Original Article

The fetal gastrointestinal tract is exposed to melatonin and superoxide dismutase rich amniotic fluid throughout prenatal development

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Amniotic fluid (AF) is the first fluid to enter the gastrointestinal tract. Preterm birth is leading to a sudden interruption of AF swallowing. Understanding the composition of amniotic fluid is crucial to implement strategies preventing intestinal injury in preterm infants. We hypothesized that the fetal gastrointestinal tract (GIT) is exposed to melatonin and antioxidant enzymes via amniotic fluid throughout prenatal development. Amniotic fluid samples from 76 pregnant women with a median (range) gestational age of 38.0 (14.3–40.1) weeks have been collected. Immediately after birth blood samples were collected from the umbilical vein ($n = 53$). Median (Interquartile range) melatonin concentration was 30.5 pg/ml (12.7–118.3) and superoxide dismutase 1 (SOD1) concentration was 84 ng/ml (59–123). Extracellular glutathione peroxidase concentration was either not detectable or exceptionally low. We found a positive correlation between melatonin concentration in amniotic fluid and gestational age (Spearman's correlation coefficient, $r = 0.570$, $p<0.001$), while SOD1 concentration in amniotic fluid was inversely correlated with gestational age ($r = –0.246$, $p = 0.032$). Compared to serum samples, melatonin concentration was statistically significantly higher in amniotic fluid ($p<0.001$). Our results indicate that the fetal gastrointestinal system is continuously exposed to melatonin and SOD1 via the amniotic fluid throughout prenatal development.

***Key Words*:** **melatonin, superoxide dismutase, amniotic fluid, fetal nutrition, intestine**

Amniotic fluid (AF) is a dynamic and complex mixture that changes with the progression of pregnancy and reflects the physiological status of the developing fetus. In contrast to the common belief that human milk is our first food, AF is the first fluid to enter the gastrointestinal tract (GIT). The embryo starts swallowing AF already around the tenth week of gestation reaching maximum values of approximately 800 ml/day at the end of gestation.[1–3] It is important to note that AF contains not only nutrients, but also many immunomodulatory peptides such as interleukin (IL)-10 and bioactive components such as growth factors including epidermal growth factor (EGF), insulin-like growth factors (IGF)-I and transforming growth factor (TGF). This indicates that AF plays a vital role in inflammatory response and development of the GIT *in utero*.[4,5] Preterm birth is leading to a sudden interruption of AF swallowing. This deficiency of AF consumption will be replaced by human milk. But mothers of preterm infants often experience insufficient milk production in the first postnatal days, resulting in mixed feeding of human milk and formula. It has been well-established that AF and human milk, but not formula, show many similarities in terms of presence of growth and immune-modulatory factors.[6]

The preterm gut is very sensitive to enteral feeding which may either promote gut adaptation and health or induce gut injury, bacterial overgrowth and inflammation resulting in necrotizing enterocolitis (NEC), an important cause of morbidity and mortality in preterm newborn infants. The pathophysiology of NEC is not completely understood. Its causes are believed to be multifactorial and affected by three primary factors: intestinal immaturity, microbial dysbiosis, and formula feeding.[7] Human milk has repeatedly been shown to prevent intestinal injury in preterm infants, while bovine milk–based infant formulas or fortifier have been suspected as a possible risk factor in the onset of NEC and systemic infections in very low-birth-weight infants.[8,9] Therefore, recent research efforts have focused on developing new feeding strategies to reduce the risk and severity of intestinal injury.[10,11]

The similarities between human milk and AF lead to the hypothesis that AF may exert protective effects against the development of NEC, as does human milk.[6] This drove subsequent studies aimed at demonstrating the NEC-protective mechanisms afforded by AF and human milk. For this purpose, previous studies mainly focused on growth factors in AF and human milk. To clarify the role of growth factors in AF and human milk on gastrointestinal adaptation, Hirai *et al.*,[4] applied AF, human milk and various growth factors contained in these fluids to human fetal small intestinal cell lines. Because the trophic effect of each recombinant growth factor or the synergistic effect of these growth factors were much lower than the effects of AF or of human milk application, the authors argued that unknown additional factors may contribute to growth-promoting activity in these fluids.

