Comparison between tryptophan methoxyindole and kynurenine metabolic pathways in normal and preterm neonates and in neonates with acute fetal distress

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_Abstract_

**Objective:** To analyze the kynurenine and methoxyindole metabolic pathways of tryptophan in order to identify changes in premature neonates and in neonates suffering from fetal distress.

**Methods:** One hundred and twelve neonates were assigned to three groups: normal neonates (control group), preterm neonates (neonates born before the 37th gestational week) and neonates suffering from fetal distress. Each of these groups was then divided into two subgroups according to the time of birth corresponding with the time of blood sampling: a diurnal subgroup, comprising neonates whose blood was sampled between 0900 and 2100 h, and a nocturnal subgroup, comprising neonates whose blood was sampled between 2100 and 0900 h. Blood samples from the umbilical artery and vein were taken in the delivery room at birth from each neonate for measurement of melatonin, the main methoxyindole pathway metabolite. Urine samples were collected from 0900 to 2100 h (diurnal groups) and from 2100 to 0900 h (nocturnal groups), and the presence of kynurenic acid, xanturenic acid, 3-hydroxyantranilic acid, L-kynurenine and 3-hydroxykynurenine determined.

**Results:** The results show the existence of diurnal/nocturnal differences in the concentration of melatonin in cord blood and in the urinary excretion of kynurenines. In normal neonates, the production of methoxyindoles (determined as melatonin) is decreased during the day and increases at night, whereas production of kynurenines is high during the day, decreasing at night. In the fetal distress group, a significant increase in the umbilical artery concentration of melatonin was found. This group also showed a reduction in L-kynurenine concentrations in the diurnal and nocturnal groups, and an increase in xanturenic acid and 3-hydroxyantranilic acid during the day. Correlation and regression studies confirmed that the differences in the day/night pattern of the tryptophan metabolic pathways were greater in normal neonates than in the preterm and fetal distress groups.

**Conclusions:** The results indicate the existence of an imbalance in tryptophan metabolites in preterm infants and those with fetal distress, blunting the normal diurnal/nocturnal rhythm of both melatonin and kynurenines.

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**Introduction**

Tryptophan metabolism, including its main brain pathways such as the methoxyindole and kynurenine routes, has been extensively studied in children (1–3). However, the interrelationships between some of these metabolites, and their participation in neonatal pathology, are as yet less well known. Melatonin (N-acetyl-5-methoxytryptamine), the main metabolite of the methoxyindole pathway, displays neuroprotective capacities against convulsive episodes in humans (4–7) and in experimental animals (8–10). Melatonin inhibits brain excitability, regulating both γ-aminobutyric acid and benzodiazepine receptors and Na⁺, K⁺-ATPase activity (11–13). A relationship between melatonin, corticotrophic and opioid peptides and brain benzodiazepine receptors has been also reported (14, 15). Recent studies have shown the ability of melatonin to counteract both kainate- and N-methyl-D-aspartate (NMDA)-induced excitotoxicity (16–18). Inhibition of the NMDA receptor activity by melatonin depends, at least partially, on its effect to reduce nitric oxide synthase activity, thus decreasing the amount of nitric oxide produced by the NMDA activation (19). Melatonin responds to stress (20), suggesting a role for this indoleamine during the neonatal period (21, 22). A melatonin circadian rhythm in the newborn umbilical cord has been reported (22), although it seems to depend on maternal melatonin, as this rhythmicity disappeared from neonate plasma 3 days after birth.
(23). Although definitive establishment of a normal melatonin circadian rhythm occurs between the 3rd and 6th month of life (24), the pineal gland is active in a newborn infant. This gland significantly increases the production of melatonin when a physiological stimulus, such as the darkness produced after eye-covering in neonatal phototherapy treatment, is applied (25).

