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Fate of melatonin orally administered in preterm newborns: Antioxidant performance and basis for neuroprotection

Francesca Garofoli[1] | Valentina Franco[2,3] | Patrizia Accorsi[4] | Riccardo Albertini[5] | Micol Angelini[1] | Carlo Asteggiano[6,7] | Salvatore Aversa[8] | Elena Ballante[9,10] | Renato Borgatti[6,11] | Raffaella F. Cabini[12] | Camilla Caporali[6] | Luisa Chiapparini[13] | Sara Cociglio[6] | Elisa Fazzi[4,14] | Stefania Longo[1] | Laura Malerba[4] | Valeria Materia[8] | Laura Mazzocchi[7] | Cecilia Naboni[6,11] | Michela Palmisani[2,3] | Anna Pichiecchio[6,7] | Lorenzo Pinelli[15] | Camilla Pisoni[1] | Lorenzo Preda[13,16] | Alice Riboli[17] | Francesco M. Risso[8] | Vittoria Rizzo[5] | Elisa Rognone[7] | Anna M. Simoncelli[13] | Paola Villani[5] | Chryssoula Tzialla[18] | Stefano Ghirardello[1] | Simona Orcesi[6,11]

Correspondence
Valentina Franco, Clinical and Experimental Pharmacology Unit, Department of Internal Medicine and Therapeutics, University of Pavia, ViaFerrata 9, Pavia, Italy.
Email: valentina.franco@unipv.it

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Abstract

Preterm infants cannot counteract excessive reactive oxygen species (ROS) production due to preterm birth, leading to an excess of lipid peroxidation with malondialdehyde (MDA) production, capable of contributing to brain damage. Melatonin (ME), an endogenous brain hormone, and its metabolites, act as a free radical scavenger against ROS. Unfortunately, preterms have an impaired antioxidant system, resulting in the inability to produce and release ME. This prospective, multicenter, parallel groups, randomized, double-blind, placebo-controlled trial aimed to assess: (i) the endogenous production of ME in very preterm infants (gestational age ≤ 29 + 6 WE, 28 infants in the ME and 26 in the placebo group); (ii) the exogenous hormone availability and its metabolization to the main metabolite, 6-OH-ME after 15 days of ME oral treatment; (iii) difference of MDA plasma concentration, as peroxidation marker, after treatment. Blood was collected before the first administration (T1) and after 15 days of administration (T2). ME and 6-OH-ME were detected by liquid chromatography tandem mass spectrometry, MDA was measured by liquid chromatograph with fluorescence detection. ME and 6-OH-ME were not

For affiliations refer to page 7.

Stefano Ghirardello and Simona Orcesi share senior authorship.

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detectable in the placebo group at any study time-point. ME was absent in the active group at T1. In contrast, after oral administration, ME and 6-OH-ME resulted highly detectable and the difference between concentrations T2 versus T1 was statistically significant, as well as the difference between treated and placebo groups at T2. MDA levels seemed stable during the 15 days of treatment in both groups. Nevertheless, a trend in the percentage of neonates with reduced MDA concentration at T2/T1 was 48.1% in the ME group versus 38.5% in the placebo group. We demonstrated that very preterm infants are not able to produce endogenous detectable plasma levels of ME during their first days of life. Still, following ME oral administration, appreciable amounts of ME and 6-OH-ME were available. The trend of MDA reduction in the active group requires further clinical trials to fix the dosage, the length of ME therapy and to identify more appropriate indexes to demonstrate, at biological and clinical levels, the antioxidant activity and consequent neuroprotectant potential of ME in very preterm newborns.

KEYWORDS
antioxidant activity, brain maturation, melatonin, neuroprotection, preterm

