Induction of antioxidant enzyme activity by hyperoxia (60% O_2) in the developing chick embryo

Jolanda C. van Golde, Paul J. Borm*, Marion C. Wolfs, Els H. Rhijnsburger* and Carlos E. Blanco

*Department of Neonatology, University Hospital and *Department of Health Risk Analysis and Toxicology, Maastricht University, Maastricht, The Netherlands*

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1. At premature birth, man and animals are exposed to relatively high oxygen levels, compared with intra-uterine conditions, at a time when their antioxidant enzyme (AOE) system is still immature. Using the chick embryo as a study model, we investigated changes in the AOE system in response to hyperoxia applied at different time points during the incubation period. Relations between hyperoxia and AOE activity were studied in selected organs (brain, heart, liver, intestine and lungs) of developing chick embryos (during the second half of the incubation period).

2. Incubated White Leghorn eggs were divided into four groups: control ($n = 100$) and three test groups exposed for 48 h to 60% O_2 on day 10 (test group 1, $n = 80$), day 14 (test group 2, $n = 60$) and day 18 (test group 3, $n = 30$). Superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) enzyme activities were measured in homogenates of the brain, heart, liver, intestine and lungs.

3. Exposure to hyperoxia at different time points during incubation resulted in a 2- to 10-fold increase in SOD activity in all organs except the brain. Catalase and GPx enzyme activities were only induced in test group 1, 48 h after initiation of hyperoxia.

4. In the developing chick embryo, hyperoxia can produce a temporary induction of AOE activity, which is dependent on the AOE, organ, incubation time and time point of exposure.

When born prematurely, the fetus is abruptly exposed to a much higher arterial oxygen level (P_{a,O_2}, 50–100 mmHg) compared with intra-uterine conditions (P_{a,O_2}, 25 mmHg), which results in an increased production of reactive oxygen species (ROS) (Frank, 1985). ROS such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (HO•) are normal products of the aerobic metabolic processes in the cell (Saugstad, 1990; Varsila, Hallman & Andersson, 1994; Yu, 1994). Under physiological conditions, these ROS are detoxified by various antioxidant enzyme (AOE) defence systems, including specific enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (Saugstad, 1990; Varsila *et al.* 1994; Yu, 1994). If ROS are not detoxified efficiently, interactions with cell components such as lipids, protein or DNA may occur, causing cellular damage or even cell death (Frank, 1985; Janssen, Borm, Van Houten & Mossman, 1993; Yu, 1994). Production and activity of AOEs increase markedly in the final days before birth, and even more so after birth (Frank & Sosenko, 1987).

However, when the organism is immature, the response from the AOE system may be insufficient, thereby confronting the premature newborn infant with oxidative stress (Frank, 1985; Varsila *et al.* 1994). The latter has been associated with bronchopulmonary dysplasia, retinopathy, necrotizing enterocolitis and intracranial haemorrhage (Saugstad, 1990).

In order to study the ability of hyperoxia to induce the AOE system in immature organ systems, we used the developing chick embryo model. In this study the chick embryo was exposed to hyperoxia on days 10, 14 and 18 of the incubation period. Since gas exchange in the chick embryo occurs through micropores in the eggshell, hyperoxia can be easily induced (Bissonnette & Metcalfe, 1987). We describe the developmental changes of SOD, GPx and catalase in fetal brain, heart, liver, intestine and lungs under physiological conditions as well as after exposure to hyperoxia at three different time points during the second half of the total incubation period.

METHODS

Chemicals

Purified standards for SOD (bovine erythrocytes, EC 1.15.1.1) and catalase (bovine, EC 1.11.1.6) were obtained from Boehringer Mannheim and for glutathione peroxidase (bovine erythrocytes, lyophilized) from Sigma. All other chemicals and reagents were of analytical grade.

