BRAIN DAMAGE AND OXIDATIVE STRESS IN THE PERINATAL PERIOD: MELATONIN AS A NEUROPROTECTIVE NEW DRUG

Perrone S, Bertrando S, Cornacchione S, Negro S, Tataranno ML, Tei M, Stazzoni G, Buonocore G.

Department of Pediatrics, Obstetrics and Reproductive Medicine, University of Siena

Background: Prenatal factors represent the main determinants of hypoxic-ischemic encephalopathy (HIE) rather than intra- or post-partum conditions in perinatal period. Oxidative stress (OS) plays a key role in perinatal brain damage. The development of therapeutic strategies to improve the outcomes of babies with HIE is still mandatory.
Aim: to evaluate the effectiveness of melatonin as a neuroprotective drug. To investigate the influence of Melatonin on the OS biomarkers production in an animal model of cerebral hypoxia-ischemia.
Methods: 30 rat pups were subjected to ligation of the right common carotid artery and exposed for 2.5 hours at an hypoxic condition. A group of 15 rats was administered melatonin at a dose of 15 mg/kg 5 minutes after the procedure (Mel GROUP). At the same time 15 rats received placebo (HI GROUP). A group of 5 healthy rats was used as sham operated (S GROUP). Isoprostanes (IsoPs), neuroprostanes (NPs) and neurofurans (NFs), all markers of OS were measured at 1, 24 and 48 h from ischemic injury in homogenized cerebral cortex of the two sides, right (hypoxia and ischemia) and left (hypoxia).
Results: In the HI group were observed: a significant increase of IsoPs on the left side of cortex after 1 h from HI injury ($p<0.001$); a significant increase of NPs on both sides after 24 h ($p<0.05$) and a significant increase of NFs on the left ($p<0.05$) after 24 h. After 48 h in the Mel group was observed a significant increase of IsoPs on the left ($p<0.05$) and of NPs on both sides of cerebral cortex ($p<0.05$).
Conclusions: Melatonin reduces OS biomarkers in cerebral cortex of HI rats after 24 h from its administration. The drug is no longer effective after 48 h. These results lay the groundwork for future clinical studies in infants.
Keywords: oxidative stress, hypoxia-ischemia, animal model, melatonin.

INTRODUCTION

Despite recent advances in preterm newborns healthcare, today hypoxic-ischemic encephalopathy (0.5-1/1000 live birth) is still the main cause of acute perinatal mortality (10-60%) [1].
The majority of survivors (25%) shows neurological deficits, such as cerebral palsy, mental retardation or epilepsy, with long-term effects also on the healthcare system and the society [2, 3, 4].
Hypoxic-ischemic encephalopathy occurs when the oxygen brain intake and/or blood flow is reduced (hypoxia/ischemia) [5]. Consequently the neonatal brain converts its aerobic metabolism into anaerobic in an effort to sustain itself activity. The inability to perform the normal metabolic pathway results in an increased free radicals (FRs) generation, cell apoptosis and cell death [6]. A direct relation has been demonstrated between the degree of hypoxia and the amount of FRs production [7, 8]. Neonatal brain is characterized by a high rate of oxygen consumption and low concentration of antioxidants, making it very susceptible to the hypoxic-ischemic injury [9, 10].
The enormous burden of human suffering and financial cost caused by perinatal brain damage make neuroprotection a major health care priority and potential targets of neuroprotective interventions has been studied over time. Currently there are no established therapies for the treatment of hypoxic-ischemic injury aside from hypothermia, but many clinical trials and animal studies showed that hypothermia alone is not able to provide a complete protection or to a normal neurodevelopmental outcome [11]. Presumably the association between moderate hypothermia and the use of antioxidants can lead to an improvement in neonatal outcome.
Among antioxidants, Melatonin (N-acetyl-5-methoxytryptamine) has high antioxidant and anti-inflammatory properties and it is the main secretory product of the pineal gland [12, 13]. It can work as a direct antioxidant, scavenging dangerous FRs, such as OH•, O2−, H2O2 and ONOO−, and as an indirect one inducing the production of antioxidant enzymes, including glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase, and superoxide dismutase. Moreover, melatonin has no pro-oxidant effects unlike many other antioxidants [14].
Melatonin is particularly interesting for its ability to cross all physiological barriers, and to be widely distribute in tissues, cells and subcellular compartments. This drug is not currently available for neonatal thera-

