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Oxidative Stress in Newborn Infants with and without Asphyxia as Measured by Plasma Antioxidants and Free Fatty Acids

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A rapid perfusion of oxygen in infants at birth may cause an increase of oxidative stress. To assess this possibility, we measured levels of blood plasma antioxidants and free fatty acids in 20 normal infants at 0, 1, 3, and 5 days after birth. Plasma levels of the most reactive antioxidant, ascorbic acid, decreased daily to equilibrium values at days 3 and 5. Percentages of oxidized form of coenzyme Q-10 (%CoQ-10) in total coenzyme Q, another good marker of oxidative stress, in infants (25–31%) were significantly higher than those in healthy young adults (4.5%). Plasma levels of total free fatty acids (FFA) in normal infants were highest at day 1 and decreased rapidly thereafter. The content of polyunsaturated fatty acids (PUFA) in total FFA was lowest at day 1 and then increased. Since PUFA are susceptible to oxidation, these changes in FFA composition suggest that oxidative stress is most evident at the initial day of neonatal life. Furthermore, it appears that mono-unsaturated fatty acids such as oleic and palmitoleic acids increase in response to the oxidative loss of PUFA. Similar changes in plasma antioxidants, FFA levels, and FFA compositions were observed in 9 infants with asphyxia. Values of %CoQ-10 in infants with asphyxia were significantly greater than those in normal infants, suggesting that infants with asphyxia have elevated oxidative stress.

Key Words: **oxidative stress; newborn infants; ascorbic acid; ubiquinol; ubiquinone; polyunsaturated fatty acids; palmitoleic acid.**

Infants have less protection against oxidation. In comparison with healthy adults, lower levels of plasma antioxidants such as vitamin E, β-carotene, and sulfhydryl group (1), lower levels of plasma metal binding proteins such as caeruloplasmin and transferrin (2), and reduced activity of erythrocyte superoxide dismutase (3) are typical of newborn infants. Furthermore, infants frequently contain higher plasma levels of non-transferrin-bound iron (4) and higher erythrocyte of free iron (5) than adults. In addition, a rapid perfusion of oxygen in infants at birth may cause an increase of oxidative stress since ischemia/reoxygenation is considered as one of the major causes of oxidative stress (6).

In fact, infants have higher plasma level (130 pg/ml) of F_2-isoprostanes, free radical oxidation products of arachidonic acid, than do adults (38 pg/ml) (7). Similarly, a greater ratio of allantoin (the major end product of urate oxidation) to uric acid was observed in infant plasma (3.5%) as compared to that in adult plasma (1.2%) (8). Newborn babies have a higher percentage (13%) of dehydroascorbic acid (oxidation product of ascorbic acid) in total ascorbate than do adults (6.3%) (8). It has also been reported that plasma ratio of α-tocopheryl quinone (oxidation product of vitamin E) to α-tocopherol in infants (2.0%) is significantly greater than that of their mothers (0.6%) (9). However, these data were not evaluated over a daily course of neonatal progression.

To obtain further evidence of oxidative stress in newborn infants we measured daily change in the redox status of plasma coenzyme Q using our recently developed methods (10). It has been reported that ascorbic acid and the reduced form of coenzyme Q are the most sensitive antioxidants against oxidative stress (11–13). We also measured the plasma composition of free fatty acids as well as concentrations of the antioxidants, ascorbic acid, uric acid, and unconjugated bilirubin. We demonstrate an increase of oxidative stress in infants

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Abbreviations: HPLC, high pressure liquid chromatography; ECD, electrochemical detector; FFA, free fatty acids; %CoQ-10, percentage of oxidized form of coenzyme Q-10 in total coenzyme Q-10; PUFA, polyunsaturated fatty acids; %PUFA, percentage of PUFA in total FFA; 16:1, palmitoleic acid; 18:1, oleic acid; %16:1, percentage of 16:1 in total FFA; %18:1, percentage of 18:1 in total FFA.

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born with asphyxia reflecting a longer period of ischemia than normal delivery.