Protocol

Fertile White Leghorn eggs were incubated at 38 °C and 60% humidity. The oxygen concentration in the incubator could be increased up to 60%. The gas composition in the incubator was continuously monitored during incubation. Exposure of the egg to hyperoxia (inspired O_2 fraction (F_{I,O_2}) = 0·6) for 3 h led to a significant change in P_{a,O_2} (to 52 ± 6 mmHg; Δ28 mmHg) in the chorioallantoic artery, similar to the transition of P_{a,O_2} levels at birth (Piiper, Tazawa, Ar & Rahn, 1980). During incubation, eggs were turned hourly along their long axis. Chick embryos were divided into four groups: control (n = 100) and three test groups. In the control group, which was incubated at 21% O_2, SOD, GPx and catalase enzyme activities were measured in the brain, heart, liver, intestine and lungs from day 10 to 21 (each day, 10 chick embryos were decapitated, except on days 13 and 17). Test groups were also incubated at 21% O_2, but were exposed at different time points to 60% O_2 for 48 h. Test group 1 was exposed to 60% O_2 on days 10 and 11 (n = 80), test group 2 was exposed to 60% O_2 on days 14 and 15 (n = 60) and test group 3 was exposed to 60% O_2 on days 18 and 19 (test group 3, n = 30). In all test groups, the first measurements of SOD, GPx and catalase enzyme activities in the brain, heart, liver, intestine and lungs were begun 24 h after initiation of hyperoxia (at each chosen time 10 chick embryos were decapitated for measurements). In test group 1, measurements were performed on days 11, 12, 14, 16, 18, 19, 20 and 21; in test group 2, measurements were performed on days 15, 16, 18, 19, 20 and 21; and in test group 3 measurements were performed on days 19, 20 and 21.

Tissue preparation and enzyme determinations

On each determined day, ten chick embryos were removed from their eggs, decapitated, weighed and classified using the Hamburger–Hamilton morphological maturity index (Hamburger & Hamilton, 1951). Brain, heart, liver, intestine, and lungs were dissected from the embryo, weighed and chilled in ice-cold 0·9% NaCl solution. Subsequently, tissues were homogenized in 10 mM potassium phosphate buffer (4 °C, 1·36 g l^{-1} KH_2PO_4 and 1·78 g l^{-1} Na_2HPO_4, titrated to pH 7·4, supplemented with 30 mM KCl and 1 mM EDTA), using a Polytron Teflon homogenizer (Brinkman, Westbury, NY, USA; 2 × 60 s at the highest speed), and stored at −70 °C until AOE assays were performed.

Protein content was determined in an aliquot of tissue homogenate, according to Bradford (1976), using bovine serum albumin (BSA) as standard. Remaining homogenates were centrifuged at 12 000 r.p.m. for 5 min at 4 °C, and the resulting supernatants were analysed for SOD, GPx and catalase activity as described previously (Janssen, Marsh, Absher, Borm & Mossman, 1990), using standard spectrophotometric assays. AOE activity levels were corrected for changes in protein content in the different organs.

CuZn-SOD was measured in chloroform–ethanol (3 : 5) pre-extracted samples, using the inhibition of xantine (50 μM)-induced cytochrome C (10 μM) reduction at 415 nm. SOD activity was calculated from a semilogarithmic plot between reaction rate and the log of a standard SOD. Samples were diluted to obtain approximately 50% inhibition (Engelen, Borm, van Sprundel & Leenaerts, 1990).

Total GPx activity was measured at 340 nm for 3 min, using 0·3 mM H_2O_2-induced reduction of nicotinamide adenine dinucleotide phosphate.

Catalase was measured at 240 nm for 3 min, using the linear decrease of H_2O_2 (10 mM) at pH 7·0 in 50 mM phosphate buffer (6·8 g l^{-1} KH_2PO_4 and 8·9 g l^{-1} Na_2HPO_4, titrated to pH 7·0). Activity was calculated using the the molar extinction coefficient of 0·0394 $mmol^{-1}$ cm^{-1}.

Statistics

Data are presented as means ± S.D. Differences in AOE levels in different organs during normal development were tested using the Mann–Whitney U test. AOE levels in test groups were compared with the levels on the same day in the control group using the Mann–Whitney U test. Significance was accepted when $P < 0·05$.