Correspondence to:
Prof. Giuseppe Buonocore
Department of Pediatrics, Obstetrics and Reproductive Medicine, University of Siena
Policlinico "Santa Maria alle Scotte", Viale Bracci 2, 53100 Siena, Italy
Phone: (+39) 0577 586523 Fax: (+39) 0577 586182
e-mail: buonocore@unisi.it

peutic use but when used as a compassionate drug in neonatal sepsis, broncopulmonary dysplasia, and neonatal asphyxia [15, 16] seem to be promising, and support the possibility for its wider use in perinatal medicine.
Recently, we demonstrated the melatonin neuroprotective effects when administered before the HI insult with a reduction of the oxidative damage [17].
The aim of this study is to evaluate whether the use of melatonin after the HI insult is equally protective. The objective is to establish the effectiveness of melatonin as a neuroprotective drug, determining the influence on the production of oxidative stress (OS) and in the brain of rats suffering from hypoxia-ischemia.

EXPERIMENTAL

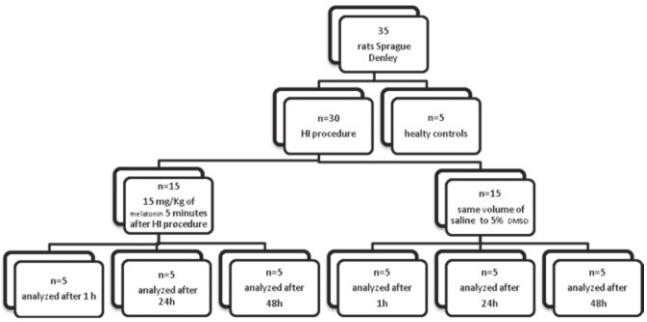
Materials

Pregnant Sprague-Dawley rats as animal model were obtained from Charles River, Calco (LC), Italy. Melatonin; Dimethyl sulphoxide (DMSO); Butylated Hydroxytoluene (BHT); 2,3,4,5,6-pentafluorobenzyl bromide (PFBB); N,N-Diisopropylethylamine (DIPEA), Bis(trimethylsilyl)trifluoro acetamide (BSTFA), N,N-Dimethylformamide (DMF); Undecane; Methanol; Tris(hydroxymethyl)aminomethane (TRIS); Ethylenediaminetetraacetic acid (EDTA); Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA); Phenylmethanesulfonyl fluoride (PMSF); Bovine Serum Albumin (BSA); Sodium dodecyl sulfate (SDS); NaCl; Tween-20; paraformaldehyde; Phosphate buffered saline (PBS); Sucrose; 3,3′-Diaminobenzidine tetrahydrochloride;hydrogen Peroxide (H2O2) were obtained from Sigma-Aldrich, Milan, Italy.
Protease inhibitor cocktail was from Boehringer Mannheim, Mannheim, Germany. Bradford dye was from Bio-Rad laboratories, Milan, Italy. SPE C-18 Sep-Pak cartrige and SPE Silica Sep-Pak cartrige were from Waters VIMODRONE (MI), Italy. Thin layer chromatography (TLC) were from VWR International s.r.l., Milan, Italy. Gascromatography capillary fused silica column DB-1701 15 meter length, 0,25 mm internal diameter and 0,25 µm film thickness were from Agilent Technologies Cernusco sul Naviglio (MI), Italy.
The deuterated internal standard d4-8-IsoPgF2α was from Cayman Chemicals, Tallinn Estonia.
ECL system (Amersham Pharmacia Biotech, UK)
Avidin-biotin peroxidase solution (Elite ABC kit) was from, Vectastain, Vector, USA.