Melatonin is a small lipophilic neurohormone that is mainly generated in the pineal gland.[12] In a variety of important physiological functions melatonin plays a key role as immuno-

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modulator, direct free radical scavenger, indirect antioxidant, and cytoprotective agent.[13] Moreover, melatonin plays an important part in gastrointestinal physiology, including regulation of gastrointestinal motility, modulation of several molecular pathways in inflammation and cellular injury, control of intestinal epithelium proliferation and migration, as well as inter-organ communication, e.g., between gut and liver.[14–16] Superoxide dismutase (SOD) and glutathione peroxidase (GPx) are key antioxidant enzymes against oxidative stress. The role of oxidative stress in the development of NEC is also known. Understanding the antioxidant composition of AF and human milk is crucial for implementing strategies to prevent NEC in formula-fed preterm infants. We have previously demonstrated the presence of melatonin, SOD and GPx with a diurnal rhythm in human milk.[17] Although the presence of melatonin and SOD1 has been previously shown in AF sample obtained in late pregnancy, there is no data about their concentration in AF during progression of gestation.[18,19] We hypothesized that the GIT of the fetus is exposed to melatonin and the antioxidant enzymes SOD and extracellular GPX (EC-GPx) via the AF throughout prenatal development. The aim of this study was to analyze the concentration of melatonin, SOD1 and EC-GPx and to determine whether their concentrations change during progression of gestation, and according to the time of day.

Materials and Methods

Subjects. Eighty-seven women were included in the study. Pregnant women with polyhydramnios and amnion infection syndrome were excluded because both factors may affect the results of our analysis.

AF was obtained from 15 pregnant women undergoing amniocentesis and from 72 pregnant women undergoing caesarean delivery under general anesthesia. AF samples were obtained by needle aspiration that was visually free of blood contamination. Eleven samples were excluded from the analysis due to sample amount or quality (blood contamination). The AF samples were centrifuged at 3,000 × *g* for 5 min, aliquoted and stored at −80°C until analysis. One minute after umbilical cord clamping, we carefully aspirated 2–5 ml fetal blood in non-heparin-containing syringes from the umbilical vein. We obtained 57 umbilical cord samples (14 samples from preterm babies and 43 from term babies). One minute after umbilical cord clamping and using a clamped segment of the umbilical cord, we carefully aspirated 2–5 ml fetal blood in non-heparin-containing syringes from the umbilical vein. Blood samples were centrifuged at 3,000 × *g* for 5 min. The plasma was pipetted and frozen at −80°C until analysis.

Ethics approval and consent to participate. In accordance with the Declaration of Helsinki, the study was approved by the Ethics Committee of the University of Bonn (REC number 077/13). We obtained written informed consent from all participants.

Melatonin assays. Plasma melatonin concentration was measured using a commercial radioimmunoassay kit (melatonin direct RIA, RE29301; IBL-International, Hamburg, Germany). According to the manufacturer`s protocol, the intra- and inter-assay coefficients of variation were 3.9–6.9% at a range of 28.8 to 266 pg/ml and 6.2–15.9% at a range of 3.5 to 281 pg/ml. The mean recovery of melatonin was 102% and the sensitivity of the assay was 0.9 pg/ml.

Measurement of melatonin concentration in AF was performed using commercial enzyme linked immunoassay (ELISA) kits (Melatonin ELISA, RE54021; IBL-International) at the laboratory of IBL-International in Hamburg, Germany (see Bagci *et al.*[20]). Standards and all AF samples were incubated in duplicate in wells of microtiter plates. According to the manufacturer's protocol, the intra- and inter-assay coefficients of variation for serum were 3.0–11.4% at the range of 8.8–151.7 pg/ml and 6.4–19.3% at the range of 5.6–134.3 pg/ml, respectively. The mean recovery for measurement of melatonin concentration in AF assayed by extraction ELISA was determined as 96.8% [coefficient of variation (CV) = 15.6%], with a range between 78.2 and 116.8%. The mean recovery of melatonin was 102.4% and the sensitivity of the assay was 1.6 pg/ml.