1 | INTRODUCTION

In recent years, advanced medical and instrumental techniques allow very preterm newborn's higher possibility of survival, nevertheless greater survival rates result has unaccompanied improvement in neurodevelopment during childhood and beyond.[1,2] To improve long-term cognitive outcomes in very preterm infants, the need to identify neuroprotective strategies that promote brain maturational events taking place in the adverse, nonphysiological, extrauterine environment has been suggested.[3] Brain oxidative stress is one of the most critical factor accountable for the so-called "encephalopathy of prematurity," involving many of the unique developmental events active in cerebral white and gray matter structures.[4,5] Interventions for amelioration/prevention of this dysmaturation process in preterm newborns are mainly based on advanced obstetric and neonatal care but there are also many attempts to identify therapeutic neuroprotective molecules able to face the oxidative stress.[3,5] Different severity of oxidative stress is related to different stages of a newborn's brain lesion, revealed by an increase of lipid peroxidation biomarkers in biological fluids.[6,7] Oxidative stress is an imbalance between excessive reactive oxygen species (ROS) production and low antioxidant activity. In particular, lipid peroxidation products in the presence of ROS are broken into fragments of aldehyde groups with high reactivity.[8] If the break occurs on both chain sides, malondialdehyde (MDA) is formed. The MDA concentration depends on the lipid saturation level, since peroxidation occurs only when lipids are in an unsaturated form. MDA is the most abundant α, β-unsaturated aldehyde generated from endogenous peroxidation of polyunsaturated fatty acids.[8,9] The neonatal brain with a high concentration of polyunsaturated fatty acids, a high rate of oxygen consumption and high levels of iron, low concentration of antioxidants is especially vulnerable to excessive amounts of ROS.[6]

Antioxidant defense mechanisms develop in fetuses during the last trimester of pregnancy.[10] Therefore, preterm newborns have a weak antioxidant defense that cannot counteract the harmful effects of free radicals; thus, they are prone to oxidative injuries, particularly harmful at brain level.[11,12]

One of the most effective endogenous antioxidant is melatonin (ME), an indoleamine hormone secreted in the brain and characterized by many activities. The best known is the regulation of the sleep-wake and circadian rhythm, but several additional biological properties have been described.[13–16] ME acts as a free radical scavenger against toxic ROS and has an antiapoptotic property.[15] ME is rapidly metabolized in the liver to its primary metabolite 6-hydroxymelatonin (6-OH-ME), which is further conjugated with sulfate to produce sulfatoxymelatonin and, to a smaller extent, with glucuronic acid.[13] ME and its metabolites, including 6-OH-ME, can neutralize several toxic oxygen derivatives part of the cascade. Via this means, one ME molecule has the capability to scavenge up to 10 ROS versus the classic antioxidants that scavenge one or fewer ROS.[14] ME is metabolized at the brain level into kynuramine-derivatives, responsible for the

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antioxidant/anti-inflammatory response.[9,16] The fetus does not secrete ME, but receives ME by trans-placental transfer. Preterm birth led to a sudden interruption of ME amniotic fluid swallowing, but also, in case of a term delivery, the newborn has a delay from birth of about 12 weeks in the functional production of ME. Infants born prematurely, mainly before 32 weeks of gestation, suffer from a further delay in endogenous production.[15] There are few in vivo studies showed that preterm newborns have an absent, lower, or delayed hormone production.[17–19] On the other hand, Carloni et al found measurable basal ME levels in preterm newborns studied pharmacokinetics of different oral doses (0.1, 0.5, and 5 mg/kg) of ME after intragastric administration, demonstrating the possibility to maintain a daily sustained ME blood concentration using a single oral administration in preterm newborns.[20] Since ME is an active bio component of human milk with concentrations higher at night, the newborn can take advantage of breastfeeding.[19] Although the use of ME as a neuroprotector appears to be a promising strategy in preterm infants, few recent studies tested the effect of ME administration on the clinical outcome in preterm populations.[21,22] By contrast, several trials reported the effectiveness of ME administration in term newborns suffering from hypoxic-ischemic encephalopathy.[23–25]

Our study aimed to assess: (i) the endogenous ME production in very preterm infants (gestational age, GA ≤ 29 + 6 WE) measuring plasma concentration within 96 h from birth; (ii) plasma ME availability and its metabolization to the main metabolite 6-OH-ME after 15 days of oral ME treatment (3 mg/kg/day or equivalent placebo); (iii) MDA plasma concentrations in the ME and the placebo groups.

2 | METHODS

2.1 | Study design

This was a prospective, multicenter, parallel groups, randomized, double-blind, placebo-controlled trial involving a 24-month assessment period.[26] Participants were randomly allocated to the ME or the placebo groups at a 1:1 ratio, in blocks of 4. The study lasts 24 months to allow neurodevelopmental evaluation, according to the published study protocol.[26] The present study describes the results of biological parameters after 15 days of treatment in ME and placebo groups through the ME, 6-OH-ME, and MDA plasma determination. The randomization was un-blinded only for the biologist and statisticians while blinding was maintained for the researchers involved in clinical assessments.