Animal model of cerebral HI

All surgical and experimental procedures were carried out in accordance with the Italian regulations for the laboratory animals care and were approved by the institutional and State authorities. Pregnant Sprague-Dawley rats were housed in individual cages and the day of delivery was considered day 0.
Neonate rats were kept in regular light/dark cycle (lights on 8 am – 8 pm) with free access to the food and water for 7 days after birth. A total of 30 7-day-old rats underwent unilateral ligation of the right common carotid artery, via a midline neck incision after anesthesia with 0.2 mL of inhaled isoflurane. The surgery usually last for 3–4 minutes for each rat, after which the rats were kept in an incubator for observation at 34°C for about 10–15 minutes. They were subsequently placed in a hypoxic chamber with 8% of O2/92% N2 maintained at 34°C for 2.5 hours.
Melatonin, dissolved in DMSO and diluted in saline solution to a final concentration of 5% DMSO, was administered to a group of 15 rats 5 minutes after the end of the ischemic-hypoxic procedure at the dose of 15 mg/kg (MEL GROUP/MELg).
15 rats underwent only surgical artery ligation and hypoxia without receiving melatonin but only a similar volume of DMSO and they served as HI controls (HI GROUP/HIg). A group of 5 healthy rats was used as sham operated (S GROUP/gS). Both the Mel Group and the HI Group were then divided into three subgroup of 5 rats each, which were sacrificed, respectively, at 1, 24 and 48 hours after surgical treatment (figure 1).

Fig.1. *Study >Population*



Preparation of brain tissue extracts

A brain tissue sample (100 mg) from the prefrontal cortex was immediately frozen in liquid nitrogen and stored at -80°C until the use. BHT 100 uM in methanol was added to the cerebral cortex homogenate to prevent oxidation during processing.

IsoPs, NPs, NFs determination

Isoprostanes (IsoPs), neuroprostanes (NPs) and neurofurans (NFs) as OS biomarkers, were measured at 1, 24 and 48 hours after the HI injury in homogenized cerebral cortex of the two sides, right (hypoxia and ischemia) and left (hypoxia) in each group.
Quantification of IsoPs, NPs, NFs requires Folch extraction (1) and two purification steps using solid-phase C-18 Sep-Pak cartridge followed by silica Sep-Pak cartridge. After extraction these molecules were converted to the corresponding pentaflourobenzyl esters to facilitate compound analysis by GC-NICI-MS. This derivatization was carried out with 40 µL of 10% PFBB in acetonitrile and 20 µL 10% DIPEA in acetonitrile. For the purification of pentaflourobenzyl esters the TLC

was used. The purified compounds were further derivatized with 20 µL BSTFA and 7 µL dry DMF to be converted to the corrisponding trimethylsilyl ether. The sample dissolved in 10 µL of undecane was injected into GC/MS. The OS markers values were expressed as ng/mg of tissue. IsoPs, NPs, NFs quantification was made through an Agilent 7890A, a gas chromatograph coupled to Agilent 5975C mass spectrometer equipped with a DB1701 fused silica capillary column programmed from 190°C to 300°C at 20°C per mm. The ions monitored were 569 m/z, for iPs, 593 m/z for nPs, 585 m/z for isoFN and 609 m/z for nFs. The corresponding ion for the deuterated internal standard d4-8-IsoPGF2α was 573 m/z [18].