Measurement of SOD1. Presence of SOD1 (EC1.15.1.1) has been previously shown in amniotic fluid from Down syndrome affected pregnancies.[21] SOD1 concentration in plasma and AF was measured using a commercially available ELISA Kit [Cu/Zn SOD ELISA, Superoxide Dismutase (SOD) (E.C.1.15.1.1.), 30150476; IBL-International] according to the manufacturer's instructions (sensitivity: 0.04 ng/ml, intra-assay precision: 5.1%, inter-assay precision: 5.8%).

Measurement of EC-GPx. EC-GPx (GPx3) is an isoform of the GPx family which is secreted into plasma, lung and milk.[22] EC-GPx concentration in AF was assayed using a commercially available ELISA Kit (ALPCO Diagnostics, Salem, NH). The intra- and inter-assay coefficients of variation for Gpx3 were 4.21–9.64% and 1.12–5.04%, respectively.

Statistical analyses. The statistical analyses were performed using SPSS 22.0 (SPSS Inc., Chicago, IL) as well as R (ver. 4.0.0) and R Studio (ver. 1.2.1335) software. All data are presented as median [interquartile range (IQR)]. The variables were tested for normality using the Kolmogorov–Smirnov and the Shapiro–Wilk tests. To analyze the difference between preterm and term pregnancy, the samples were divided into two groups based on gestational age (GA): Group 1 included samples obtained <37 weeks of gestation and group 2 included samples obtained ≥37 weeks of gestation. GA was calculated from the last menstrual period and by ultrasonographic fetal biometry. Because the melatonin, SOD1- and Gpx3 concentrations were not normally distributed, we used the two-tailed non-parametric Mann–Whitney *U* test for the group comparison. Spearman's rank correlation coefficients test was used to analyze associations among GA, time of obtaining the sample, melatonin and SOD1 concentration in AF and serum. For all analyses, p values of less than 0.05 were considered statistically significant.

Results

AF samples from 76 pregnant women, with a median age of 34 years (IQR: 30–37) and a median gestational age of 38.0 weeks (32.9–38.9) were analyzed. Melatonin concentration was 30.5 pg/ml (12.7–118.3) and SOD1 concentration was 84 ng/ml (59–123). The samples were divided into two categories according to GA: Group 1 ($n = 27$), samples obtained <37 weeks of gestation and group 2 ($n = 49$), samples obtained ≥37 weeks of gestation. The results are shown in Table 1. In 43% of the AF samples ($n = 30$) the EC-GPx concentration was not detectable. The EC-GPx concentration in the other AF samples was exceptionally low [43.5 ng/ml (16.1–118.6)].

We found a positive correlation between melatonin concentration in AF and GA (Spearman's correlation coefficient, $r = 0.570$, $p<0.001$), while SOD1 concentration in AF was inversely correlated with GA ($r = -0.246$, $p = 0.032$).

To evaluate whether the time of day of sampling affects the melatonin and SOD1 concentration in AF, we compared their concentrations in samples collected in the morning and the afternoon, both before and after 37 weeks of gestation (Table 2). We first normalize MT and SOD1 through a log-transformation in order to apply parametric methods and then estimate separately two-way-ANOVAs with GA and time as factors. The results confirm the significance of GA for melatonin ($p<0.001$), and, weakly, for SOD1 ($p<0.10$). In

Table 1. Gestational age, melatonin, and SOD concentrations in amniotic fluid in groups 1 and 2

	All patients	Group 1 (n = 27)	Group 2 (n = 49)	p value*
Age, year	34 (30–37)	32 (29–35)	34 (31–38)	0.027
Gestational age, weeks	38.0 (32.9–38.9)	26.0 (19.0–34.1)	38.6 (38.0–39.0)	<0.001
Melatonin (pg/ml)	30.5 (12.7–118.3)	10.6 (6.1–24.3)	91.6 (27.4–172.4)	<0.001
SOD1 (ng/ml)	84 (59–123)	105 (77–144)	74 (53–93)	0.003

The data are expressed as the median (IQR). SOD1; superoxide dismutase 1. Group 1 included samples obtained <37 weeks of gestation and group 2 included samples obtained ≥37 weeks of gestation. *p value present group 1 vs group 2.