RESULTS

Control group development

The normal development of SOD and GPx in the developing chick embryo (in the brain, heart, liver, intestine and lungs) was characterized by a rapid increase in activity during the final 20–30% of the incubation time (Tables 1 and 2). In contrast, catalase activity in these organs did not increase during incubation (Table 3).

SOD enzyme activity increased from day 15 onwards, and continued to increase until the end of the measured period in all organs, except the liver. In the liver, the maximum level of SOD enzyme activity was reached on day 18 of incubation. The enzyme activity in brain and heart was significantly higher than that in lungs and intestine ($P < 0·05$).

GPx enzyme activity increased steadily from day 12 until the end of the measured period. The highest levels of GPx enzyme activity were found in liver tissue ($P < 0·05$). All other organs showed similar levels of GPx enzyme activity.

Catalase enzyme activity showed no change during the measured period and the activity was much lower than that of SOD and GPx enzymes ($P < 0·05$).

The maximum SOD, GPx and catalase enzyme activities were reached earliest in the liver.

Test groups: changes induced by hyperoxia

An increase in SOD enzyme activity was observed in all organs except the brain (Table 1). Observations were remarkably similar in heart, liver, intestine and lungs. A significant increase in SOD enzyme activity was reached 24 h after initiation of hyperoxia in test group 1 and after 48 h in test groups 2 and 3. The increase in SOD enzyme activity on days 16–18 was significantly higher in test group 2 compared with test group 1 ($P < 0·05$). An increase in SOD enzyme activity was observed in embryos in test group 3 on days 19 and 20. However, because all embryos died when hyperoxia was stopped, SOD levels could not be measured on day 21. It follows therefore that maximum SOD levels and duration of the significant increase could also not be measured.

Interestingly, in brain tissue hyperoxia induced an increase in SOD enzyme activity in test group 1 only. In more developed chick embryos, no increase in enzyme activity was observed after initiation of hyperoxia. In fact, contrary to what was observed in all other organs, a significant

Table 1. SOD enzyme activity (units (mg protein)$^{-1}$) in the brain, heart, liver, intestine and lung of chick embryos in control and test groups