RESULTS

Treatment with melatonin reduces OS markers level
HI Group and in the Mel Group medians of OS biomarkers (IsoPs, NPs and NFs) were respectively compared to the S Group at 1, 24 and 48 hours after the HI injury. OS biomarkers levels were measured in both right and left cerebral cortex and were analyzed separately for each side [table 1].
IsoPs showed a significant decrease ($p<0.001$) on the left side of cortex in the Mel Group after 1 h from HI injury (gS 3.52 ng/mg of tissue; gHI 5.99; gMel 3.15) and a significant increase after 48 h (gHI 3.51; gMel 4.89).
NPs showed a significant decrease ($p<0.05$) on the both side of the cerebral cortex of the rats treated with melatonin after 24 h from HI injury (right: gS 3.42; gHI 24.08; gMel 11.61; left: gS 4.85; gHI 18.08; gMel 9.45) and a significant increase after 48 h (right: gHI 11.11; gMel 22.35; left: gHI 15.23; gMel 18.23).
NFs showed a significant decrease ($p<0.05$) on the right side of cortex in the Mel Group after 24 h from HI injury (gS 0.11; gHI 1.56; gMel 0.84).

DISCUSSION

Oxidative stress plays a key role in the neonatal brain injury [19], depending on the excessive production of FRs and on the lack of antioxidant system. Antioxidant strategies could be useful tools in brain injury treatment.
Melatonin has several metabolic functions as antioxidant, anti-inflammatory, chronobiotic and even as an epigenetic regulator, via mechanisms including nuclear receptors, co-regulators, histone acetylating and DNAmethylating enzymes [20]. Melatonin has been used in adulthood to correct insomnia and jet lag [21] and in Alzheimer disease [22]. It appears particularly interesting as a neuroprotectant in the newborn because of its efficacy, safety profile and its possibility to act at different levels in the mechanisms responsible for the progression of the neurodegenerative process. We have investigated its antioxidant and anti-inflammatory property in the brain.
The evaluation of lipid peroxidation is a useful means to evaluate brain OS damage. We measured Isoprostanes, Neuroprostanes and Neurofuranes in brain tissues. Isoprostanes (IsoPs), markers of lipid peroxidation, are a prostaglandin-like compounds formed in vivo by nonenzymatic free radical-catalyzed peroxidation of arachidonic acid [23]. They are considered a very reliable means to assess OS status in vivo, providing an important tool to explore the role of OS in the pathogenesis of human diseases [24]. Moreover, a new oxygen insertion step diverts IsoPs intermediates pathway to instead form compounds, termed Isofurans (IsoFs), containing a substituted tetrahydrofuran ring [25]. This different method of formation explains why oxygen tension can affect the lipid peroxidation profile. The same can happen in neuronal cells where docosahexaenoic acid (DHA), a major component of neuronal membranes, is oxidized both in vitro and in vivo to form IsoPs-like compounds termed Neuroprostanes (NPs) [26, 27]. The NPs are the only quantitative in vivo biomarkers of oxidative damage that is selective for neurons. An alternative pathway of oxidation of DHA results in the formation of IsoF-like compounds termed neurofurans (NFs), which are sensitive to changes in oxygen tension [28]. The abundance of DHA in the brain makes the NFs analysis particularly valuable in the quantitative assessment of lipid peroxidation after brain damage. IsoPs, IsoFs, NPs and NFs are chemically and metabolically stable and so are well-suited to act as in vivo biomarkers of OS.
In this study we have a significant reduction, in Mel Group, of IsoPs after 1 hour from HI injury and of NPs and NFs after 24 h from HI injury. We also have a significant increase, in Mel Group, of NPs after 48h probably due to short half-time of exogenous melatonin [29]. The increase of OS biomarkers at 48 hours in the treated group suggest that the effect of Melatonin persists for 24 h, so repeated treatments are necessary to ensure a long lasting protective effect.
This study shows the protective role of melatonin when administered 5 minutes after the hypoxic-ischemic insult. We speculate Melatonin may be an important and complete neuroprotectant, but clinical trials are necessary to assess some issues, such as the dose and the therapeutic regimen to be used and the availability of controlled formulations suitable for these patients.