2.2 | Study population

Preterm newborns have been recruited within 96 h from birth in two Neonatal Intensive Care Units (NICUs): at the coordinating center, Fondazione IRCCS Policlinico San Matteo, together with IRCCS Mondino Foundation in Pavia, Italy, and at the collaborator center Children's Hospital "Spedali Civili" in Brescia, Italy. The trial complies with the Helsinki Declaration and was approved by each involved ethical committees and registered at https://clinicaltrials.gov (accessed on March 27, 2023, NCT04235673). Both parents signed written informed consent before the enrollment.

2.3 | Inclusion and exclusion criteria

GA ≤ 29 + 6 (weeks + days); ability to receive enterally (via an orogastric tube) 20 mL/kg/day maternal or formula milk, within 96 h from birth; written informed consent by both the parents. Infants with genetic, congenital, metabolic, and/or TORCH diseases, periventricular/intraventricular hemorrhage > grade III and/or periventricular leukomalacia ≥ grade II within 96 h from birth were excluded.

2.4 | Study medication and sample collection

Preterm infants were enrolled to randomly receive 3 mg/kg/day ME drops or equivalent amount of placebo drops for 15 days (ME, and placebo were supplied free of charge by a commercial company, FI, Italy). Blood (0.8 mL) was collected through an indwelling arterial catheter, before the administration of ME/placebo (T1) and after the last ME/placebo dose on Day 15 (T2) were collected by catheter, if available, or peripheral blood withdrawal. Heel punctures were not performed for dosing ME. Doses were administered at 11 p.m. Blood samples were collected in plastic vials with EDTA at 7.00 in the morning and centrifuged within 3 h. All samples were stored at − 20°C until analysis.

2.5 | ME and 6-OH-ME measurement by LC-MS/MS

ME and 6-OH-ME were extracted from plasma using a modified procedure from a previously published method.[27] Briefly, 100 μL of plasma sample were mixed with 100 μL of the internal standard *N*-acetyltryptamine 3000 ng/mL (Sigma-Aldrich) and 100 μL of sulfosalicylic acid 10% (Sigma–Aldrich). Samples were then placed in an ice bath for 10 min, the mixture was centrifuged (17 000 g for 10 min)

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and 20 μL of 1 M NaOH were added. The sample was loaded into Chem Elut solid phase extraction cartridges (Agilent) under vacuum and adsorbed for 30 min. Extraction was performed with 4 mL of dichloromethane collected into glass tubes, evaporated to dryness using nitrogen and reconstituted with 50 μL of water containing 0.1% of formic acid. An aliquot of 20 μL was injected into the liquid chromatography tandem mass spectrometry (LC-MS/MS) system for analysis. The analytical assay was validated with an LC-MS/MS system that comprised an HPLC ExionLC 100 integrated system coupled to a 3200 QTRAP® triple-quadrupole mass spectrometer (Applied Biosystems Sciex) equipped with an electrospray ionization source operating in the positive mode. Multiple reaction monitoring was used for data acquisition. The monitored transitions were *m/z* 233.1 → 174.1 for ME, *m/z* 249.1 → 190.1 for 6-OH-ME and *m/z* 203.1 → 144.1 for *N*-acetyltryptamine. Chromatographic separation was performed with an Onyx monolithic C18 column, 100 × 3 mm (Phenomenex) maintained at 40°C. The mobile phase consisted of water containing 0.1% of formic acid (A) and acetonitrile containing 0.1% of formic acid (B) at a flow rate of 0.6 mL/min. Calibration curves showed good linearity over the tested range (0.1–1600 ng/mL) with correlation coefficients >0.9996 for all the calibration curves constructed for both analytes. Precision and accuracy were evaluated at four concentration levels by assaying replicates ($n = 5$) of samples spiked with ME and 6-OH-ME 0.1, 30, 300, and 1300 ng/mL. The limit of quantitation was 0.1 ng/mL for ME and 6-0H-ME. Intraday and interday precision was <13.9% for ME and <11.9% for 6-OH-ME. Intraday and interday accuracy were within 94.9%–100.8% for ME and 94.1%–103% for 6-OH-ME.