Tryptophan metabolites of the kynurenine pathway may be classified into two groups. Quinolinic acid is an agonist of the brain NMDA receptor (26), and its administration induces epileptiform seizures (27). L-Kynurenine and 3-hydroxykynurenine have also been reported to be convulsants (2, 3, 28, 29). Kynurenic acid is an antagonist of the brain NMDA receptor, and may protect it against excitotoxicity (30, 31), whereas xanthurenic acid displays some lesser neuroprotective properties.

In the light of these considerations, an equilibrium between the excitotoxic (L-kynurenine and 3-hydroxykynurenine) and neuroprotective (kynurenic acid, xanthurenic acid and melatonin) tryptophan metabolites would be expected to exist, as an imbalance between them seems to participate in changes in brain homeostasis. Thus we considered it worthwhile to investigate the relationship between melatonin, the main metabolite of the methoxyindole pathway, and the metabolites of the kynurenine pathway in normal neonates. Our aim was to identify the normal association between these metabolites in neonates, and to determine whether alterations in brain tryptophan metabolism could be related to prematurity or fetal distress.

Infants and methods

One hundred and twelve newborns from the University of Granada Hospital were studied. Their parents were fully informed and gave their authorization for inclusion of their infants in the study, as did the hospital’s Ethics Committee, in accordance with the Helsinki Declaration of 1975, as revised in 1983. A history was prepared and a complete clinical examination carried out for all the babies involved; anthropometric measurements were noted and a routine biochemical analysis was made. Depending upon their clinical diagnosis, the neonates were divided into three main groups: control, preterm and fetal distress groups. Each of these groups was then divided into two subgroups (diurnal and nocturnal) depending upon the time of birth that correspond with the time of blood sampling. If the time of sample was in the period between 0900 and 2100 h the neonate was assigned to the diurnal group, otherwise the neonate was included in the nocturnal group (samples obtained between 2100 and 0900 h).

Experimental procedure

The control group contained 42 neonates (body weight between 2500 and 4000 g) born after 37 weeks of gestation. Thus we considered it worthwhile to investigate the relationship between melatonin, the main metabolite of the methoxyindole pathway, and the metabolites of the kynurenine pathway in normal neonates. Our aim was to identify the normal association between these metabolites in neonates, and to determine whether alterations in brain tryptophan metabolism could be related to prematurity or fetal distress.

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Methods

The plasma and urine concentrations of melatonin were
determined by RIA (WHB, Bromma, Sweden). This method has been validated elsewhere for the direct measurement of melatonin in human plasma and urine (32). Pooled human plasma serially diluted with assay buffer gave displacements parallel to those of melatonin standards. The intra- and interassay coefficients of variation were 11.3% and 16.3% respectively. Recovery of melatonin, as assessed by the standard addition method, was 84.4% and sensitivity was 5 ng/l.

Standard reagents (1-kynurenine, 3-hydroxykynurenine, kynurenic acid, xanturenic acid and 3-hydroxyantranilic acid) of the highest purity available were bought from Sigma (Madrid, Spain) for determinations of kynurenine metabolites. These metabolites were determined by thin-layer chromatography (TLC) described elsewhere (33, 34), using 60 F254 silica gel plates (Merck, Madrid, Spain). Briefly, 20 μl standard mixture containing 4 μg each standard or 100 μl urine were applied to the chromatographic plate and quickly dried to avoid diffusion of the sample. The plate was then developed with the eluent (butanol : formic acid : distilled water, 40 : 5 : 55) without previous saturation. After a development time of 4 h, the solvent front reached 10 cm and the chromatography was stopped. The chromatogram was then dried in hot air and the spots were exposed by ultraviolet light (360 nm) to allow reproducible results. The spots were identified by their respective Rf values. Peak in a photomatic Uvatom 75 (Atom, Madrid, Spain). The spots were exposed by ultraviolet light (360 nm) and the spots were exposed by ultraviolet light (360 nm) and the chromatogram was then dried in hot air to avoid diffusion of the sample. The plate was then developed with the eluent (butanol : formic acid : distilled water, 40 : 5 : 55) without previous saturation. After a development time of 4 h, the solvent front reached 10 cm and the chromatography was stopped. The chromatogram was then dried in hot air and the spots were exposed by ultraviolet light (360 nm) to allow reproducible results. The spots were identified by their respective Rf values. Peak areas for appropriate standards covering the dynamic range, spotted in duplicate on each TLC plate, were used to construct a standard curve. Peak areas calculated for samples of urine were converted into the quantity of each kynurenine metabolite via the corresponding standard curve. Peak areas were always calculated during the night (P < 0.01), whereas the urinary excretion of 1-kynurenine (P < 0.001) and kynurenic acid, xanturenic acid and 3-hydroxyantranilic acid resulted in correlation coefficients greater than 0.99.