REFERENCES

1. Jacobs S, Hunt R, Tarnow-Mordi W, Inder T, Davis P, Cooling for newborns with hypoxic ischaemic encephalopathy. Cochrane Database Syst Rev. (4): CD003311; 2007.
2. Dilenge ME, Majnemer A, Shevell MI. Long-term developmental outcome of asphyxiated term neonates. J Child Neurol. 16(11): 781–792; 2001.
3. Ferriero DM. Neonatal brain injury. N Engl J Med. 351(19): 1985–1995; 2004.
4. Fernando F. Gonzalez, M.D.a and Donna M. Ferriero, M.D. Neuroprotection in the Newborn Infant Clin Perinatol. 36(4): 859–880; 2009.
5. Gunn AJ, Thoresen M Hypothermic neuroprotection NeuroRx. 3(2): 154-69; 2006.
6. Perlman JM Intervention strategies for neonatal hypoxic-ischemic cerebral injury. Clin Ther, Review. 28(9): 1353-65; 2006.

IsoPs ng/mg of tissue	Medians value on right cortex				Medians value on left cortex			
	gS	gHI	gMel	p value	gS	gHI	gMel	p value
1h	2.90 (2.7-5.25)	7.43 (4,77-9.21)	4.20 (3.31-6.66)	n.s.	3.52 (3.16-4.03)	5.99 (5.49-7.45)	3.15 (2.82-4.14)	.009
24h		5.73 (3.61-7.03)	4.44 (3.46-5.07)	n.s.		4.01 (3.30-6.43)	4.08 (3.18-6.35)	n.s.
48h		3.44 (2.25-4.23)	5.20 (0.00-6.40)	n.s.		3.51 (3.07-3.68)	4.89 (4.58-6.46)	.009
NPs ng/mg of tissue	Medians value on right cortex				Medians value on left cortex			
	gS	gHI	gMel	p value	gS	gHI	gMel	p value
1h	3,42 (1,12-8,53)	18.03 (5.23-39.82)	9.59 (2.99-19.29)	n.s.	4.85 (3.19-7.64)	14.81 (7.46-24.90)	14.60 (5.20-16.73)	n.s.
24h		24.08 (11.72-27.11)	11.61 (9.13-20.28)	.018		18.08 (13.21-34.73)	9.45 (5.50-16.25)	.022
48h		11.11 (6.90-15.39)	22.35 (10.68-30.30)	.021		15.23 (10.20-21.46)	18.23 (14.69-21.74)	.008
NFs ng/mg of tissue	Medians value on right cortex				Medians value on left cortex			
	gS	gHI	gMel	p value	gS	gHI	gMel	p value
1h	0,11 (0,10-0,67)	0,65 (0,27-0,79)	0,61 (0,34-2,10)	n.s.	0,26 (0,16-0,75)	0,30 (IR: 0,18-0,65)	0,53 (IR: 0,33-2,28)	n.s.
24h		0,84 (0,57-1,34)	1,56 (0,82-2,73)	.030		0,46 (IR: 0,32-0,94)	0,85 (IR: 0,30-1,79)	n.s.
48h		0,81 (0,74-2,13)	0,49 (0,22-1,11)	n.s.		0,74 (IR: 0,41-0,94)	0,54 (IR: 0,38-1,83)	n.s.

Table1. *Medians value and IR of OS biomarkers, performed at 1, 24 and 48 hours after the HI injury in homogenized cerebral cortex of the two sides, right (hypoxia and ischemia) and left (hypoxia) in Mel GROUP (gMel), HI GROUP (gHI) and S GROUP (gS).*

7. Mishra OP, Delivoria-Papadopoulos M. Lipid peroxidation in developing fetal guinea pig brain during normoxia and hypoxia. Dev Brain Res. 45: 129-135; 1989.

8. Mishra OP, Delivoria-Papadopoulos M. Cellular mechanisms of hypoxic injury in the developing brain. Brain Res Bull. 48: 233-238; 1999.

9. Buonocore G, Perrone S, Bracci R. Free radicals and brain damage in the newborn. Biol Neonate. 79(34): 180–186; 2001.

10. Halliwell B. Oxidative stress and neurodegeneration: where are we now? J Neurochem Jun. 97 (6): 1634–1658; 2006.

11. Cilio MR, Ferriero DM. Synergistic neuroprotective therapies with hypothermia. Semin Fetal Neonatal Med. 15(5): 293-8; Epub 2010.