2.6 | MDA assay

MDA determination was performed using a reagent kit for HPLC analysis with fluorescence detection (Chromsystems Instruments and Chemicals GmbH). Assays were performed on a liquid chromatograph (HPLC Agilent 1260 Infinity Series LC System) connected to a fluorescence detector (Fluorescence Detector, Spectra G1321B, Agilent Technologies). According to the manufacturer's instructions flow rate was set at 1.0 mL/min, the injection volume was 20 μL, the chromatographic run was 5 min, and working excitation and emission wavelengths were 515 and 553 nm, respectively.

2.7 | Sample size determination and statistical analyses

The sample size was determined based on a single available similar study.[28] It was calculated that 26 patients/group were sufficient to detect (power greater than 80%), a statistically significant difference equal to 8 nmol/mL (standard deviation in the two groups equal to 7) between the null hypothesis (H0) of the same concentration of MDA 16 nmol/mL in the two groups and the alternative hypothesis (H1) of an MDA concentration reduction to 8 nmol/mL in group 2 (*t*-test for independent groups with alpha error = 5%). Considering a dropout rate of 15%, a total of 30 newborns/group should have been screened for enrollment.

The Shapiro–Wilk test was used to determine if datasets were well-modeled by a normal distribution. Categorical variables were described as counts and percentages and compared between treatment groups by chi-squared test. Continuous variables were expressed with mean and standard deviation or median and interquartile range. Comparisons between treatment groups were provided with a *t*-test for independent groups or a Wilcoxon test when variables are not normally distributed. A two-sided *p*-value < .05 was considered statistically significant. Statistical analyses were performed at the BioData Science Unit of the Coordinating Centre by using R 4.0.2 software (R Core Team, 2020; R Foundation for Statistical Computing, Vienna, Austria; https://www.R-project.org/ March 15, 2021).

3 | RESULTS

A total of 60 patients were screened for enrollment in the NICUs of Pavia and Brescia. Six preterm infants were excluded (1 for parents' linguistic problems, five because they were seriously compromised, whose three died). Finally, 28 and 26 infants were enrolled and evaluated until the end of the administration period.

Maternal and neonatal descriptive data, relevant to the 15 days of the study period are reported in Tables 1 and 2.

ME and 6-OH-ME were not measurable in the entire cohort (mean GA below 28 weeks; Table 3) before the first ME/placebo dose administration (T1). Plasma ME and 6-OH-ME were still undetectable after 15 days (T2, corresponding to a range of preterm neonate post-natal life from 16 to 20 days) in the placebo group. In contrast, in the ME group, the hormone and the metabolite resulted detectable and the difference T2/T1 was statistically significant for both compounds (Table 3), as well as the difference of levels comparing the treated and the placebo group at T2 (Table 3). MDA remained stable during the 15 days of ME or placebo treatment. Inter- and intra group differences resulted not statically significant. The percentage of neonates reporting a reduced MDA concentration at T2 in the ME group was 48.1% versus 38.5% in the placebo, but the difference was not significant (Figure 1). All the results were unchanged at the regression analysis considering

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TABLE 1 Maternal characteristics.

Maternal variables	Melatonin group $N = 28$	Placebo group $N = 26$	p-Value
Age (years)			
Mean (SD)	31.46 (5.27)	32.54 (5.27)	0.50
Delivery			
Vaginal N (%)	8 (29%)	7 (27%)	0.62
Caesarian N (%)	20 (71%)	19 (73%)	
Preterm premature rupture of membranes > 18 h (PPROM)			
Yes N (%)	9 (32)	8 (31)	0.28
No N (%)	19 (68)	18 (69)	
Respiratory distress syndrome prophylaxis			
Yes N (%)	28 (100%)	23 (88%)	0.23
No N (%)	0 (0%)	3 (12%)	

Abbreviation: SD, standard deviation.

TABLE 2 Neonatal characteristics.

Neonatal variables	Melatonin group $N = 28$	Placebo group $N = 26$	p-Value
Gestational age (weeks)			
Mean (SD)	27.49 (1.68)	27.57 (1.68)	0.91
Birthweight (g)			
Mean (SD)	994.9 (301.3)	993.6 (301.3)	0.99
Length (cm)			
Mean (SD)	35.41 (3.3)	35.33 (3.3)	0.99
Head circumference (cm)			
Mean (SD)	25.05 (2.2)	24.68 (2.2)	0.99
Sex			
Male N (%)	14 (50%)	13 (50%)	0.99
First born			
Yes N (%)	17 (61%)	16 (62%)	0.99
Twins			
Yes N (%)	8 (29%)	7 (27%)	0.99
Apgar 5′ min			
N (%) ≥ 7	20 (71.2)	21 (80.8)	
N (%) > 4 < 7	2 (7.1)	2 (7.7)	
N (%) ≤ 4	6 (21.4)	3 (11.5)	0.38
Surfactant administration			
Yes N (%)	11 (39)	11 (42)	0.99
Sepsis[a]			
Yes N (%)	5 (18)	7 (27)	0.52
Mechanical ventilation[a]			
Hours median (range)	13.50 (0–120)	24 (0–118)	0.75

Abbreviation: SD, standard deviation.