EXPERIMENTAL

Human plasma. Blood was collected with an aliquot of heparin from umbilical cord vein of infants at time of delivery and at 1, 3, and 5 days of age with the condition of parental consent. In this study samples were collected from 20 infants born normally at full term (gestational age was between 37 to 41 weeks) having body weights of 2345–3940g. 9 infants were born with asphyxia (gestational age was from 31 to 41 weeks) having lower than 5 points in their Apgar scores. Approximately 1.5 ml of blood was collected from the heel of infants. Heparinized blood was drawn from young healthy volunteers. Plasma was separated from blood by centrifugation at 1500 *g* for 10 min and was stored at −80°C until analysis.

Analytical procedures. Plasma levels of ascorbic acid, uric acid, and unconjugated bilirubin were determined by high pressure liquid chromatography (HPLC) on a bonded-phase aminopropylsilyl column (Type Supelcosil LC-NH_2, 5 μm, 250 × 4.6 mm i.d., Supelco, Tokyo) with UV detection (275 nm) as previously described (14). The mobile phase consisted of methanol/40 mM sodium monobasic phosphate (=9/1, v/v) delivered at a flow rate of 1.0 ml/min.

Plasma levels of reduced and oxidized forms of coenzyme Q, vitamin E (mixture of α- and γ-tocopherols), lycopene, β-carotene, free cholesterol, and cholesteryl esters were determined as previously reported (10). In brief, plasma (50 μl) was mixed vigorously with 250 μl of cold methanol and 500 μl of cold hexane in a 1.5 ml-polypropylene tube. After centrifugation at 10,000 *g* for 3 min at 4°C, 5 μl of the hexane layer (corresponding to 0.5 μl of plasma) was injected immediately and directly onto HPLC equipped with two guard columns (Type Supelguard LC-ABZ, 5 μm, 20 × 4.6 mm i.d., Supelco), an analytical column (Type Supelcosil LC-8, 5 μm, 250 × 4.6 mm i.d., Supelco), a reduction column (Type RC-10-1, Irica, Kyoto), and an amperometric electrochemical detector (ECD, Model Σ985, Irica). The oxidation potential for ECD was +600 mV (vs Ag/AgCl) on a glassy carbon electrode. The mobile phase consisted of 50 mM sodium perchlorate in methanol/*tert*-butyl alcohol (85/15, v/v) delivered at a flow rate of 0.8 ml/min.

Plasma free fatty acids (FFA) were analyzed by HPLC separation after derivatization with monodansylcadaverine (15). Briefly, plasma aliquots (50 μl) were mixed with 200 μl of methanol containing 12.5 μM margaric acid (internal standard) and then centrifuged at 12000 rpm for 3 min. A sample of the supernatant (50 μl) was dried under a stream of N_2 and the residue was mixed with 1 μl of diethyl phosphorocyanidate and 50 μl of *N,N*-dimethylformamide containing monodansylcadaverine (2 mg/ml). After standing at room temperature in the dark for 20 min, 5 μl was injected onto an octadecylsilyl column (3 μm, 3.3 cm × 4.6 mm i.d., Supelco) and a pKb-100 column (5 μm, 25 cm × 4.6 mm i.d., Supelco) connected in series. FFA components were measured by fluorescence detection (Model 821-FP, Japan Spectroscopic, Tokyo, with excitation selected at 320 nm and emission measured at 520 nm). The mobile phase consisted of acetonitrile/methanol/water (=17.5/65.0/17.5, v/v/v) delivered at a flow rate of 1.5 ml/min with analytical columns maintained at 40°C.

