenediamine tetraacetic acid (EDTA; E-5134-500G)	Sigma-Aldrich
ethylene glycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA; E-4378-25G)	Sigma-Aldrich
phenylmethylsulfonyl fluoride (PMSF; P-7626)	Sigma-Aldrich
absolute ethanol (EtOH; 20821.321-2.5L)	VWR Chemicals
protease inhibitor cocktail (1 697 498)	Roche
paraformaldehyde, 4% in PBS (22023-20ML)	Biotium
phosphate buffered saline (PBS; P5368-10PAK)	Sigma-Aldrich
sucrose (S0389-500G)	Sigma-Aldrich
toluidine blue (89640-25G)	Sigma-Aldrich

INSTRUMENTATION	PROVISIONER
Centrifuge 5417R	Eppendorf
Centrifuge 4237R	A.L.C. International
Ultrasonic Liquid Processor XL Sonicator	Heat System Ultrasonic Inc.
ABIPRISM 7500 Real Time PCR System	Applied Biosystems

KIT	PROVISIONER
mirVana™ - miRNA Isolation Kit	Applied Biosystems
TaqMan™ MicroRNA Assay	Applied Biosystems
TaqMan™ MicroRNA Reverse Transcription Kit	Applied Biosystems

3.3 Cerebral hypoxia-ischemia (HI)

The procedure used was a slight modification of the one described by Rice et al. (1981). On postnatal day 7 (PN7) pups were anesthetized with 3% isoflurane in oxygen and the right common carotid artery of each pup was exposed, isolated from nerve and vein, and ligated with surgical silk. The wound was then sutured and the animal allowed to recover for 3 hours under a heating lamp. Pups were then placed in airtight jars and exposed for 2.5 hours to a humidified nitrogen–oxygen mixture (92% and 8%, respectively) delivered at 5–6 L/min (HI). The jars were partially submerged in a 37°C water bath to maintain a constant thermal environment (Carloni et al., 2008).

3.4 Melatonin treatment

Melatonin was dissolved in dimethyl sulfoxide (DMSO, vehicle) and diluted in normal saline to a final concentration of 5% DMSO.

Animals used for brain damage assessment received a single, two, or three intraperitoneal administrations of melatonin (15 mg/kg) or vehicle, respectively. Each pup weights 16 g and the volume of melatonin solution or vehicle applied equals 240 μL. Three doses were applied starting 5 min after the hypoxic-ischemic insult and repeated after 24 h and 48 h (Carloni et al., 2014).

For RT-qPCR analysis, the melatonin solution was injected intraperitoneally to pup rats 5 minutes after HI, at the dose of 15 mg/kg (Carloni et al., 2017b).

3.5 Assessment of brain damage

Animals were sacrificed on postnatal day 14, i.e. 7 days after HI (5 days after the administration of the third dose of 15 mg/kg melatonin or vehicle), by being anesthetized with 3% isoflurane in oxygen and perfusion-fixed with 4% paraformaldehyde in 0.1 mol/L PBS. Brains were rapidly removed on ice, immersion-fixed in 4% paraformaldehyde at 4°C for 4 h and cryoprotected with 8% sucrose/PBS (72h, 4°C). To evaluate tissue injury, coronal sections (40 μm thick) of the brain of each animal were cut on a cryostat and thaw-mounted onto acid-washed subbed slides (gelatine and chrome alum). Sections were then stained with toluidine blue, as described by Carloni et al. (2008). A computerized videocamera-based image analysis system (NIH Image software) was used to measure cross-sectional areas from the level of the anterior genu of the corpus callosum to the end of the gyrus dentatus. Measurements, based on the intensity and uniformity of the staining, were performed by an experimenter that was blinded to the conditions of the treatment and included only intact tissue. Regional volumes were estimated by summing areas and multiplying by the distance between sections (40 μm) (Carloni et al., 2014).

3.6 Sample preparation for RT-qPCR

The pups were sacrificed by decapitation in three different time points: 1h, 6h and 24h after HI and melatonin treatment.

3.6.1 Serum

After sacrification, the trunk blood was collected from the site where the pups were decapitated, and stored overnight at room temperature. Next morning the blood was centrifuged for 10 minutes at 2500 rpm. The serum was aspirated and subjected to the miRNA extraction.

3.6.2 Cerebral cortex lysates

After sacrification, the brains were rapidly removed and the right cerebral cortex of each of the pups was separated from the left. They were further sonicated in 0.4 mL lysis buffer containing 1M Tris, 0.25M EDTA, 0.025M EGTA, 10mM phenylmethylsulfonyl fluoride (PMSF) in absolute EtOH and f-complete protease inhibitor cocktail, using an Ultrasonic Liquid Processor XL Sonicator. Homogenates were centrifuged for 10 minutes at 15000 rpm (4°C) and the supernatants aspirated and stored at -20°C until the RNA extraction.

3.7 Quantitative real-time PCR for mature miRNA analysis

The miRNAs were isolated from the serum and brain homogenate supernatants using the mirVana isolation kit and following the manufacturer's recommended protocol.

