Analgesic and Antioxidant Effects of Melatonin in Pediatric Surgery

Protocol Version

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1. Introduction

Pediatric patients undergoing surgery are at significant risk for experiencing postoperative pain. Studies have shown a high prevalence of pain and behavioral problems that can persist for several weeks after surgery. The memory of perioperative pain in children can be distressing and have lasting consequences. Children who develop negatively biased pain memories are more likely to experience poorer pain outcomes in the future. Research has shown that children with such memories experience increased postoperative pain several days after surgery. In addition, inadequate management of acute postoperative pain may contribute to problems such as postoperative bleeding and impaired recovery. These findings underscore the importance of effectively managing postoperative pain to potentially prevent the formation of negatively biased pain memories in young children. In addition, acute pain activates the nociceptive system and triggers a stress response involving the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis. This cascade leads to cerebral, autonomic, and hormonal responses and induces changes in several biochemical processes. Pain has been shown to be associated with oxidative stress (OS) in response to tissue injury, inflammation, and cytokine production.

Melatonin, a neurohormone produced by the pineal gland, plays a role in the nociceptive modulation of various types of pain. Melatonin has anti-inflammatory and antioxidant properties. Early oral

administration of melatonin in infancy has been shown to significantly reduce plasma levels of OS biomarkers such as total hydroperoxides and advanced oxidation protein products.

High levels of these OS biomarkers have been observed in neonates with high pain scores, demonstrating a strong relationship between pain and OS. Melatonin shows a potential role in protecting neonates undergoing surgery from the deleterious effects of OS. After administration of exogenous melatonin, a significant reduction in lipid and protein peroxidation in the postoperative period has been reported in neonates. Recently, Song et al. reported that sirtuin 1 (SIRT1) also plays a critical role in the pathogenesis of pain, showing analgesic effects in chronic pain conditions such as neuropathic and inflammatory pain.

Several studies have shown that lipid peroxidation is involved in different types of pain, such as cancer pain, fibromyalgia, and neuropathic pain. 4-HNE plasma levels are good indices of free radical damage on lipids. Lipid damage from free radical exposure leads to lipid hydroperoxide generation from lipids and to carbonyl formation and protein hydroperoxide generation from proteins. Lipid peroxidation reaction leads to increased endogenous production of reactive aldehydes and their derivatives, such as malondialdehyde and 4-HNE20. Malondialdehyde is the most mutagenic by-product of lipid peroxidation, while 4-HNE is the most toxic. 4-HNE is often used as a marker of OS and has been implicated in the pathogenesis of cancer, neurodegenerative diseases, diabetes, and other diseases. 4-HNE is involved in many pathophysiological signaling pathways SIRT1, acting as a NAD+-dependent deacetylase, plays a crucial role in critical cellular functions such as energy metabolism, genomic stability, inflammation, and immune response. Due to its multifunctional role, SIRT1 has been considered a promising target for therapies aimed at treating various conditions, including metabolic disorders and age-related diseases. Decreased SIRT1 levels have been observed in the spinal cord of various pathological pain models, in which SIRT1 activation helped alleviate chronic pain by regulating inflammation, oxidative stress, and mitochondrial dysfunction.

Recent animal studies have demonstrated that miRNAs are involved in pain processing pathways. Ikuma et al. found the increased extracellular release of microRNAs from dorsal root ganglion cells in a rat model of neuropathic pain.

In this prospective, randomized, double-blind pilot study, we test the hypothesis that melatonin, administered as premedication before induction of anesthesia, reduces OS and postoperative pain involving the sirtuin pathway in children undergoing minor abdominal surgery.

The relationship between pain and 4-HNE, between 4-HNE and melatonin levels, and between microRNAs and SIRT1 levels will be investigated.

2. Methods

2.1 Recruitment and Randomization

The trial is approved by the local Ethics Committee (LocalNo.125222022) according to the Helsinki Declaration of 1964. Patients' legal guardians will provide written informed consent.

Fifteen Children between 3 and 5 years of age scheduled for elective surgery will be prospectively enrolled at the Department of Pediatric Surgery, University Hospital of Messina, Italy. Inclusion criteria are the need for elective surgery, in the morning time. Exclusion criteria are children with cerebral malformations and/or injuries, or surgery in the afternoon or at night to eliminate conditions that could affect melatonin production. Children will be also excluded in cases of withdrawal of informed consent, insufficient blood samples, and hemolysis of the blood sample, as hemolysis interferes with the biochemical determination of the OS biomarkers.

Infants matching the clinical criteria for inclusion in the study will then be randomized to be premedicated with oral melatonin (Mel group) or 5% dextrose (Control group)

Permuted, block randomization will be performed by a biostatistics using computerized sequences, and group assignments will be provided in concealed opaque envelopes. Parents of participants will be informed of their infant's group allocation at discharge.