[a]During the first 15 days of study.

confounders including sex, GA, birthweight, sepsis, and mechanical ventilation.

4 | DISCUSSION

Recent epidemiological studies attested no significant progress in last decades regarding long-term neurodevelopmental outcomes in very preterm infants, emphasizing the need to identify neuroprotective strategies. ME can be considered a prime candidate among the most promising molecules, by mean of antioxidant mechanism useful to counteract brain damage and consequent neurodevelopmental impairment.[3,5,6,13,14]

In the present study, we aimed to assess the antioxidant activity of ME because the hormone is claimed to mitigate the neurodevelopmental damage due to brain oxidative stress.[13–16] In particular, very preterm newborns, due to the premature end of the gestational period, are early deprived of the maternal contribution of trans-placenta ME and are unable to synthesize ME at an effective level,[15,19] precisely in the period they would have a great need for its antioxidant activity, for the maturation of their central nervous system.

The results of our ME trial confirmed that very preterm infants (mean GA < 28 weeks) cannot produce endogenous detectable plasma levels of ME during their first 20 days of life. Following 15 days of oral administration (T2) of exogenous ME, we measured an appreciable amount of ME and its primary metabolite, 6-OH-ME in the treated group. At the same time, ME and 6-OH-ME were still undetectable in the plasma of the placebo group. The oral administered ME was metabolized to 6-OH-ME. The large amount of 6-OH-ME detected in the ME group aligns with the described extensive first liver metabolism operated by the cytochrome P450,[29–31] confirming that the metabolism of the orally administered ME took place. The intra-group difference T2/T1 of ME and 6-OH-ME concentrations was statistically significant in the ME group. Since, in the placebo group, ME and 6-OH-ME were not detectable at T1 and T2, no intra-group difference was found. For the same reason, at T2, the intergroup difference of ME and 6-OH-ME concentrations between treated and non-treated infants resulted highly significant. ME plasma concentrations are comparable with findings from other

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TABLE 3 Melatonin, 6-hydroxymelatonin, and malondialdehyde levels in the melatonin and placebo groups at T1 and T2 time points.

Biological parameters	ME ($n = 28$)	Placebo ($n = 26$)	p-value
T1 ME concentration, ng/mL			
Median (IQR)	0 (0–0)	0 (0–0)	
T2 ME concentration, ng/mL			
Median (IQR)	0.82 (0.29–1.25)	0 (0–0)	0.0001
T1 6-OH-ME concentration, ng/mL			
Median (IQR)	0 (0–0)	0 (0–0)	
T2 6-OH-ME concentration, ng/mL			
Median (IQR)	0.34 (0.09–1.62)	0 (0–0)	0.009
T1 MDA concentration, nmol/mL			
Median (IQR)	0.19 (0.13–0.29)	0.18 (0.12–0.26)	0.33
T2 MDA concentration, nmol/mL			
Median (IQR)	0.2 (0.09–0.28)	0.18 (0.16–0.29)	0.97
Difference T2/T1 ME, ng/mL			
Median (IQR)	0.82 (0.29–1.25)	0 (0–0)	0.0001
Difference T2/T1 6-OH-ME, ng/mL			
Median (IQR)	−0.34 (−1.62 to 0.09)	0 (0–0)	0.009
Difference T2/T1 MDA, nmol/mL			
Median (IQR)	0 (−0.03 to 0.06)	−0.01 (−0.05 to 0.04)	0.21

Note: No differences between ME and placebo groups were detected in maternal and neonatal clinical characteristics at birth.

Abbreviation: IQR, interquartile range.