After the miRNA extraction, rat miR-126, miR-146a and U6 (reference miRNA) expressions were evaluated, using the TaqMan MicroRNA assay. The TaqMan MicroRNA reverse transcription kit was used to reverse transcribe microRNA. Subsequently, RT-qPCR was performed in 20 μL of PCR mix containing 1 μL of 20× TaqMan MicroRNA assay, which contained PCR primers and probes (5′-FAM), 10 μL of 2× TaqMan Universal PCR Master Mix No Amp Erase UNG and 5 μL of reverse-transcribed product. The reaction was first incubated at 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute. The quantitative real-time PCR (RT-qPCR) was performed on a ABIPRISM 7500 Real Time PCR System. Data were analyzed by a 7500 system software (1 1.4.0) with the automatic comparative threshold (Ct) setting for adapting baseline. Detection thresholds were set at 35 Ct. The relative amounts of miR-34a, miR-146a, and miR-126 were calculated using the Ct method:

$\Delta Ct = Ct(\text{miR-146a/ miR-126}) - Ct(U6);\ 2^{\Delta Ct}$

Results are expressed in the figures as fold induction relative to control values (Carloni et al., 2016).

3.8 Data analyses

Statistical analyses were performed by one-way ANOVA using the Prism Computer program (GraphPad Software Inc.). The Newman–Keuls multiple comparison test was used to determine differences between single treatment groups. $P < 0.05$ was considered significant (Carloni et al., 2016; Carloni et al., 2014).

4. RESULTS AND DISCUSSION

4.1 Brain damage

In accordance with the previous findings (Carloni et al., 2008), HI induced a severe damage in the side of the brain ipsilateral to the occluded carotid. Therefore, the left side of the brain in this model is non-ischemic and can be considered an internal control. Administration of melatonin (15 mg/kg) to neonatal rats subjected to HI in 5 min, 24 h and 48 h after HI, resulted in a significant reduction of brain injury (Fig. 7) compared to vehicle-treated ischemic animals. Brain injury in the whole hemisphere, the cerebral cortex and the hippocampus of melatonin-treated animals was 60%, 47% and 78% lower, respectively (Carloni et al., 2014).

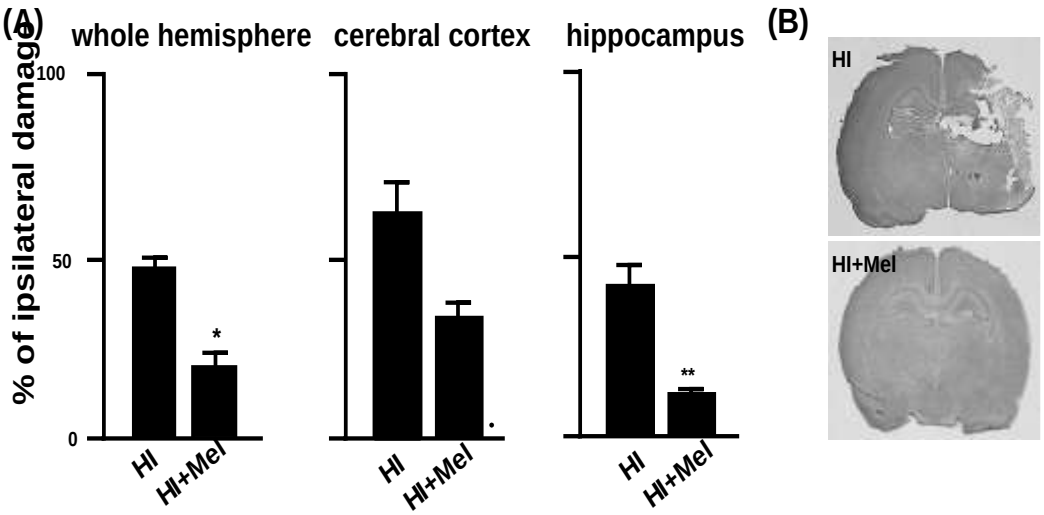

Figure 7. (A) Infarct volume measured in vehicle-treated (HI) or melatonin-treated ischemic animals (HI+Mel). Results are expressed as percentage of ipsilateral damage calculated from bilateral regional volumes using the formula: 100(L-R)/L, where L is the volume of the left side, i.e. contralateral region and R the volume of the the right side, ipsilateral to the occluded carotid artery (N = 10/group). * $p < 0.05$, ** $p < 0.01$, One-way ANOVA followed by Newman-Keuls Multiple Comparison Test. (B) Representative photomicrographs of each experimental group (Carloni et al., 2014).

4.2 RT-qPCR analysis of miR-126 and miR-146a

4.2.1 miR-126

Studies with microarray and realtime PCR revealed that miR-126 is the most frequently expressed vascular miRNA in primary human endothelial cells (ECs) from veins, arteries, skin, and brain. One study showed that, in mice, miR-126 is located within the intron 7 of the Egf7 gene and its deletion leads to a delayed vascular development in retina and brain, and impairment of adult VEGF-dependent corneal angiogenesis (Kuhnert et al., 2008). Another study confirmed the role of miR-126 in vascular development by showing that the deletion of miR-126 in mice causes defects in EC proliferation, migration, and angiogenesis, thereby leading to vascular integrity impairment, hemorrhaging, and partial embryonic lethality (Ma and Zhang, 2015). Mechanistically, the actions of miR-126 appear to reflect, at least in part, its potentiation of MAP kinase signaling downstream of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), which act as potent inducers of angiogenesis (Fig. 8). Spred-1, an intracellular inhibitor of the Ras/MAP kinase pathway, serves as a target for repression by miR-126.