	Day	Brain	Heart	Liver	Intestine	Lung
Control	10	n.d.	n.d.	n.d.	n.d.	n.d.
($n = 100$)	11	5·8 ± 1·1	n.d.	1·0 ± 0·4	n.d.	n.d.
	12	13·5 ± 2·4	1·3 ± 0·2	3·2 ± 1·3	1·1 ± 0·6	0·3 ± 0·2
	14	15·3 ± 2·1	14·8 ± 1·8	13·6 ± 1·4	6·9 ± 0·8	13·9 ± 3·2
	15	52·4 ± 2·9	32·8 ± 6·4	26·1 ± 5·7	8·9 ± 3·8	24·5 ± 3·1
	16	125·6 ± 9·9	78·4 ± 4·6	102·3 ± 10·3	19·4 ± 2·6	33·9 ± 2·8
	18	167·8 ± 8·7	130·7 ± 11·5	365·6 ± 64·2	50·4 ± 3·1	83·9 ± 13·7
	19	218·5 ± 22·2	190·8 ± 16·5	335·0 ± 23·7	96·4 ± 14·7	87·1 ± 9·6
	20	228·4 ± 18·4	316·9 ± 45·8	298·5 ± 28·1	118·6 ± 21·9	82·0 ± 19·9
	21	302·6 ± 28·4	430·4 ± 111·0	226·3 ± 35·5	137·6 ± 18·8	81·9 ± 21·8
Test group 1	10	—	—	—	—	—
($n = 80$)	11	6·1 ± 2·8	3·9 ± 2·4*	4·6 ± 0·8*	2·1 ± 1·3*	1·8 ± 1·1*
	12	21·6 ± 2·1*	14·9 ± 5·7*	8·7 ± 2·8*	6·8 ± 3·4*	13·2 ± 1·4*
	14	88·2 ± 7·6*	60·9 ± 11·9*	61·4 ± 9·0*	16·2 ± 6·8*	24·6 ± 10·5*
	15	—	—	—	—	—
	16	149·7 ± 13·2*	123·8 ± 12·3*	289·0 ± 18·9*	45·8 ± 5·1*	73·4 ± 14·4*
	18	213·1 ± 12·5*	186·5 ± 45·8*	335·7 ± 31·7	88·1 ± 17·4*	91·6 ± 20·0
	19	264·2 ± 16·7*	215·4 ± 21·7*	293·8 ± 5·0†	105·2 ± 16·6	85·0 ± 11·8
	20	235·7 ± 25·8	300·1 ± 37·1	235·8 ± 27·3	112·6 ± 26·8	87·1 ± 9·9
	21	280·3 ± 22·2	398·5 ± 32·4	208·9 ± 28·1	129·9 ± 12·7	83·5 ± 15·5
Test group 2	10	—	—	—	—	—
($n = 60$)	11	—	—	—	—	—
	12	—	—	—	—	—
	14	—	—	—	—	—
	15	10·9 ± 2·4†	20·5 ± 7·3†	19·7 ± 10·7	10·8 ± 2·3	8·5 ± 1·1†
	16	29·9 ± 7·1†	380·9 ± 45·2*	310·9 ± 23·4*	180·5 ± 48·3*	130·9 ± 21·8*
	18	3·8 ± 1·9†	519·6 ± 38·7*	1485·2 ± 53·8*	410·1 ± 23·9*	277·9 ± 32·1*
	19	20·9 ± 5·3†	189·5 ± 41·8	312·1 ± 32·1	43·5 ± 3·2	38·0 ± 4·7
	20	76·9 ± 13·3†	326·8 ± 29·1	440·5 ± 17·3*	95·7 ± 19·8	115·5 ± 18·2*
	21	125·7 ± 21·5†	445·3 ± 35·3	425·7 ± 28·4*	130·6 ± 16·4	110·2 ± 13·3*
Test group 3	10	—	—	—	—	—
($n = 30$)	11	—	—	—	—	—
	12	—	—	—	—	—
	14	—	—	—	—	—
	15	—	—	—	—	—
	16	—	—	—	—	—
	18	—	—	—	—	—
	19	21·4 ± 5·6†	588·0 ± 45·7*	102·6 ± 28·6	100·3 ± 12·9	56·6 ± 8·6
	20	43·3 ± 10·0†	1133·3 ± 63·9*	491·5 ± 32·6*	315·2 ± 38·5*	130·3 ± 19·9*
	21	—	—	—	—	—

Test group 1, hyperoxia on days 10 and 11; test group 2, hyperoxia on days 14 and 15; test group 3, hyperoxia on days 18 and 19. Each data point is the mean ± S.D. of ten chick embryos. n.d., not detectable (AOE activity level < 0·001 units (mg protein)$^{-1}$). * $P < 0·05$, SOD enzyme activity significantly increased compared with control group; † $P < 0·05$, SOD enzyme activity significantly decreased compared with control group.

decrease in SOD enzyme activity was observed in the brain ($P < 0·05$).

An increase in GPx enzyme activity was observed in all organs only in test group 1 (Table 2). This increase occurred 48 h after the initiation of hyperoxia ($P < 0·05$) and its duration was 3 days in the heart, liver and intestine and 6 days in the brain. In the lung tissue a significant increase in GPX enzyme activity was observed 24 h after initiation of hyperoxia. However, the level of enzyme activity in test group 1 decreased from day 19 to 21 in the brain, from day 16 to 21 in the heart, liver and intestine and from day 14 to 21 in the lungs ($P < 0·05$). In contrast, in test group 2, in the heart, liver, intestine and lungs a significant decrease in

Table 2. GPx enzyme activity (units (mg protein)$^{-1}$) in the brain, heart, liver, intestine and lung of chick embryos in control and test groups