RESULTS AND DISCUSSION

Plasma antioxidants and FFA concentrations in 20 normal infants and in 9 infants with asphyxia were monitored until 5 days after birth and compared with plasma from 31 normal adults having an average age of 21.8 ± 7.5 (± S.D.) years; the results are summarized in Table 1. Figure 1 highlights the time course changes of selected parameters in the daily progression of neonatal infants. In normally born infants, plasma levels of Vitamin C (the most reactive antioxidant in plasma (11, 13)) decreased significantly after birth, consistent with previous observation (8, 16, 17), and thereafter concentrations equilibrated by day 3 (Fig. 1). Infants with asphyxia showed a similar decline in vitamin C levels yet a significant difference remained between the two groups at days 3 and 5 (Fig. 1). Uric acid levels in both groups peaked at day 1 and subsequently declined. There was, however, no significant difference observed between the two groups. Unconjugated bilirubin, another important antioxidant (18), increased daily as observed previously (16) and overall levels were much higher than those measured in the plasma from young adults. There was no difference in plasma levels of unconjugated bilirubin between normal infants and infants with asphyxia.

Vitamin E and the reduced form of coenzyme Q-10 (ubiquinol-10), are both important lipid soluble antioxidants which increased after birth with greatest increases being observed at day 1 for ubiquinol-10 and at day 3 for vitamin E. This difference in accumulation may reflect the fact that ubiquinol-10 is independently biosynthesized whereas vitamin E must be trophically acquired.

Our group and others proposed that the redox status of coenzyme Q-10 should serve well as a marker of oxidative stress (10, 19–22) since oxidative stress is defined as as a disturbance in the prooxidant-antioxidant balance in favor of the former (23) and plasma ubiquinol-10 is vulnerable to oxidation (12, 13). The percentage of the oxidized form of coenzyme Q-10 (%CoQ-10) in total coenzyme Q-10 was found to be in the range of 25–32% during the initial 5 days of life and significantly greater than values generally observed in young healthy adults (4.5%), potentially indicating a condition of oxidative stress at birth. In contrast, %CoQ-10 in infants with asphyxia was found to be significantly elevated (39–62%) than values associated with infants having a normal birth (Fig. 1). While these results indicate that oxidative stress is more evident in infants with asphyxia, it is worthy to note that total levels of coenzyme Q-10 in both groups were equivalent (Fig. 1) showing that the measurement of %CoQ-10 offers a true indication of redox status.

Plasma levels of total FFA in normal infants increased by more than 4-fold at day 1 and then decreased daily (Fig. 1). This and the fact that newborn infants have higher levels of plasma creatine kinase and lactate dehydrogenase (24) suggest an initial increase in cell lysis followed by atypical release of hydrolyzed cell membrane phospholipids. Similar and dramatic changes in FFA concentrations have been observed subsequent to ischemia-reperfusion in the brain (25). By this comparison we suggest that the

TABLE 1

Changes in Plasma Antioxidants and Free Fatty Acids in Newborn Infants without and with Asphyxia and Comparison with Normal Adults