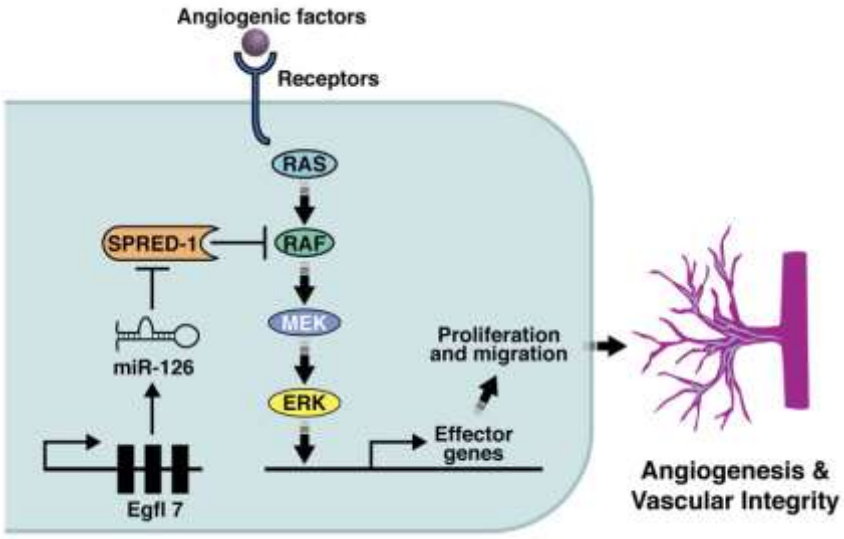

Figure 8. A model for the function of miR-126 in angiogenesis (Wang et al., 2008)

The seed region of miR-126 is completely complementary to the 3'UTR sequence of Spred-1. Thus, miR-126 overexpression relieves the repressive influence of Spred-1 on the signaling pathways activated by VEGF and FGF, favoring angiogenesis. Conversely, in the absence of miR-126, Spred-1 expression is elevated, resulting in repression of angiogenic signaling (Wang et al., 2008).

Following HI, injured vessels at the site of the infarct initiate neoangiogenesis to restore blood flow to the brain. VEGF and FGF expression increases in response to ischemia and is critical for the development of collateral vessels in the ischemic brain. miR-126 is expressed in hematopoietic stem cells, and might therefore contribute to the regenerative functions of this cell population. The discovery that miR-126 is required for vascular integrity and angiogenesis, as well as survival post-HI, suggests that strategies to elevate miR-126 in the ischemic brain could enhance tissue repair.

The results reported in Fig. 9 show that HI reduced the expression of miR-126 in the cerebral cortex compared to the CTRL group, both 1 hour and 24 hours after the insult (Fig. 9A and E, respectively), while its expression increased 6 hours after HI (Fig. 9C). Furthermore, melatonin treatment significantly increased the cortical expression of miR-126 both 1 hour (Fig. 9A) and 6 hours (Fig. 9C) after HI, suggesting that melatonin could enhance tissue repair in the ischemic brain by increasing miR-126 levels. Twenty-four hours after HI, however, the melatonin-induced increased expression of miR-126 was no longer observed (Fig. 9E). Considering the data collected from animal and human studies, which indicate that pharmacokinetic profiles in newborns differ from those in adult humans and animals, and the fact that the neuroprotective action of melatonin occurs at doses much higher than those needed to replace the physiological values (ranging from 5 to 15 mg/kg) (Carloni et al., 2017a), this finding is not surprising.
Interestingly, the serum levels of miR-126 were increased at all time points analyzed – i.e., 1 hour 6 hours and 24 hours after HI (Fig. 9B, D and F, respectively). Melatonin markedly reduced miR-126 levels in serum 1 hour and 6 hours after HI (Fig. 9B and D, respectively). Conversely, the miR-126 levels were significantly higher in the serum of melatonin-treated group compared to CTRL 24 hours after the insult (Fig. 9F).