12. Esposito E, Cuzzocrea S. Antiinflammatory activity of melatonin in central nervous system. Current Neuropharmacology. 8: 228-42; 2010.

13. Reiter RJ, Tan DX, Qi W, Manchester LC, Karbownik M, Calvo JR. Pharmacology and physiology of melatonin in the reduction of oxidative stress in vivo. Biological Signals and Receptors. 9: 160-71; 2000.

14. Deng WG, Tang ST, Tseng HP, Wu KK. Melatonin suppresses macrophage cyclooxygenase-2 and inducible nitric oxide synthase expression by inhibiting p52 acetylation and binding. Blood. 108: 518-24; 2006.

15. Gitto E, Karbownik M, Reiter RJ, Tan DX, Cuzzocrea S, Chiu-razzi P, Cordaro S, Corona G, Trimarchi G, Barberi I. Effects of melatonin treatment in septic newborns. Pediatr Res. 50(6): 756-60; 2001.

16. Gitto E, Reiter RJ, Amodio A, Romeo C, Cuzzocrea E, Sabatino G, Buonocore G, Cordaro V, Trimarchi G, Barberi I. Early indicators of chronic lung disease in preterm infants with respiratory distress syndrome and their inhibition by melatonin. J Pineal Res. 36(4): 250-5; 2004.

17. Carloni S, Perrone S, Buonocore G, Longini M, Proietti F, Balduini W. Melatonin protects from the long-term consequences of a neonatal hypoxic-ischemic brain injury in rats. J Pineal Res. 44: 157-64; 2008.

18. Milne GL, Sanchez SC, Musiek ES, Morrow JD. Quantification of F2-isoprostanes as a biomarker of oxidative stress. Nat Protoc.2(1):221-6; 2007.

19. Ferriero DM. Oxidant mechanisms in neonatal hypoxia-ischemia. Dev Neurosci. 23(3): 198–202; 2001.

20. Korkmaz A, Reiter RJ. Epigenetic regulation: a new research area for melatonin? J Pineal Res, Review. 44(1): 41-4; 2008.

21. Haimov I, Shochat T, Lavie P. Melatonin: a possible link between sleep and the immune system. Isr J Med Sci, Review. 33(4): 246-50; 1997.

22. He H, Dong W, Huang F. Anti-amyloidogenic and anti-apoptotic role of melatonin in Alzheimer disease. Curr Neuropharmacol. 8(3):211-7; 2010.

23. Nyska A, Kohen R. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. Toxicol Pathol. 30: 620-30; 2002.

24. Milne GL, Yin H, Brooks JD et al. Quantification of F2-isoprostanes in biological fluids and tissues as a measure of oxidant stress. Methods Enzymol. 433: 113-26; 2007.

25. Fessel JP, Porter NA, Moore KP, Sheller JR, Roberts LJ 2nd. Discovery of lipid peroxidation products formed in vivo with a substituted tetrahydrofuran ring (isofurans) that are favored by increased oxygen tension. Proc Natl Acad Sci USA. 99: 16713-18; 2002.

26. Arneson KO, Roberts LJ. Measurement of products of docosahexaenoic acid peroxidation, neuroprostanes, and neurofurans. Methods Enzymol. 433: 127-43; 2007.

27. Musiek ES, Brooks JD, Joo M et al. Electrophilic cyclopentenone neuroprostanes are anti-inflammatory mediators formed from the peroxidation of the omega-3 polyunsaturated fatty acid docosahexaenoic acid. J Biol Chem. 283: 19927-35; 2008.

28. Song WL, Lawson JA, Reilly D et al. Neurofurans, novel indices of oxidant stress derived from docosahexaenoic acid. J Biol Chem.283:6-16; 2008.

29. Cardinali DP, Srinivasan V, Brzezinski A, Brown GM. Melatonin and its analogs in insomnia and depression. J Pineal Res. 2011.