Group	Age (days)	Vitamin C (μM)	Uric acid (μM)	Unconjugated Bilirubin (μM)	Vitamin E (μM)	$CoQH_2$-10 (μM)	CoQ-10 (μM)	Total Q-10 (μM)	%CoQ-10
Normal	0	73.4 ± 23.8	304 ± 58	13 ± 3	7.2 ± 2.3	0.10 ± 0.05	0.03 ± 0.02	0.13 ± 0.06	25.7 ± 11.0
(n = 20)	1	**49.5 ± 12.2**	**396 ± 62**	**53 ± 20**	7.7 ± 2.1	**0.21 ± 0.08**	**0.07 ± 0.04**	**0.28 ± 0.09**	25.3 ± 11.8
		<0.001	*<0.001*	*<0.001*	*0.478*	*<0.001*	*<0.001*	*<0.001*	*0.901*
	3	**35.3 ± 11.6**	**209 ± 91**	**105 ± 38**	**14.2 ± 6.5**	**0.27 ± 0.12**	**0.12 ± 0.09**	**0.39 ± 0.19**	29.1 ± 12.3
		<0.001	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*0.366*
	5	**36.1 ± 17.9**	**184 ± 68**	**127 ± 61**	**17.4 ± 7.7**	**0.24 ± 0.08**	**0.11 ± 0.04**	**0.34 ± 0.10**	31.2 ± 11.4
		<0.001	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*0.132*
Asphyxia	0	61.7 ± 54.5	278 ± 65	17* ± 4	9.2 ± 3.7	0.06* ± 0.04	0.10* ± 0.07	0.12 ± 0.01	61.1** ± 24.7
(n = 9)	1	53.3 ± 41.8	423 ± 218	**71 ± 38**	9.1 ± 2.3	**0.15* ± 0.04**	0.10 ± 0.03	**0.25 ± 0.06**	**39.0** ± 8.3**
		0.366	*0.087*	*0.002*	*0.945*	*<0.001*	*1.000*	*<0.001*	*0.029*
	3	25.1* ± 10.4	226 ± 81	**107 ± 40**	**15.4 ± 7.3**	**0.16* ± 0.07**	**0.19** ± 0.03**	**0.40 ± 0.08**	40.9* ± 14.5
		0.079	*0.151*	*<0.001*	*0.042*	*0.001*	*0.004*	*<0.001*	*0.050*
	5	20.9* ± 12.0	**151 ± 91**	**112 ± 57**	**20.4 ± 8.9**	**0.21 ± 0.08**	**0.17** ± 0.07**	**0.38 ± 0.10**	45.1** ± 14.1
		0.055	*0.004*	*0.001*	*0.005*	*<0.001*	*0.049*	*<0.001*	*0.110*
Normal adult		**41.6 ± 32.7**	**370 ± 52**	**7 ± 3**	**21.8 ± 7.5**	**0.70 ± 0.27**	0.03 ± 0.01	**0.74 ± 0.28**	**4.5 ± 1.4**
(n = 31)		*(<0.001)*	*(<0.001)*	*(<0.001)*	*(<0.001)*	*(<0.001)*	*(1.000)*	*(<0.001)*	*(<0.001)*

Group	Age (days)	Vitamin E/ Total Q-10	Total FFA (μM)	PUFA (μM)	%PUFA	18:1 (μM)	%18:1	16:1 (μM)	%16:1
Normal	0	59.4 ± 18.2	148 ± 52	33 ± 13	21.9 ± 9.0	35 ± 16	23.5 ± 10.5	6 ± 6	4.3 ± 3.8
(n = 20)	1	**28.8 ± 6.1**	**619 ± 234**	**71 ± 25**	**11.5 ± 4.0**	**170 ± 87**	27.5 ± 14.0	**65 ± 27**	**10.5 ± 4.3**
		<0.001	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*0.313*	*<0.001*	*<0.001*
	3	**39.5 ± 16.7**	**344 ± 83**	**84 ± 18**	24.6 ± 5.3	**92 ± 19**	26.8 ± 5.5	**16 ± 15**	4.5 ± 4.2
		<0.001	*<0.001*	*<0.001*	*0.265*	*<0.001*	*0.227*	*0.015*	*0.878*
	5	50.0 ± 16.5	159 ± 64	**56 ± 24**	**35.3 ± 15.0**	37 ± 16	23.3 ± 9.9	**2 ± 1**	**0.9 ± 0.9**
		0.117	0.584	<0.001	0.001	0.670	0.957	0.001	<0.001
Asphyxia	0	69.8 ± 21.1	145 ± 24	27 ± 3	18.4 ± 2.3	27 ± 5	18.8 ± 3.7	4 ± 2	2.8 ± 1.1
(n = 9)	1	**36.7** ± 5.0**	**359*** ± 83**	**60 ± 18**	16.8** ± 5.1	**89*** ± 25**	**24.8 ± 7.0**	**33*** ± 10**	**9.1 ± 2.8**
		0.001	<0.001	<0.001	0.408	<0.001	0.044	<0.001	<0.001
	3	**38.5 ± 15.9**	133*** ± 35	27*** ± 16	20.3 ± 11.9	**35*** ± 6**	**26.3 ± 4.8**	**7* ± 1**	**5.4 ± 1.1**
		0.003	*0.422*	*0.939*	*0.644*	*0.012*	*0.001*	*<0.001*	*<0.001*
	5	52.4 ± 13.7	158 ± 43	**41* ± 13**	**26.0* ± 8.1**	**43 ± 16**	**27.1 ± 9.9**	3* ± 1	1.9* ± 0.8
		0.055	*0.447*	*0.009*	*0.023*	*0.018*	*0.040*	*0.111*	*0.052*
Normal adult		**29.6 ± 27.2**	**278 ± 277**	**60 ± 55**	22.2 ± 5.6	**95 ± 118**	34.3 ± 42.6	9 ± 15	2.3 ± 1.2
(n = 31)		*(<0.001)*	*(<0.001)*	*(0.016)*	*(0.894)*	*(0.008)*	*(0.053)*	*(0.177)*	*(0.132)*