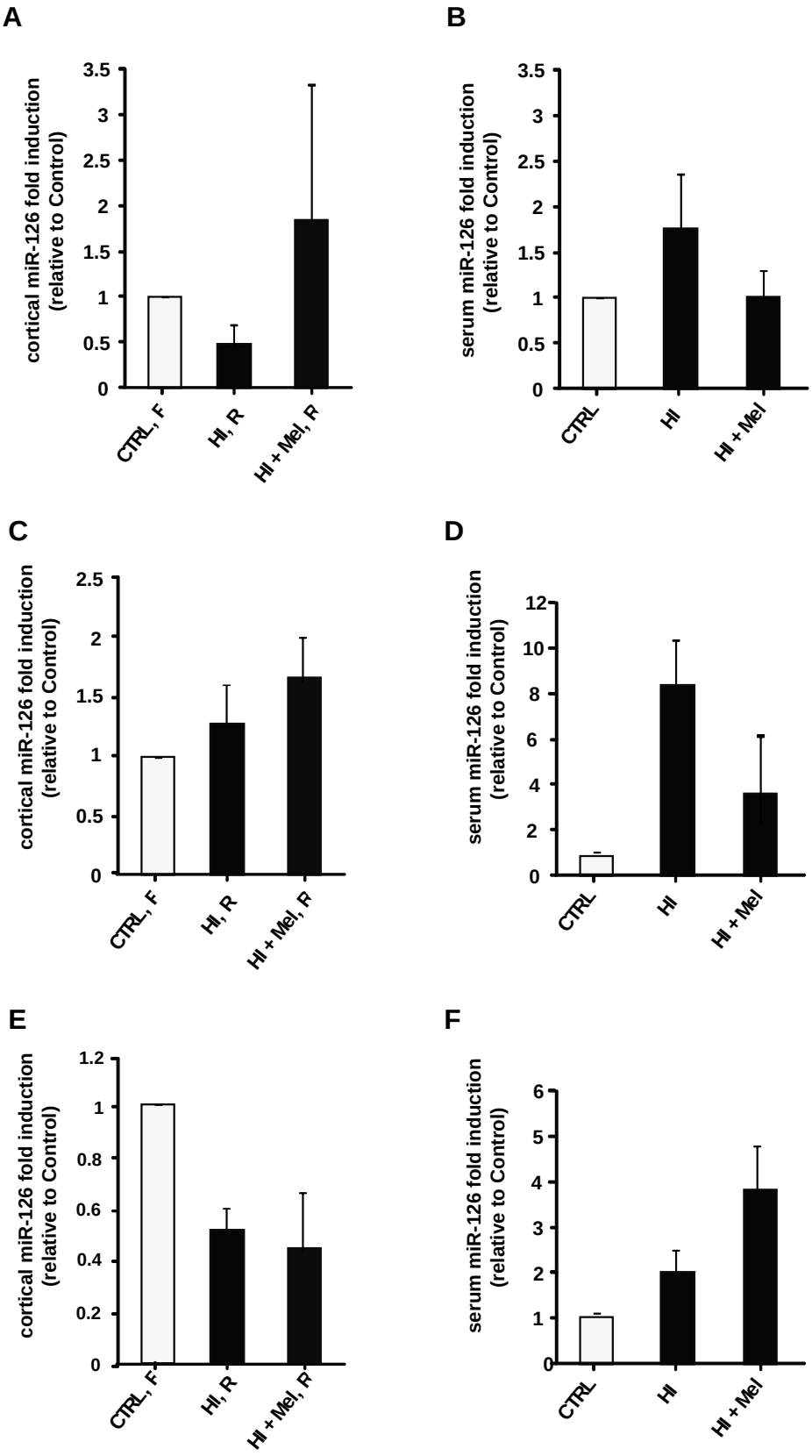

Figure 9. Expression of miR-126a in the cerebral cortex (A, C, D) and serum (B, D, F) of neonatal rats 1 h (A, B), 6 h (C, D) or 24 h (E, F) after HI and melatonin treatment. Legend: R, right ischemic cerebral cortex; Mel, single dose of melatonin (15 mg/kg). Results are reported

as fold induction related to control values and are the mean ± SEM (N=4). P < 0.05 compared to the control group, one-way ANOVA followed by Newman–Keuls multiple comparison test.

4.2.2 miR-146a

MicroRNA-146a, a negative regulator of inflammation, is characteristically upregulated in the pathogenesis of various neurological conditions and considered to play a key role in the regulation of cell survival responses by negative regulation of Toll-like receptor 4 (TLR4) through targeting tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) and interleukin-1 receptor-associated kinase 1 (IRAK1) genes (Fig. 10) in innate and adaptive immune cells (Cho et al., 2019; Gaudet AD et al., 2018; Slota and Booth, 2019). miR-146a expressed by brain endothelia reduces NFκB activation and T-cell adhesion by targeting NFκB pathway activators RhoA, Nfat5, IRAK1, and TRAF6, which could limit immune cell infiltration and neuroinflammation during a pathological condition.

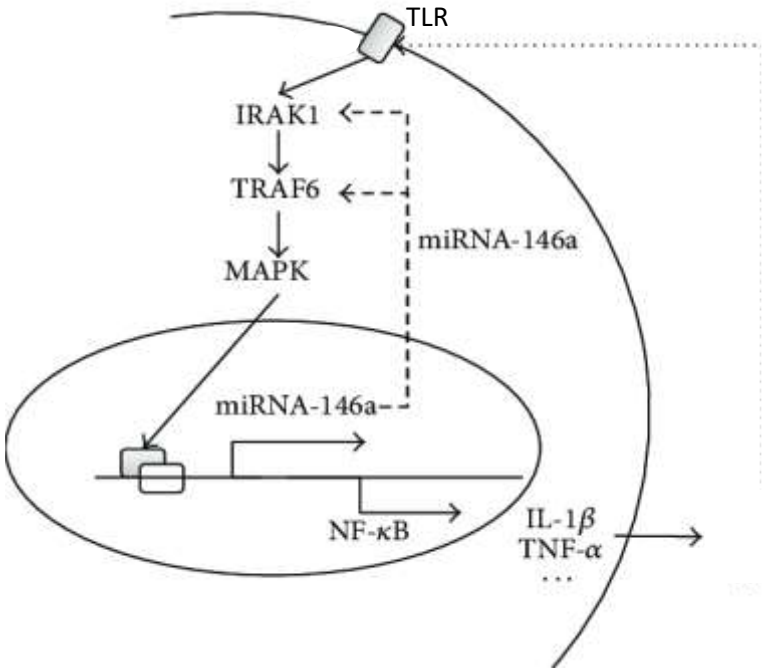

Figure 10. Schematic representation of miR-146a regulating inflammation by a negative feedback mechanism in various neurological conditions (Wei et al., 2016)

