**Brain Damage and Oxidative Stress in the Perinatal Period: Melatonin as a Neuroprotective New Drug**


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**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.
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 sulfoxide (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).

**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 pentafluorobenzyl esters to facilitate compound analysis by GC-NICI-MS. This derivation was carried out with 40 μL of 10% PFBB in acetonitrile and 20 μL 10% DIPEA in acetonitrile. For the purification of pentafluorobenzyl 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 corresponding trimethylsilyl ether. The sample dissolved in 10 μL of undecon 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 Iso-

**REFERENCES**