The chromatographic method was highly sensitive, even when there were marked concentration variations among the five metabolites studied. The TLC method was subject to quality control (34). Within-run (within-plate) analytical variation for the kynurenine standards of eight replicates at every concentration of each metabolite was determined, and the coefficients of variation thus obtained were between 6.5% and 8.5%. In precision studies on the urine samples from a control group (eight replicates in each case) the coefficients of variation were similar to those obtained for the within-run analytical variation of the standards (6.7% to 8.9%). The limit of detection – that is, the minimum amount of a compound that must be present in the chromatogram to allow reproducible results (sensitivity) – was 0.3 μg for xanturenic acid and 1.0 μg for 1-kynurenine, 3-hydroxykynurenine, kynurenic acid and 3-hydroxyantranilic acid. The values obtained for each compound (in μg/100 ml urine) were multiplied by 10 and divided by the child’s weight (in kg). Thus the results were expressed in μg/ml·kg.

**Statistical analysis**

All results are expressed as means ± S.E.M. Plasma melatonin is expressed in ng/l. Data were analyzed by two-way analysis of variance, correlation and regression techniques, and Fisher’s transformation.

**Results**

Table 1 shows the results obtained in the control and experimental groups. Melatonin concentration in both the umbilical artery and vein increased significantly during the night (P < 0.001), whereas the urinary excretion of 1-kynurenine (P < 0.001) and kynurenic acid, xanturenic acid and 3-hydroxyantranilic acid.

Table 1: Diurnal and nocturnal production of melatonin and kynurenine metabolites in normal and preterm neonates and in neonates suffering from fetal distress. Data are expressed as means ± S.E.M.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Control group (Diurnal)</th>
<th>Control group (Nocturnal)</th>
<th>Preterm group (Diurnal)</th>
<th>Preterm group (Nocturnal)</th>
<th>Fetal distress group (Diurnal)</th>
<th>Fetal distress group (Nocturnal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methoxyindoles (ng/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>aMT-A</td>
<td>26.1 ± 2.9</td>
<td>75.1 ± 2.9***</td>
<td>32.8 ± 4.9</td>
<td>75.8 ± 8.2***</td>
<td>54.4 ± 3.5</td>
<td>100.9 ± 6.9***</td>
</tr>
<tr>
<td>aMT-V</td>
<td>35.6 ± 5.5</td>
<td>84.3 ± 7.3*</td>
<td>34.4 ± 2.5</td>
<td>65.1 ± 9.1</td>
<td>40.8 ± 3.4</td>
<td>97.9 ± 11.6*</td>
</tr>
<tr>
<td>Kynurenines (μg/ml·kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KYN</td>
<td>24.6 ± 1.8</td>
<td>5.7 ± 0.5***</td>
<td>34.3 ± 6.2</td>
<td>20.7 ± 2.4**</td>
<td>7.3 ± 1.1</td>
<td>11.2 ± 7.5</td>
</tr>
<tr>
<td>3HK</td>
<td>84.9 ± 27.9</td>
<td>40.1 ± 4.8</td>
<td>86.4 ± 25.4</td>
<td>69.6 ± 6.3</td>
<td>85.5 ± 21.1</td>
<td>54.9 ± 6.7</td>
</tr>
<tr>
<td>KYNA</td>
<td>42.2 ± 4.6</td>
<td>26.4 ± 3.2**</td>
<td>63.0 ± 17.5</td>
<td>27.2 ± 4.2</td>
<td>31.3 ± 3.9</td>
<td>46.3 ± 5.01*</td>
</tr>
<tr>
<td>XA</td>
<td>36.8 ± 6.6</td>
<td>14.9 ± 1.4**</td>
<td>36.7 ± 10.1</td>
<td>28.8 ± 5.9</td>
<td>57.2 ± 5.1</td>
<td>20.4 ± 4.8**</td>
</tr>
<tr>
<td>3HANA</td>
<td>6.41 ± 0.5</td>
<td>3.6 ± 0.3**</td>
<td>10.8 ± 3.1</td>
<td>38.9 ± 8.2*</td>
<td>11.1 ± 0.4</td>
<td>4.1 ± 0.4***</td>
</tr>
</tbody>
</table>