Its upregulation suggests that cells are compensating for pathological inflammation and attempting to restore homeostasis (compensatory anti-inflammatory response) (Gaudet et al.,

2018). Moreover, miR-146a is a key regulator of oligodendrogenesis both in the normal and ischemic brain and a negative-feedback regulator of astrocyte-mediated inflammation (Cho et al., 2019).

Macrophage polarization is also modulated by miR-146a through the Notch1 pathway, with miR-146a expression promoting M2 polarization. Therefore, miR-146a serves to diminish inflammatory signaling within the CNS following stimulation by NF-κB, helping to limit excessive neuroinflammation (Slota and Booth, 2019).

The results obtained in this study show a reduced expression of miR-146a in the cerebral cortex 1 hour after HI (Fig. 11A). Conversely, 6 hours after HI the expression of miR-146a was notably upregulated (Fig. 11C), while after 24 hours there was no significant difference of miR-146a expression in the HI group compared to CTRL (Fig. 11E). Taking into account these rapidly occurring changes in miR-146a expression after HI over the time, the logical assumption would be that the compensatory anti-inflammatory response aiming to restore homeostasis and prevent secondary energy failure, is naturally the most active 6 hours after the hypoxic-ischemic insult. Furthermore, the fact that 24 hours after HI no major difference in miR-146a levels was noted between the HI and CTRL group, suggests that the pathways involved in innate immunity, such as TLR signalling pathway, were no longer highly active at that time.

In melatonin-treated groups, the expression of miR-146a was increased 1 hour (Fig 11A) as well as 24 hours (Fig. 11E) after HI, while the effect of melatonin on miR-146a levels was minimally visible 6 hours after the insult (Fig. 11C). Considering that miR-146a was underexpressed 1 hour after HI in comparison with CTRL, melatonin may have helped rise this miRNA's expression to a level required for the adequate defensive immune response, as well as the reduction of inflammation and, consequently, brain damage. Regarding the results observed 6 hours after the insult, i.e. no differences beetween HI and HI+Mel groups, they could be explained by the pharmacokinetic profile of melatonin in newborns, which is still not well known.

Similar to miR-126, the serum levels of miR-146a were increased at all time points analyzed after HI (Fig. 11B, D and F, respectively). Furthermore, melatonin markedly reduced miR-146a levels in serum 1 hour and 6 hours after HI (Fig. 11B and D, respectively), whereas miR-146a levels significantly increased 24 hours after the drug administration (Fig. 11F).