Note. Average ± S.D. are shown. Numbers in italic and bold show P values and significant differences compared to the values at birth, respectively, as determined by *t*-test. *, **, *** indicate significant differences ($P < 0.05$, 0.01, and 0.001, respectively) compared to normal infant at the same age as analyzed by *t*-test. Numbers in parenthesized italic and underlined bold show P values and significant differences compared to normal infant at birth, respectively, as determined by *t*-test. Average age of normal adult (all male) is 21.8 ± 7.5 (± S.D.). Abbreviations: $CoQH_2$-10, ubiquinol-10; CoQ-10, ubiquinone-10; total Q-10=$CoQH_2$-10+CoQ-10; %CoQ-10(%)=CoQ-10/total Q-10; FFA, free fatty acids; PUFA, polyunsaturated fatty acids; %PUFA (%) = PUFA/FFA; 18:1, oleic acid; %18:1 (%) = 18:1/FFA; 16:1, palmitoleic acid; %16:1 (%) = 16:1/FFA.

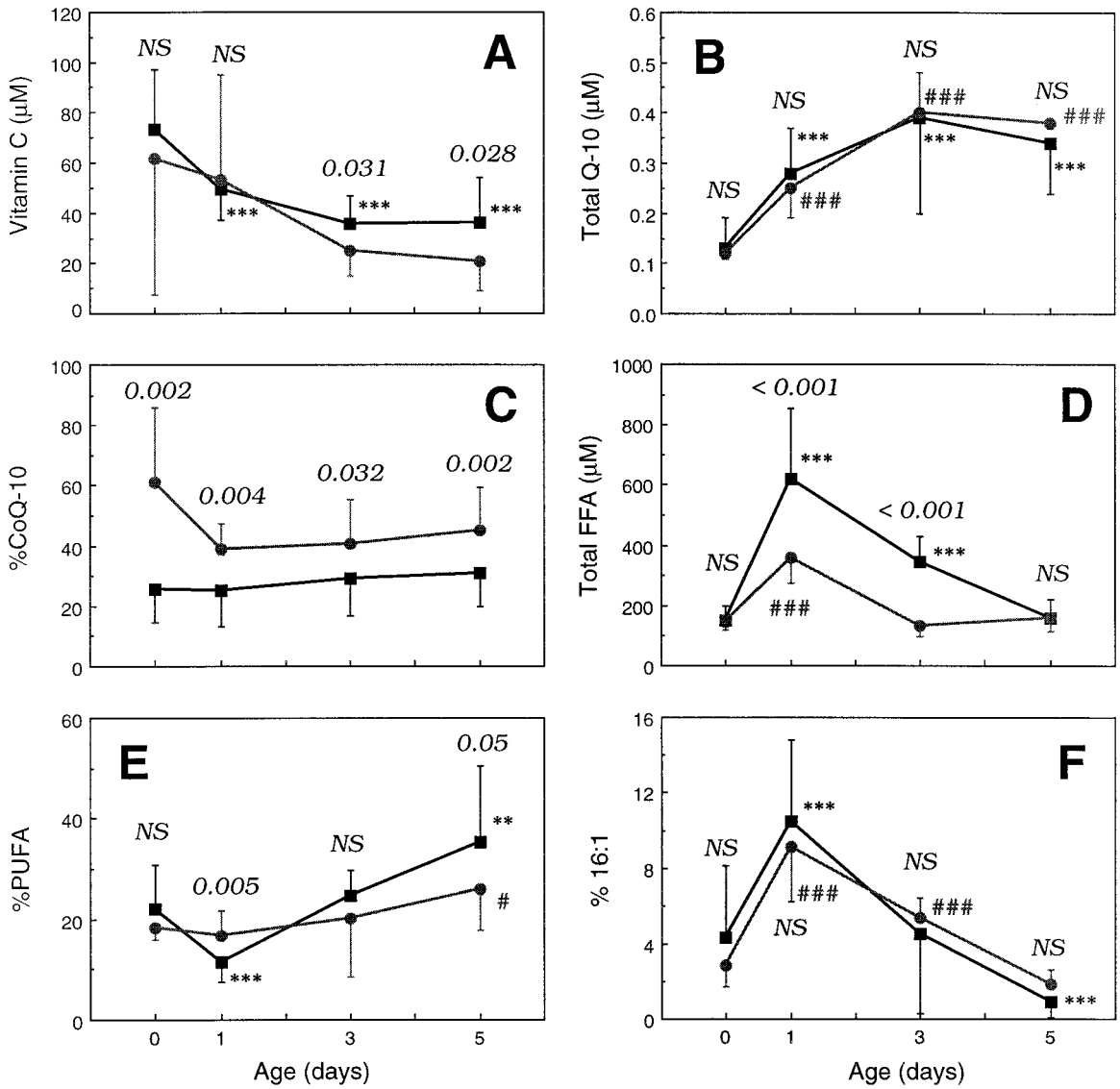

FIG. 1. Changes in plasma levels of vitamin C (**A**), total coenzyme Q-10 (total Q-10) (**B**), oxidized form of coenzyme Q (CoQ-10) percentage in total Q-10 (%CoQ-10) (**C**), total free fatty acids (FFA) (**D**), polyunsaturated fatty acids (PUFA) percentage in total FFA (%PUFA) (**E**), and palmitoleic acid (16:1) percentage in total FFA (%16:1) (**F**) in normal (■) and asphyxiated (●) infants after birth. *, **, *** indicate significant differences ($P < 0.05$, 0.01, and 0.001, respectively) compared to values of normal infants at birth; # and ### indicate significant differences ($P < 0.05$ and 0.001, respectively) compared to the values of infants with asphyxia at birth, as determined by *t*-test. Italic numbers show P values between normal infants and infants born with asphyxia at the same age, as determined by *t*-test. NS stands for not significant.

process of birth causes a similar physiological condition of ischemia-reperfusion.