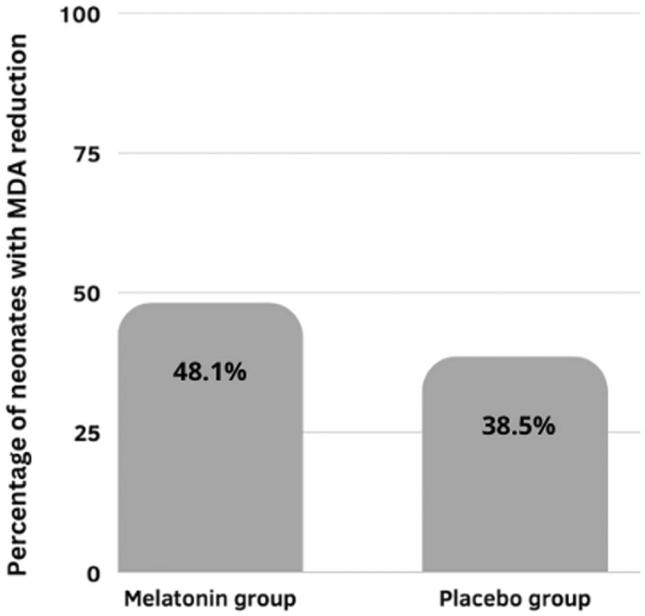


FIGURE 1 Percentage of neonates reporting a reduced malondialdehyde concentration at T2 compared to T1.

studies after oral administration in infants.[20,32] Previous studies showed that naturally secreted ME is not detectable or barely measurable in preterm infants. A study of the early 90s reported a delay of approximately 9 weeks in the excretion of urine 6-sulfatoxymelatonin in premature infants compared to full-term infants, who can reach the amount of 25% excretion of adult at 24 weeks of age.[18] Biran et al confirmed that ME circulating levels are significantly lower in preterm compared to term infants.[17] Carloni et al studied pharmacokinetics of oral doses of ME after intragastric administration measuring basal ME levels.[20] In our placebo group, plasma ME was not detectable at baseline and after 15 days, indicating that our infants were still unable to produce ME. We underline that the enrolled infants were very preterm and the literature describes a delay in the pineal gland secretion more extended and marked compared to term infants.[17] Moreover, NICU patients suffer from immaturity-related ME deficiency due to continuous noisy and nonrhythmic lighting conditions of the ward environment with potentially lasting effects on ME production.[33] Additionally, all the preterm patients received a variable quantity of maternal milk mixed with formula or were supported by parenteral nutrition. ME is a natural component of breastmilk, characterized by different concentrations, depending on GA, circadian rhythm, and other maternal variables.[34] Unfortunately, we have no precise data regarding the amount of maternal milk our patients received. In any case, the amount of ME received by breast milk did not achieve a detectable quantity in neonate's plasma, confirmed by its absence in placebo group's plasma at T2.

Moreover, all the mothers expressed breastmilk during daylight, while it has been reported that it should be better to pump milk during night, because ME concentrations are higher during nighttime.[34] For these reasons, ME of breast milk could not contribute to achieving a measurable amount of ME in plasma of infants. This functional limit may indicate that the maternal lactogenic compensatory mechanisms may activate after 30 WE GA, as demonstrated for other immune components, such as IgA from human milk.[35]

To our knowledge, this is the first study quantifying a sizable amount of 6-OH-ME, endorsing the metabolism of orally administered ME, and confirming the availability of the hormone and its main metabolite. Since also ME metabolites can neutralize numerous toxic oxygen derivatives, they represent an additional help to the antioxidant activity of the primary compound.[14] One ME