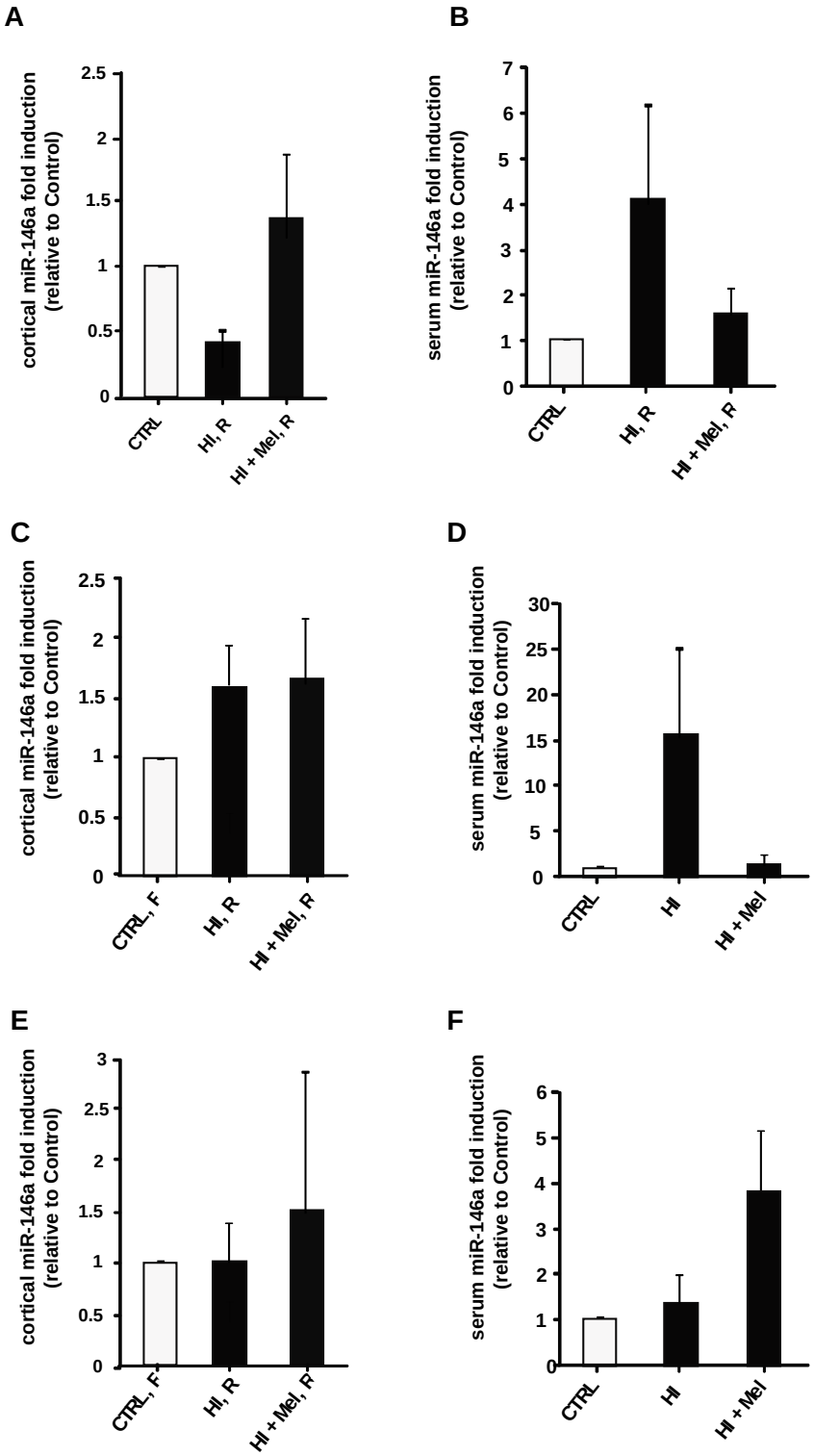

Figure 11. The expression of miR-146a in the cerebral cortex (A, C, D) and serum (B, D, F) of neonatal rats 1 h (A, B), 6 h (C, D) or 24 h (E, F) after HI and melatonin treatment. Legend: R, right ischemic cerebral cortex; Mel, single dose of melatonin (15 mg/kg). Results are reported

as fold induction related to control values and are the mean ± SEM (N=4). $P < 0.05$ compared to the control group, one-way ANOVA followed by Newman–Keuls multiple comparison test.

5. CONCLUSIONS

The results reported in this thesis confirm that melatonin significantly reduces the brain damage induced by neonatal hypoxia-ischemia and provide preliminary evidence of the modulation of miR-126 and miR-146a after the ischemic insult and melatonin treatment. The RT-qPCR analysis revealed a significant modulation of both miR-126 and miR-146a in the early phase of the damage, i.e. one hour after HI. This modulation is observed both in the cerebral tissue and in the serum, indicating that miRNA dysregulation in the circulation may reflect similar changes in the ischemic tissue. Melatonin markedly modulated miR-126 and miR-146a in both tissue and blood. Therefore, the results of this study may support the idea of miRNAs as potential biomarkers for perinatal brain injury.

MicroRNAs variations in blood could potentially lead to early diagnosis and well-timed treatment of HIE in neonates, and could be useful for evaluating the efficacy of neuroprotective drugs. However, further work is in progress to better assess this hypothesis.

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7. SUMMARY

Birth asphyxia, i.e. impaired intrapartum gas exchange, is the most common cause of brain damage in perinatal period. The clinical cerebral entity observed after a severe global asphyxia is termed hypoxic-ischemic encephalopathy (HIE).

Four million newborn infants experience birth asphyxia each year, which leaves them with significant brain injuries and long-lasting consequences, such as cerebral palsy, epilepsy and sensory deficits. To date, the diagnosis and prognosis of an ischemic insult rely on clinical manifestations, which are highly variable, and neuroimaging. However, with this approach it is difficult to predict outcomes early during the course, when the intervention with targeted therapies would be the most efficient. Therefore, on a global scale, finding quantitative biomarkers that could measure the degree of injury, assist in triage to therapy and give prognostic information, will contribute significantly to the management of neonatal HIE. MicroRNAs (miRNAs) have an immense potential as biomarkers of perinatal brain injury, considering that a lot of them have been detected both in brain tissue and in serum/plasma. Additionally, ischemic insult is showed to affect their levels in both of these types of samples.

Furthermore, hypothermia is currently the only therapy for perinatal HIE, indicating that there is a strong need for alternative and supplementary neuroprotective agents. Melatonin has emerged as a promising molecule in that aspect due to its neuroprotective properties, low toxicity and ability to readily cross the blood-brain barrier.

Focus of this thesis is set on the changes in miR-126 and miR-146a levels induced by cerebral hypoxia-ischemia (HI) and melatonin in cerebral cortex, as well as in the serum of newborn rats. MicroRNA miR-126 is involved in angiogenesis, while miR-146a negatively regulates innate and adaptive immune responses. These processes are activated during HI and seem to play a major role in brain damage and/or neuronal protection.

The results reported in this thesis confirm that melatonin significantly reduces the brain damage induced by neonatal HI and provide preliminary evidence of the modulation of miR-126 and miR-146a after the ischemic insult and melatonin treatment. The RT-qPCR analysis revealed a significant modulation of both miR-126 and miR-146a in the early phase , i.e. one hour after the insult. This modulation is observed both in the cerebral tissue and in the serum, indicating that miRNA dysregulation in the circulation may be the reflection of similar changes in the ischemic tissue. Therefore, the results of this study may support the idea of miRNAs as potential biomarkers for perinatal brain injury.