	Day	Brain	Heart	Liver	Intestine	Lung
Control	10	21·6 ± 9·5	n.d.	103 ± 10·4	0·03 ± 0·04	9·75 ± 7·1
($n = 100$)	11	27·7 ± 9·4	0·23 ± 0·6	139 ± 19·1	4·89 ± 4·24	17·6 ± 8·5
	12	32·7 ± 11·8	1·49 ± 1·5	147 ± 10·4	31·7 ± 18·3	64·3 ± 5·4
	14	46·5 ± 6·7	1·68 ± 1·5	157 ± 12·9	66·3 ± 17·5	135 ± 17·9
	15	49·5 ± 9·8	39·9 ± 12·4	192 ± 17·6	83·4 ± 8·5	146 ± 28·4
	16	54·7 ± 7·5	75·4 ± 10·2	255 ± 15·3	92·2 ± 17·4	178 ± 32·2
	18	56·1 ± 10·3	124 ± 29·9	252 ± 18·6	95·3 ± 14·5	126 ± 23·1
	19	49·5 ± 10·9	98·8 ± 21·7	252 ± 13·2	93·1 ± 22·2	115 ± 24·3
	20	36·2 ± 9·39	76·8 ± 11·8	247 ± 26·7	92·1 ± 12·6	111 ± 16·2
	21	32·2 ± 14·1	42·9 ± 16·9	192 ± 25·3	43·1 ± 12·3	72·1 ± 15·1
Test group 1	10	—	—	—	—	—
($n = 80$)	11	26·9 ± 8·0	10·1 ± 10·6	149 ± 18·5	6·91 ± 11·5	45·8 ± 17·5*
	12	46·5 ± 12·7*	34·3 ± 18·4*	206 ± 22·3*	69·5 ± 7·12*	65·6 ± 31·9
	14	64·6 ± 12·7*	66·1 ± 19·2*	267 ± 26·2*	94·3 ± 12·5	69·5 ± 8·5 †
	15	—	—	—	—	—
	16	69·6 ± 14·9*	40·2 ± 10·9†	227 ± 31·1	83·8 ± 11·9	52·6 ± 14·7†
	18	79·7 ± 11·9*	42·6 ± 13·7†	200 ± 12·2†	45·7 ± 10·2†	42·7 ± 4·0†
	19	27·8 ± 9·4†	54·8 ± 19·9†	176 ± 11·8†	38·5 ± 12·6†	33·3 ± 4·6†
	20	12·9 ± 4·4†	44·7 ± 7·8†	175 ± 23·1†	26·5 ± 5·8†	24 ± 17·3†
	21	19·9 ± 5·9†	30·8 ± 5·5†	166 ± 9·1†	17·4 ± 6·5†	22·4 ± 7·6†
Test group 2	10	—	—	—	—	—
($n = 60$)	11	—	—	—	—	—
	12	—	—	—	—	—
	14	—	—	—	—	—
	15	55·1 ± 7·5	37·5 ± 11·2	165 ± 15·1†	48·9 ± 10·3†	87·1 ± 7·5†
	16	63·9 ± 9·1	38·7 ± 7·4†	170 ± 22·9†	45·9 ± 11·3†	91·5 ± 12†
	18	52·1 ± 10·3	39·2 ± 9·0†	172 ± 14·9†	33·3 ± 11·9†	95·5 ± 7·1†
	19	51·2 ± 7·1	37 ± 9·5†	183 ± 12·5†	43·9 ± 10·8†	100 ± 15†
	20	38·9 ± 8·9	33·2 ± 8·6†	213 ± 21·8†	42·7 ± 5·1†	94·6 ± 13·4
	21	33·9 ± 6·1	24·7 ± 6·8†	211 ± 20·4†	52·1 ± 10·2†	80·2 ± 10·2
Test group 3	10	—	—	—	—	—
($n = 30$)	11	—	—	—	—	—
	12	—	—	—	—	—
	14	—	—	—	—	—
	15	—	—	—	—	—
	16	—	—	—	—	—
	18	—	—	—	—	—
	19	39·5 ± 7·1†	71·1 ± 7·8†	235 ± 28·5†	84·6 ± 27·7	89·1 ± 14·6†
	20	30·9 ± 10·5	64·2 ± 9·5†	194 ± 31·1†	48·8 ± 6·9†	87·8 ± 7·8†
	21	—	—	—	—	—

Test group 1, hyperoxia on days 10 and 11; test group 2, hyperoxia on days 14 and 15; test group 3, hyperoxia on days 18 and 19. Each data point is the mean ± S.D. of ten chick embryos. n.d., not detectable (AOE activity level < 0·001 units (mg protein)$^{-1}$). * $P < 0·05$, GPx enzyme activity significantly increased compared with control group; † $P < 0·05$, GPx enzyme activity significantly decreased compared with control group.