aMT-A, umbilical artery melatonin; aMT-V, umbilical vein melatonin; KYN, 1-kynurenine; 3HK, 3-hydroxykynurenine; KYNA, kynurenic acid; XA, xanturenic acid; 3HANA, 3-hydroxyantranilic acid.

* P < 0.05, ** P < 0.01, ***P < 0.001 compared with the respective diurnal groups.
(P < 0.01) decreased significantly. These results indicate a nocturnal profile for the production of methoxyindole (melatonin) and a diurnal profile for production of kynurenine in normal neonates.

Preterm neonates showed a similar pattern of production of melatonin in umbilical artery (P < 0.001). The significant day/night differences in melatonin concentration were lost in the umbilical vein, mainly because of a decrease in its concentration at night (Table 1). In this group, the diurnal production of kynurenines remained similar to that in the control group, whereas the nocturnal production increased, although only l-kynurenine (P < 0.01) and 3-hydroxykynurenine (P < 0.01) did so significantly. Thus a shift in the day/night pattern of production of kynurenines seemed to occur in preterm neonates compared with normal ones.

Neonates suffering from fetal distress showed a significant day/night difference in melatonin production in both the umbilical artery and vein (Table 1). Moreover, there was a decrease in the diurnal production of l-kynurenine, leading to loss of the significant day/night differences compared with control, whereas such significant differences were found for kynurenic acid (P < 0.05), xanturenic acid (P < 0.001) and 3-hydroxykynurenine (P < 0.001). It is interesting to note the significant increase in the nocturnal production of kynurenic acid, shifting the normal day/night differences.