8. STRUKTURIRANI SAŽETAK

Uvod u područje istraživanja: Asfiksija novorođenčeta, koju karakterizira nedostatna opskrba krvi i tkiva kisikom, jedan je od najčešćih uzroka oštećenja mozga u perinatalnom razdoblju. Klinička manifestacija, tj. lezija mozga koja nastaje kao posljedica manjka kisika i poremećaja cirkulacije naziva se hipoksično-ishemijska encefalopatija (HIE). Prevalencija asfiksije novorođenčadi doseže brojku od četiri milijuna godišnje, rezultirajući dugotrajnim posljedicama po dijete, kao što su cerebralna paraliza, epilepsija i gubitak osjetnih živaca. Dijagnostika i prognostika novorođenačke HIE danas se oslanja na kliničke manifestacije, koje znatno variraju od pojedinca do pojedinca, te na tehnike slikanja mozga. Nedostatak ovakvoga pristupa je nemogućnost rane detekcije patoloških promjena, što je ključno kako bi se na vrijeme primijenila ciljana terapija. Iz tog razloga očigledno je da postoji potreba za pronalaskom kvantitativnih biomarkera pomoću kojih bi se mogao izmjeriti stupanj oštećenja, procijeniti potreba za terapijom i vrsta potrebne terapije te pratiti njena učinkovitost. Svojstva biomarkera prepoznata su u mikroRNA molekulama s obzirom da ih je moguće kvantificirati i u moždanom tkivu i u serumu/plazmi te s obzirom da asfiksija, kao i posljedična HIE, utječu na njihove razine u oba tipa uzorka. Nadalje, terapijska je hipotermija trenutno jedina opcija za liječenje HIE, što dovodi do zaključka da je pronalaženje alternativnih i/ili suplementarnih neuroprotektivnih molekula prioritet u perinatalnoj medicini. Melatonin je obećavajuća molekula u tom smislu, budući da pokazuje antioksidativna svojstva, niske je toksičnosti te lako prijelazi krvno-moždanu barijeru.

Obrazloženje teme: Tema ovog diplomskog rada promjene su u razinama dvaju mikroRNA, miR-126 i miR-146a, izazvane hipoksijom-ishemijom (HI), u mozgu i u serumu novorođenih štakora. MikroRNA miR-126 važna je za angiogenezu, dok miR-146a igra ulogu u negativnoj regulaciji urođenih i stečenih imunosnih mehanizama. Ovi su procesi narušeni u stanju hipoksije-ishemije, stoga bi njihova adekvatna modulacija mogla zaštititi još nerazvijeni mozak od neželjenih posljedica.

Rezultati: Rezultati ovog istraživanja potvrdili su da melatonin značajno smanjuje oštećenje mozga uzrokovano novorođenačkom asfiksijom te pruža preliminarne dokaze o modulaciji mikroRNA miR-126 i miR-146a nakon hipoksije-ishemije i tretiranja melatoninom mladunčadi štakora. Točnije, RT-qPCR analizom utvrđeno je da su razine obaju mikroRNA znatno promijenjene već u ranoj fazi, tj. sat vremena nakon izazivanja HI. Navedena promjena vidljiva je kako u moždanoj kori, tako i u serumu, što upućuje na to da poremećaj razina mikroRNA u cirkulaciji može odražavati slične promjene u ishemičnom tkivu.

Zaključak: Rezultati ovoga diplomskog rada govore u korist endogenih mikroRNA kao potencijalnih biomarkera HIE s obzirom na njihovu tkivnu selektivnost, stabilnost u plazmi te na činjenicu da su vjerni pokazatelji određenih patoloških promjena u moždanom tkivu.

9. BASIC DOCUMENTATION CARD /
TEMELJNA DOKUMENTACIJSKA KARTICA

Basic documentation card

University of Zagreb
Faculty of Pharmacy and Biochemistry
Study: Master of Pharmacy
Department of Pharmacology
Domagojeva 2, 10000 Zagreb, Croatia

Diploma thesis

The microRNAs as potential biomarkers of neonatal brain ischemia and their alterations following the melatonin treatment

Marta Dobrić

SUMMARY

Birth asphyxia, i.e. impaired intrapartum gas exchange, is the most common cause of brain damage in perinatal period. The clinical cerebral entity observed after a severe global asphyxia is termed hypoxic-ischemic encephalopathy (HIE). Four million newborn infants experience birth asphyxia each year, which leaves them with significant brain injuries and long-lasting consequences, such as cerebral palsy, epilepsy and sensory deficits. To date, the diagnosis and prognosis of an ischemic insult rely on clinical manifestations, which are highly variable, and neuroimaging. However, with this approach it is difficult to predict outcomes early during the course, when the intervention with targeted therapies would be the most efficient. Therefore, on a global scale, finding quantitative biomarkers that could measure the degree of injury, assist in triage to therapy and give prognostic information, will contribute significantly to the management of neonatal HIE. MicroRNAs (miRNAs) have an immense potential as biomarkers of perinatal brain injury, considering that a lot of them have been detected both in brain tissue and in serum/plasma. Additionally, ischemic insult is showed to affect their levels in both of these types of samples.