Table 2. Melatonin and SOD1 concentrations in amniotic fluid at different sampling times

		Time		p value*
		0800–1159	1200–1759	
Melatonin (pg/ml)	Group 1	14.9 (8.7–25.1)	10.1 (5.9–22.3)	0.792
	Group 2	93.4 (32.8–230.7)	84.9 (19.6–132.7)	
SOD1 (ng/ml)	Group 1	105 (72–194)	96 (80–140)	0.841
	Group 2	67 (50–93)	75 (63–90)	

*Two-way-ANOVAs was used to consider main and interactive effects of gestational age and time as factors. The data are expressed as the median (IQR). SOD1; superoxide dismutase 1. Group 1 included samples obtained <37 weeks of gestation and group 2 included samples obtained ≥37 weeks of gestation.

Table 3. Melatonin and SOD1 concentrations in serum and amniotic fluid from 57 patient

	Serum (n = 57)	Amniotic fluid (n = 57)	p value
Melatonin (pg/ml)	11.2 (6.1–20.7)	58.4 (14.7–135.5)	<0.001
SOD1 (ng/ml)	88 (69–131)	77 (51–94)	0.090

The data are expressed as the median (IQR). SOD1; superoxide dismutase 1.

contrast, we detect no significant difference regarding the melatonin (p = 0.792) and SOD1 concentration (p = 0.841) in AF between samples obtained in the morning and in the afternoon. Neither are the interaction effects between the two factors significant.

Compared to serum samples (n = 57), melatonin concentration was statistically significantly higher in AF, while SOD1 concentration was not statistically significantly different between serum and AF (Table 3). Neither melatonin nor SOD1 concentration in AF were significantly correlated with their concentration in serum (p = 0.810 and p = 0.799, respectively).

Discussion

As understanding the composition of AF is crucial for implementing strategies to prevent intestinal injury in formula-fed preterm infants, we purposed to evaluate concentrations of melatonin, SOD1 and EC-GPx in AF during the fetal period. Our study demonstrates for the first time that the fetal GIT is continuously exposed to melatonin and SOD1 rich AF throughout prenatal development. Melatonin concentration was significantly higher in AF than in the umbilical vein. Neither melatonin nor SOD1 concentration in AF were correlated with their concentrations in the umbilical cord. In contrast to human milk, EC-GPx concentration was found to be very low or below the limit of detection in many samples of AF.[17]

To date, melatonin concentration in AF was analyzed in two studies including only AF samples above 35 weeks of gestation.[18,19] In AF samples collected during spontaneous vaginal deliveries above 37 weeks of gestation, the melatonin concentration was found to be higher than those in our study (mean ± SEM 262 ± 22 and 190 ± 15 pg/ml, during labor at term and induction of labor at term, respectively).[18] In contrast to that study, we collected our samples only from pregnant women undergoing

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elective cesarean delivery or amniocentesis without labor. We have previously demonstrated that melatonin concentration in pregnant women depends on both mode and time of labor.[23] As AF samples in the first trimester of pregnancy could be collected only during amniocentesis during daytime, we collected all samples only at daytime. This enables a comparison between the two groups. Therefore, our results present only daytime melatonin concentration in AF (same as the results from Kiveala *et al.*[19]). According to data from these three studies it is important to point out that daytime melatonin concentration in AF at term is 50–70-fold higher than its daytime concentration in human milk.[17,24] Moreover, daytime AF samples below 37 weeks of gestation contain significantly more melatonin than daytime as well as nighttime preterm and term human milk.[17,24]

Although the presence of SOD1 and GPx in AF has been previously demonstrated, there was no comparable data on the concentration of SOD1 and EC-GPx considering GA.[25] In our analysis concentration of EC-GPx was found to be very low or below the limit of detection, which was also reported by Mihaliovic *et al.*[26] Although both preterm and term human milk contained enough EC-GPx [1,591 ng/ml (1,174–2,208) and 1,505 ng/ml (799–2,269), respectively], EC-GPx concentration in AF was found 30–40 times lower, if at all detectable, in some samples.[17]