Interventions

- *Mel group*: Melatonin (Dicoson, Dicofarm, Italy, 5 drops = 1 mg) will be administered orally. The product is listed in the Register of Dietary Supplements on the website of the Ministry of Health (http://www.ministerosalute.it/alimenti/dietetica) and is classified with the following code: 943314283. This product is subject to the European Directive on Foodstuffs according to DL n. 169 of May 21, 2004, and not to the European Directive on Medicines 2001/20/EC transposed at the Italian level with D.L. n. 211 of June 24, 2003. Melatonin administration has a good safety profile, with no known adverse effects. Approximately 1 hour before induction of anesthesia, patients will receive oral melatonin or placebo. Mel treated children received a single dose of oral melatonin 0.5 mg/kg (for a max 10 mg). Melatonin (Dicoson, Dicofarm, Italy) will be prepared by a dedicated resident in a fixed volume of 5 mL by adding water to a syringe without a needle.
- *Control group*: Approximately 1 hour before induction of anesthesia, patients will receive 5 mL of 5% dextrose (placebo), used to simulate the sweet taste of the melatonin formulation.

  The contents of the syringe will be blindly administered to the patients by the attending nurse, who will not be involved in the study.

2.3 Perioperative management of children, analgesia and anesthesia

The induction of general anesthesia will be obtained in all children through bolus doses of 1 mg/kg over 20 s of intravenous propofol, followed by similar bolus doses of propofol until the patient will be anesthetized, and anesthesia was maintained with inhaled sevoflurane.

Children will be considered anesthetized when they are asleep and unarousable, and the eyelash reflex disappear. Thereafter, anesthesia will be maintained with inhaled sevoflurane.

At the end of the surgery, after returning to the ward, all patients will be assessed for postoperative pain using the Face, Legs, Activity, Cry, and Consolability (FLACC) scale. If FLACC score is greater than 3, the analgesic drug paracetamol will be administered at the dose of 15 mg/kg, every six hours at most. Clinical and research personnel will be unaware of the group assignments until the completion of data analysis. The number of doses of paracetamol administered to children of both treatment conditions within 48 hours after surgery, even if discharged, will be recorded.

2.4 Melatonin, 4-Hydroxynonenal, and SIRT1 Measurements

Samples of 0.2 mL of plasma will be collected at T0 (pre-hospitalization, 1 day before surgery), T1 (after anesthesia, immediately before surgery) and T2 (at 1 h after the end of the surgery and awakening after anesthesia), and biochemical analyses will be performed.

Melatonin plasma levels will be assessed at each experimental time point (T0, T1, T2) using a competitive enzyme-linked immunosorbent assay (cELISA) kit from Antibodies.com (A87093) according to the manufacturer's instructions. T1 and T2 plasma samples from Mel treated children suspected of containing concentrations higher than the highest standard (500 pg/mL) will be diluted 1:100 (v/v) with sample diluent prior to analysis. Color development will be monitored at 450 nm in a Thermo Scientific (MultiSkan FC) microplate reader, and a standard curve (range 7.813-500 pg/mL) will be generated using a four-parameter logistic (4-PL) curve fit.

4-Hydroxynonenal (4-HNE) as a marker of lipid peroxidation will be measured to evaluate OS by using a cELISA kit from Antibodies.com (A86962). Plasma concentrations will be calculated by reading the absorbance at 450 nm and referring to the standard curve (range 31.25-2000 pg/mL).

SIRT1 will be quantified using an ELISA kit from Invitrogen (EH427RB). Plasma samples will be diluted 1:2 as indicated by the Manufacturer before analysis. SIRT1 concentrations will be calculated by absorbance reading at 450 nm and referring to the standard curve (range 1.23-300 ng/mL).

2.5 Quantitative real-time PCR for mature microRNAs analysis

MicroRNA analyses will be performed based on the quali-quantitative plasma sample available (N=3 at each time point, T0, T1, and T2). MicroRNAs (miR-34 and miR-124a) will be isolated from plasma using the Norgen total RNA isolation kit[1].The Plasma microRNAs and spike-in cel-miR-39 expressions will be evaluated using the TaqMan miRNA assay. The TaqMan miRNA reverse transcription kit will be used to reverse transcribe miRNAs. Subsequently, RT-qPCR will performe in 20 μL of PCR mix containing 1 μL of 20× TaqMan miRNA 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 will be first incubated at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The quantitative real-time PCR (RT-qPCR) will be performed on a ABIPRISM 7500 Real Time PCR System. Data will be 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-34 and miR-124a will be calculated using the Ct method: ΔCt = Ct (miR-34/miR-124a) − Ct (refence miRNA); $2^{-\Delta Ct}$.

2.6 Data analysis

Data will be expressed as mean (standard error, SE) or median (interquartile range, Q1-Q3) as appropriate. Differences between T0, T1, and T2 will be analyzed by repeated measures ANOVA followed by Tukey's multiple comparison test for melatonin, 4-HNE, miR34 and miR124a plasma levels, while by Friedman test followed by Dunn's test in the case of SIRT1 levels. At each experimental time point, comparisons between the Control and Mel treated children will be performed using Student's t-test for unpaired data or Mann-Whitney U test as appropriate. Linear regression analyses will also be performed to examine correlations between variables (Pearson correlation). All data with $P < 0.05$ will be considered statistically significant. GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA, USA) will be used for statistical analysis.

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