GPx enzyme activity was observed for 6 days ($P < 0·05$). Also, in test group 3, a significant decrease in GPx enzyme activity was observed 24–48 h after the initiation of hyperoxia in the brain, heart, liver and intestine.

An increase in catalase enzyme activity was observed in all organs, except the heart, only in test group 1 (Table 3). This occurred 24–48 h after the initiation of hyperoxia ($P < 0·05$) and its duration was 24 h in the intestine, 2 days in the brain, and 5 days in the lungs and the liver. In test group 1, a decrease in catalase enzyme activity in the heart was observed from day 11 to 16 ($P < 0·05$). In test group 2, a significant decrease in catalase enzyme activity in the heart, liver and intestine was observed for 5–6 days ($P < 0·05$). In test group 3, a significant decrease in catalase

Table 3. Catalase enzyme activity (units (mg protein)$^{-1}$) in the brain, heart, liver, intestine and lung of chick embryos in control and test groups

	Day	Brain	Heart	Liver	Intestine	Lung
Control	10	0·5 ± 0·1	9 ± 2·3	74 ± 11·2	5 ± 0·2	0·3 ± 0·1
($n = 100$)	11	0·7 ± 0·2	11 ± 3·2	75 ± 14·8	7 ± 1·8	0·4 ± 0·2
	12	3 ± 0·5	21 ± 4·4	77 ± 21·5	8 ± 3·3	0·5 ± 0·1
	14	2·8 ± 0·6	22 ± 4·6	70 ± 18·2	13 ± 3·6	0·4 ± 0·2
	15	2·1 ± 0·6	23 ± 3·7	95 ± 13·8	14 ± 2·8	2·5 ± 0·4
	16	2·3 ± 0·4	22 ± 4·6	102 ± 23·7	11 ± 3·8	2·6 ± 0·4
	18	2·5 ± 0·6	17 ± 4·6	117 ± 19·3	12 ± 2·8	3·7 ± 0·4
	19	2·5 ± 0·6	14 ± 4·0	82 ± 9·6	14 ± 3·6	4·2 ± 0·5
	20	3·1 ± 0·8	11 ± 2·7	55 ± 14	14 ± 3·5	5·5 ± 0·7
	21	3·2 ± 0·6	12 ± 3·2	63 ± 8	13 ± 3·2	5·2 ± 0·6
Test group 1	10	—	—	—	—	—
($n = 80$)	11	1·9 ± 0·5*	7 ± 1·8†	43 ± 11·9†	5 ± 0·8†	0·9 ± 0·2*
	12	3·7 ± 0·5*	9 ± 2·8†	142 ± 19·4*	12 ± 2·0*	1·6 ± 0·3*
	14	1·4 ± 0·4†	12 ± 2·7†	134 ± 14·1*	9 ± 3†	2·1 ± 0·1*
	15	—	—	—	—	—
	16	1·9 ± 0·3	15 ± 3·8†	124 ± 12*	6 ± 1·1†	1·7 ± 0·5†
	18	2·3 ± 0·8	15 ± 3·1	103 ± 15·3	8 ± 2·2†	2·2 ± 0·8†
	19	2·2 ± 0·9	13 ± 3·4	54 ± 6·1†	9 ± 2·8†	1·9 ± 0·4†
	20	2·4 ± 0·7	10 ± 0·7	54 ± 7·9	13 ± 2·3	2·9 ± 0·6†
	21	3·4 ± 0·8	12 ± 2·4	63 ± 13·4	13 ± 2·5	2·3 ± 0·6†
Test group 2	10	—	—	—	—	—
($n = 60$)	11	—	—	—	—	—
	12	—	—	—	—	—
	14	—	—	—	—	—
	15	2·7 ± 1	12 ± 3·4†	44 ± 4†	9 ± 2·6†	2·9 ± 0·9
	16	2·9 ± 0·9	8 ± 1·1†	52 ± 6·4†	8 ± 1·2†	3 ± 0·5†
	18	3·3 ± 0·6	8 ± 1·7†	62 ± 9·8†	9 ± 2·1†	3·4 ± 1
	19	2·9 ± 0·8	9 ± 2†	67 ± 10·1†	9 ± 2·9†	4·5 ± 0·6
	20	3·1 ± 0·4	9 ± 1·9	55 ± 5·1	10 ± 2·9†	4·9 ± 0·8
	21	2·9 ± 0·5	11 ± 2·5	59 ± 10·8	12 ± 2·8	4·9 ± 0·8
Test group 3	10	—	—	—	—	—
($n = 30$)	11	—	—	—	—	—
	12	—	—	—	—	—
	14	—	—	—	—	—
	15	—	—	—	—	—
	16	—	—	—	—	—
	18	—	—	—	—	—
	19	2·9 ± 0·8	12 ± 3·1	63 ± 7·5†	9 ± 1·9†	2·9 ± 0·8†
	20	3·3 ± 1	10 ± 2·4	47 ± 4·1	11 ± 2·1	3·2 ± 0·9†
	21	—	—	—	—	—