The percentage alterations in the presence of these compounds in the preterm and fetal distress groups were compared with those in the control group for both the diurnal (Fig. 1) and the nocturnal (Fig. 2) subgroups. A significant increase in diurnal concentrations of melatonin was seen in the umbilical artery of neonates suffering from fetal distress (208.4 ± 13.4%, P < 0.05), but not in preterm neonates (125.6 ± 18.7%) (Fig. 1). Changes that were not significant were found in the diurnal production of melatonin in the umbilical vein (114.6 ± 9.5% and 96.6 ± 7.1%, fetal distress and preterm groups respectively). Figure 1 also shows a significant decrease in l-kynurenine (29.6 ± 4.4%, P < 0.001) and an increase in xanthurenic acid and 3-hydroxykynurenine (155.4 ± 13.8%, P < 0.05 and 173.1 ± 6.2%, P < 0.05 respectively) in the fetal distress group, whereas in the preterm group only l-kynurenine increased significantly (139.4 ± 25.2%, P < 0.05). Other tryptophan metabolites remained unchanged (3-hydroxykynurenine, 100.7 ± 24.8% and 101.7% ± 29.9%; kynurenic acid, 74.2 ± 9.2% and 149.3 ± 41.5%, for the fetal distress and preterm diurnal groups respectively). Comparisons between groups showed that changes in concentrations of l-kynurenine (P < 0.001) and xanthurenic acid (P < 0.05) in the preterm group were significantly different from those in the fetal distress group. During the night (Fig. 2) the fetal distress group showed an increase in melatonin concentrations in the umbilical artery (134.3 ± 9.2%, P < 0.001), but not in the umbilical vein (116.1 ± 13.7%). No significant changes were observed in nocturnal melatonin concentrations in the preterm group (100.9 ± 10.9% and 77.2 ± 10.8%, umbilical artery and vein respectively). During the night, there also was a significant increase in l-kynurenine (363.2 ± 42.1%, P < 0.001), xanthurenic acid (193.3 ± 39.5%, P < 0.05) and 3-hydroxykynurenine (1080.5 ± 227.7%, P < 0.05) in the preterm group, but no significant change in the fetal distress group (l-kynurenine, 196.5 ± 131.5%; 3-hydroxykynurenine, 136.9 ± 16.7%; kynurenic acid, 175.3 ± 18.9%; xanthurenic acid, 136.9 ± 32.2%;
3-hydroxyantranilic acid, 113.8 ± 11.1%). Comparisons between groups showed that the changes in melatonin (P < 0.001), L-kynurenine (P < 0.001) and 3-hydroxyantranilic acid (P < 0.05) in the preterm group were significantly different from those in the fetal distress group.

Table 2 shows the correlation coefficients and their levels of significance between umbilical artery and vein concentrations of melatonin and the various kynurenines measured. The results show a closer relationship between melatonin and kynurenines in normal neonates than in preterm neonates and those suffering from fetal distress.

**Discussion**

This study analyzed two brain metabolic routes of tryptophan in the body: the kynurenine pathway, in which tryptophan pyrrolase clears the pyrrolic ring of the amino acid, producing formylkynurenine, which is rapidly hydrolyzed to L-kynurenine and its metabolites (2), and the methoxyindole pathway, which produces melatonin. The action of N-acetyltransferase on 5-hydroxytryptamine is the limiting step in this synthesis. Although melatonin is produced in other brain structures such as the retina, the pineal gland is the main source of circulating melatonin and is responsible for its circadian rhythm.

Activation of either one or other metabolic pathway depends on the time of day, light intensity and substrate availability and reaches an equilibrium in normal conditions. Kynurenine metabolites show a day/night difference in normal neonates, with greater concentrations during the day, whereas melatonin increases at night. Thus an increase in exploitation of the kynurenine

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**Table 2** Correlation between tryptophan hydroxyindole (kynurenines) and methoxyindole (melatonin) pathway metabolites in the groups studied.

<table>
<thead>
<tr>
<th>Kynurenine</th>
<th>Methoxyindole</th>
<th>Control group</th>
<th>Preterm group</th>
<th>Fetal distress group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Diurnal</td>
<td>Nocturnal</td>
<td>Diurnal</td>
</tr>
<tr>
<td>KYN</td>
<td>aMT-A</td>
<td>–0.53***</td>
<td>–0.92***</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>aMT-V</td>
<td>–0.76***</td>
<td>–0.73***</td>
<td>–0.08</td>
</tr>
<tr>
<td>3HK</td>
<td>aMT-A</td>
<td>–0.65***</td>
<td>–0.66***</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>aMT-V</td>
<td>–0.78***</td>
<td>–0.92***</td>
<td>0.04</td>
</tr>
<tr>
<td>KYNA</td>
<td>aMT-A</td>
<td>–0.38**</td>
<td>0.28</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>aMT-V</td>
<td>–0.47***</td>
<td>0.35*</td>
<td>0.18</td>
</tr>
<tr>
<td>XA</td>
<td>aMT-A</td>
<td>–0.03</td>
<td>–0.73***</td>
<td>0.90*** 0.10</td>
</tr>
<tr>
<td></td>
<td>aMT-V</td>
<td>–0.04</td>
<td>–0.90***</td>
<td>0.92*** 0.06</td>
</tr>
<tr>
<td>3HANA</td>
<td>aMT-A</td>
<td>–0.35*</td>
<td>0.04</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>aMT-V</td>
<td>–0.55***</td>
<td>–0.05</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Abbreviations as in Table 1.