The main source of melatonin, SOD1 and EC-GPx in AF remains unclear. AF is a mixture of mainly fetal urine, the transudate of maternal plasma through the uterine decidua and fluids secreted from the lower fetal respiratory and alimentary tract, with dynamic changes in its composition.[27] Melatonin displays high lipid and water solubility and gains access to various fluids, tissues, and cellular compartments. Hence it is detected in the placenta, saliva, urine, and milk.[17,28,29] In an examination of endogenous nocturnal melatonin secretion in adolescents, an average amount of some 30 ng unmetabolized melatonin was excreted with the urine.[29] This roughly amounts to 1/1,000 of the 30 μg melatonin produced per night according to pharmacokinetic calculations.[30] It is also clearly established that melatonin is rapidly transferred from the maternal to the fetal circulation in humans which enables transmission of photoperiodic information to the fetus.[31] Because maternal melatonin levels increase throughout pregnancy, reaching maximum levels at term, this may also explain the positive correlation between GA and melatonin levels in AF.[32] However, the lack of a correlation between serum and AF melatonin concentration suggested that maternal melatonin status does not reflect its status in AF. Because melatonin is mainly metabolized in the liver, it is most likely that melatonin accumulates in AF, resulting in explicitly high melatonin concentration in AF compared to fetal circulation. Thus, the intestine is exposed to continuous high melatonin concentrations. Its effect on the intestinal development needs to be further investigated.

Like melatonin, presence of SOD1 and EC-GPx in urine has been previously demonstrated.[33,34] It is known that kidney proximal tubular cells are the main source of GPx and EC-GPx activity in plasma.[35] Decreased plasma, glomerular and urinary GPx levels are reported in patients with renal disorders, indicating an impaired synthesis of GPx and EC-GPx in the damaged kidneys.[34,36] Because there have been no previous reports on renal synthesis of GPx in fetal life, we speculate that very low GPx levels in AF may be due to impaired synthesis of extracellular GPx in the immature kidneys of the fetus.

Kidneys are also known to have all SOD types, with the highest concentration for extracellular SOD (SOD3).[37] Marklund *et al.*[38] measured higher SOD level in patients with renal impairment and found a positive correlation between serum creatinine and serum SOD levels, which assume that SOD is eliminated by the kidneys. Inayat *et al.*[39] found no differences between preterm and term urinary SOD, which suggests that also early in pregnancy urine could contain relevant SOD levels. Moreover, it is known that placental cells secrete all three forms of SOD.[40] Therefore, placenta may be also one of main sources of SOD1 in AF.

One limitation of our study is that our results present only daytime melatonin concentration and SOD1 concentration. Furthermore, we were restricted because AF samples in the first trimester of pregnancy could only be collected during amniocentesis during daytime. However, this enables a comparison between the two groups because all samples were collected during daytime.

In conclusion, our results provide first evidence that the fetal gastrointestinal system is continuously exposed to melatonin and SOD rich AF throughout its prenatal development, as by postnatal feeding of preterm infants with human milk. Any imbalance in the relative levels of these components during development process of preterm gastrointestinal tract may have deleterious effects on the structure and function of preterm GIT. These results broad our knowledge about fetal nutrition and support the importance of exclusive breastfeeding for postnatal development process of preterm gastrointestinal tract.

Author Contributions

All authors have read and complied with the authorship criteria based on ICMJE, and they have made a significant contribution to the manuscript and accept the responsibility for the study protocol and the presented results. SB, DK, PB, and AM designed research; SB, ÖA, and CB conducted research; SB, DK, LR, and EAA analyzed data; SB and DK wrote the paper; SB, PB, LR, and AM critically reviewed and revised the manuscript and approved the final manuscript as submitted. All authors read and approved the final manuscript.

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Availability of Data and Material

The data that support the findings of this study are available on request from the corresponding author, Dr. Soyhan Bagci.

Abbreviations

AF	amniotic fluid
CV	coefficient of variation
SOD1	superoxide dismutase 1
EC-GPx	extracellular glutathione peroxidase
ELISA	enzyme linked immunoassay
GA	gestational age
GIT	gastrointestinal tract
NEC	necrotizing enterocolitis
RIA	radioimmunoassay

Conflict of Interest

All authors report no conflict of interest. The authors alone are responsible for the content and writing of the papers.

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