It is interesting to note that the percentage of PUFA (%PUFA) in the total amount of FFA was lowest at day 1 and increased thereafter (Fig. 1). This indicates that phospholipid turnover at day 1 had the lowest of PUFA content. Since PUFA is very susceptible to oxidation, the cellular integrity should be oxidatively damaged at the initial stage of birth (0–1 day). On the other hand, levels of monounsaturated fatty acids such as oleic acid (18:1) and palmitoleic acid (16:1) were correspondingly high at day 1 and subsequently decreased as shown in Fig. 1. Production of these fatty acids seem to compensate for the initial loss of PUFA and may be the result of enhance activity in the action of Δ^9 desaturase (15,26). Changes in the percent composition of 16:1 (%16:1) in the total amount of FFA were more significant than those observed for %18:1. This may be expected since initial levels of 16:1 were much less than the initial concentrations of 18:1. We have previously hypothesized that monounsaturated fatty acid composition may also provide an indication of oxidative stress (15). Related changes in total FFA levels, %PUFA, and %16:1 were observed in infants with asphyxia (Fig. 1)

but it is not clear why such changes except %16:1 were not as pronounced as those measured in normal infants.

In summary, we demonstrated the use of the redox status of plasma coenzyme Q-10 as a method to evaluate oxidative stress in newborn infants, especially infants born with asphyxia. Our findings that plasma %PUFA values were lowest at day 1 with corresponding the highest values of %16:1 provide strong evidence that oxidative stress occurs as a physiological condition at birth and the initial stage of neonate life.

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