Furthermore, hypothermia is currently the only therapy for perinatal HIE, indicating that there is a strong need for alternative and supplementary neuroprotective agents. Melatonin has emerged as a promising molecule in that aspect due to its neuroprotective properties, low toxicity and ability to readily cross the blood-brain barrier.

Focus of this thesis is set on the changes in miR-126 and miR-146a levels induced by cerebral hypoxia-ischemia (HI) and melatonin in cerebral cortex, as well as in the serum of newborn rats. MicroRNA miR-126 is involved in angiogenesis, while miR-146a negatively regulates innate and adaptive immune responses. These processes are activated during HI and seem to play a major role in brain damage and/or neuronal protection.

The results reported in this thesis confirm that melatonin significantly reduces the brain damage induced by neonatal HI and provide preliminary evidence of the modulation of miR-126 and miR-146a after the ischemic insult and melatonin treatment. The RT-qPCR analysis revealed a significant modulation of both miR-126 and miR-146a in the early phase , i.e. one hour after the insult. This modulation is observed both in the cerebral tissue and in the serum, indicating that miRNA dysregulation in the circulation may be the reflection of similar changes in the ischemic tissue. Therefore, the results of this study may support the idea of miRNAs as potential biomarkers for perinatal brain injury.

The thesis is deposited in the Central Library of the University of Zagreb Faculty of Pharmacy and Biochemistry.

Thesis includes:	36 pages, 11 figures, 2 tables and 35 references. Original is in English language.
Keywords:	neonatal, rats, brain damage, HIE, miRNA, biomarkers, melatonin, miR-126, miR-146a
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Temeljna dokumentacijska kartica

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Studij: Farmacija
Zavod za farmakologiju
Domagojeva 2, 10000 Zagreb, Hrvatska

Diplomski rad

MikroRNA molekule kao potencijalni biomarkeri ishemijske ozljede mozga kod novorođenčadi i njihove modulacije nakon primjene melatonina

Marta Dobrić

SAŽETAK

Uvod u područje istraživanja: Asfiksija novorođenčeta, koju karakterizira nedostatna opskrba krvi i tkiva kisikom, jedan je od najčešćih uzroka oštećenja mozga u perinatalnom razdoblju. Klinička manifestacija, tj. lezija mozga koja nastaje kao posljedica manjka kisika i poremećaja cirkulacije naziva se hipoksično-ishemijska encefalopatija (HIE). Prevalencija asfiksije novorođenčadi doseže brojku od četiri milijuna godišnje, rezultirajući dugotrajnim posljedicama po dijete, kao što su cerebralna paraliza, epilepsija i gubitak osjetnih živaca. Dijagnostika i prognostika novorođenačke HIE danas se oslanja na kliničke manifestacije, koje znatno variraju od pojedinca do pojedinca, te na tehnike slikanja mozga. Nedostatak ovakvoga pristupa je nemogućnost rane detekcije patoloških promjena, što je ključno kako bi se na vrijeme primijenila ciljana terapija. Iz tog razloga očigledno je da postoji potreba za pronalaskom kvantitativnih biomarkera pomoću kojih bi se mogao izmjeriti stupanj oštećenja, procijeniti potreba za terapijom i vrsta potrebne terapije te pratiti njena učinkovitost. Svojstva biomarkera prepoznata su u mikroRNA molekulama s obzirom da ih je moguće kvantificirati i u moždanom tkivu i u serumu/plazmi te s obzirom da asfiksija, kao i posljedična HIE, utječu na njihove razine u oba tipa uzorka. Nadalje, terapijska je hipotermija trenutno jedina opcija za liječenje HIE, što dovodi do zaključka da je pronalaženje alternativnih i/ili suplementarnih neuroprotektivnih molekula prioritet u perinatalnoj medicini. Melatonin je obećavajuća molekula u tom smislu, budući da pokazuje antioksidativna svojstva, niske je toksičnosti te lako prijelazi krvno-moždanu barijeru.
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Rezultati: Rezultati ovog istraživanja potvrdili su da melatonin značajno smanjuje oštećenje mozga uzrokovano novorođenačkom asfiksijom te pruža preliminarne dokaze o modulaciji mikroRNA miR-126 i miR-146a nakon hipoksije-ishemije i tretiranja melatoninom mladunčadi štakora. Točnije, RT-qPCR analizom utvrđeno je da su razine obaju mikroRNA znatno promijenjene već u ranoj fazi, tj. sat vremena nakon izazivanja HI. Navedena promjena vidljiva je kako u moždanoj kori, tako i u serumu, što upućuje na to da poremećaj razina mikroRNA u cirkulaciji može odražavati slične promjene u ishemičnom tkivu.
Zaključak: Rezultati ovoga diplomskog rada govore u korist endogenih mikroRNA kao potencijalnih biomarkera HIE s obzirom na njihovu tkivnu selektivnost, stabilnost u plazmi te na činjenicu da su vjerni pokazatelji određenih patoloških promjena u moždanom tkivu.

Rad je pohranjen u Središnjoj knjižnici Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta.

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Ključne riječi: novorođenčad, štakori, oštećenje mozga, HIE, mikroRNA, biomarkeri, melatonin, miR-126, miR-146a

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