* P < 0.05, ** P < 0.01, *** P < 0.001; significant correlation level between kynurenines and methoxyindole pathway metabolites.
pathway during the day and of the methoxyindole pathway during the night reflects the normal conditions at equilibrium. This equilibrium is lost under conditions of stress (5, 6, 34, 36), and the degree of deviation depends on both the type of stress experienced and the patient. Two types of neonatal stress, prematurity and fetal distress, were studied here.

Prematurity and redox status (hypoxia–acidosis) are the main factors influencing activation/inactivation of the enzymes involved in tryptophan metabolism. Pyridoxal phosphate is the most important coenzyme involved in the tryptophan metabolism and kynurenase (the enzyme that catalyzes the transformation of 3-hydroxykynurenine to 3-hydroxyxanthilinic acid) is the enzyme most sensitive to alterations in this coenzyme (37). The decrease in pyridoxal concentrations partially depends on its deficiency during the last 3 months of pregnancy (38). Thus premature neonates may accumulate t-kynurenine, a finding further supported by the increase in nocturnal concentrations of t-kynurenine in the preterm neonate group.

Pyridoxal phosphate also affects the pineal production of melatonin in children (39), and the low levels of the coenzyme during prematurity may explain, at least partially, the reduction in melatonin concentration seen in the umbilical vein of preterm neonates. Melatonin shows a circadian rhythm in both umbilical artery and umbilical vein in normal neonates. This rhythm depends on the maternal melatonin crossing the placenta, as these day/night differences in melatonin concentration disappeared 72 h after birth (23). Nevertheless, the pineal of the neonate actively secretes melatonin and responds to physiological stimuli such as darkness (25). Therefore, the decrease in melatonin concentrations seen in the umbilical vein in preterm neonates may also reflect a decrease in maternal melatonin that crosses the placenta, a lack of pineal maturation, or both.

Alterations in tryptophan metabolism in children with neonatal pathology including fetal distress have been reported (40, 41). These patients also show a deficit in cellular oxidation-reduction and riboflavin systems (1), which may lead to an accumulation of 3-hydroxyxanthilinic acid precursor metabolites, resulting in the formation of kynurenic acid and xanturenic acid. These compounds act as endogenous anticonvulsants, counteracting the proconvulsant effect of l-kynurenine (42). Therefore, the decrease in l-kynurenine, with an increase in kynurenic acid, xanturenic acid and 3-hydroxyxanthilinic acid, in the group of patients with fetal distress could be interpreted as an endogenous compensatory mechanism. The increase in umbilical vein concentration of melatonin in this group of neonates may contribute to this compensatory mechanism, as a result of the antistress and anticonvulsant properties of the indoleamine (7).

It therefore seems that, in normal conditions, brain tryptophan, controlled by the pineal gland as a function of environmental light and by several enzymatic gates, has distinct functions depending on the time of day. At night, tryptophan degradation would occur via the methoxyindoles, thus explaining increased nocturnal melatonin concentrations whereas, during the day, because production of melatonin would not be necessary, degradation of the amino acid precursor would take place via the kynurenine pathway. The tryptophan metabolic disorders studied here and those reported elsewhere (34) suggest that the changes in the tryptophan metabolic pathways may be attributable to a so-called 'enzymatic gate' mechanism, in which the substrate is used according to the factors regulating it. Under situations of acute stress such as fetal distress or convulsions, the melatonin pathway would predominate, because of its known antistress and neuroprotective effect (43). Once the crisis is overcome, the pathways would readjust and the initial situation be restored (34).

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