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molecule has the capacity to scavenge up to 10 ROS compared to the other antioxidants that scavenge one or less ROS. The ME antioxidant activity was superior compared to vitamin C, vitamin E, glutathione, and NADH.[14] Preterm birth may cause brain injury because newborns are prone to environmental stress, producing an excess of ROS, primarily due to the exposure to high-oxygen condition compared to fetal life, with a marked increase of arterial oxygen tension. Thus, the preterm brain is exposed to hyperoxia, which induces oxidative stress and impairs neuronal cell development.[36] In particular, lipid peroxidation with reactive ROS leads to MDA production that can be used as a marker of the endogenous peroxidation of polyunsaturated fatty acids.[8,9] It is also known that the preterm newborn's immature enzymatic oxidative stress defense system is incompetent to neutralize the increased formation of reactive ROS.[37] Thus, the administration of exogenous ME could be useful to reinforce the system. For this reason, we evaluated a possible correlation between ME administration and MDA plasma level during the exposure period. Unfortunately, the results of our study did not demonstrate that oral ME significantly decreased MDA concentration between T2 and T1 time points, and the ME group reported similar concentrations as the placebo group at T2. Nevertheless, we highlighted that the percentage of preterm newborns that decreased their MDA value after the 15 days of administration was higher in the ME group than in the placebo one (48.1% vs. 38.5%, Figure 1). Similarly, a recent study analyzed, after 1 week of oral ME (0.5 mg/die), plasma concentrations of nonprotein-bound iron, advanced oxidation protein products and F2-isoprostanes as markers of oxidative stress. Among all these markers, only F2-Isoprostanes levels, after 48 h from ME administration, were significantly lower in the ME group than in the placebo group.[32] We can speculate that in our study the number of patients was too low to show the difference in MDA concentration, considering that they were very preterm, so the enzymatic pathway entrusted to face MDA production may have been immature too. The evidence of a trend in MDA reduction, even if not significant, possibly due to the low aldehyde production, suggests the need to find different markers of oxidation to measure the antioxidant effect of the exogenous hormone. No adverse events attributable to ME were recorded during the study period, according to the literature.[16,17,20] In past experiences, ME administration took place intravenously,[19] and by oral-gastric infused galenic solution[38] while our results, in line with other recent investigations,[20] confirm that the commercial oral solution we used is safe and able to provide appreciable plasma levels. The very preterm newborns demonstrated to metabolize exogenous administered ME to 6-OH-ME, confirming the feasibility and effectiveness of the ME oral therapy. We established the basis to design further clinical trials fixing the dosage and the length of ME therapy to improve and strengthen the results. Lastly, we are analyzing the further steps of the study with a focus on clinical outcome at term age, then at 2 years, to further verify the antioxidant and neuroprotectant activity of ME in infants born very preterm.

AUTHOR CONTRIBUTIONS

Francesca Garofoli and Simona Orcesi designed the study. Francesca Garofoli and Valentina Franco wrote the manuscript. Valentina Franco and Michela Palmisani validated the methods and performed the experiments and critically evaluated the results. Salvatore Aversa participated in to study design and was responsible for the experimental and clinical management of the collaborating center. Micol Angelini performed data analysis and helped with experimental work. Raffaella F. Cabin took care of software, data curation, and statistic analysis. Sara Cociglio participated to the investigation and contributed to write the manuscript. Stefania Longo performed the clinical investigation. Camilla Pisoni participated in to study design, review, and editing. Francesco M. Risso, Stefano Ghirardello, and Simona Orcesi reviewed and critically edited the manuscript. All authors have read and agreed with the present version of the manuscript. All authors reviewed and approved the manuscript.

AFFILIATIONS

[1]Neonatal Unit and Neonatal Intensive Care Unit, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy

[2]Department of Internal Medicine and Therapeutics, Clinical and Experimental Pharmacology Unit, University of Pavia, Pavia, Italy

[3]IRCCS Mondino Foundation, Pavia, Italy

[4]Unit of Child Neurology and Psychiatry, ASST-Spedali Civili of Brescia, Brescia, Italy

[5]Laboratory of Clinical Chemistry, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy

[6]Department of Brain and Behavioral Sciences, University of Pavia, Pavia, Italy

[7]Department of Neuroradiology, IRCCS Mondino Foundation, Pavia, Italy

[8]Neonatal Intensive Care Unit, Children's Hospital, University Hospital "Spedali Civili" of Brescia, Brescia, Italy

[9]Political and Social Sciences, University of Pavia, Pavia, Italy

[10]BioData Science Center, IRCCS Mondino Foundation, Pavia, Italy

[11]Child Neurology and Psychiatry Unit IRCCS Mondino Foundation, Pavia, Italy

[12]Department of Mathematics, University of Pavia, Pavia, Italy

[13]Radiodiagnostic Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy

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[14]Department of Clinical and Experimental Sciences, University of Brescia, Brescia, Italy

[15]Neuroradiology Department, Pediatric Neuroradiology Section, Spedali Civili, Brescia, Italy

[16]Department of Clinical, Surgical, Diagnostics and Pediatric sciences, University of Pavia, Italy

[17]Hospital Pediatric Psychology, Unit of Psychology, Children's Hospital "Spedali Civili" of Brescia, Brescia, Italy

[18]Neonatal and Pediatric Unit, Polo Ospedaliero Oltrepò, ASST Pavia, Pavia, Italy

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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