Test group 1, hyperoxia on days 10 and 11; test group 2, hyperoxia on days 14 and 15; test group 3, hyperoxia on days 18 and 19. Each data point is the mean ± S.D. of ten chick embryos. * $P < 0·05$, catalase enzyme activity significantly increased compared with control group; † $P < 0·05$, catalase enzyme activity significantly decreased compared with control group.

enzyme activity in the liver, intestine and lungs was observed on day 19, and on day 20 in the lungs ($P < 0·05$).

DISCUSSION

This study describes the normal development of the primary AOE system (SOD, GPx and catalase enzyme activity) in the brain, heart, liver, intestine and lung tissue during the second half of the incubation period in the chick embryo, and the changes that hyperoxia induced in this development. To study the effect of hyperoxia, chick embryos were exposed to 60 % O_2 for 48 h at three different time points of their incubation period (days 10, 14 and 18). This level of hyperoxia exposure was chosen because the level of P_{a,O_2} obtained was similar to that of neonatal P_{a,O_2} levels (Piiper *et al.* 1980). In the control group, AOE activity changes were

measured from day 10 until day 21 of the incubation period. In all test groups, measurement of AOE activity was begun 24 h after initiation of hyperoxia, and continued until the last day of incubation. We found that exposure to hyperoxia resulted in an increased AOE activity. This response to hyperoxia depended on the AOE, organ, incubation time, and time points of exposure.

In order to be prepared for the relatively enriched O_2 world after birth, a prenatal increase and/or a rapid postnatal response in AOE activity is necessary. The cause for this increase in AOE activity is not known, but possible mechanisms involved are: (a) the level of P_{O_2}, (b) the rate of oxidative metabolism during fetal development, (c) the maturational state of the tissues, and (d) the concentration of enzyme substrate (Engelen *et al.* 1990; Wilson, Lui & Del Maestro, 1992). In the chick embryo, P_{O_2} increases from minimal levels on day 4 to much higher levels on day 10 (Freeman & Mission, 1970). However, since a gradual decline in P_{O_2} and metabolic rate occurs from day 10 or 12 onwards, these factors play a less important role in the increase in AOE activity (Freeman & Mission, 1970; Prinzinger, Schmidt & Dietz, 1995). However, it might be assumed that when the immature AOE system is exposed to hyperoxia the immature organ system might be activated to increase the AOE level.

Other studies have already shown that AOE levels increase during the final part of normal gestation in other species including guinea-pig, rabbit, rat, hamster or human fetus (Frank & Groseclose, 1984; Transwell & Freeman, 1984; Gerdin, Tyden & Eriksson, 1985; Saugstad, 1990; Janssen *et al.* 1993). However, in these studies the first measurements of AOE activity were performed 3 days before birth. In our study, in which AOE levels were measured from day 10 until day 21, we found an increase in AOE activity during the final 20–30% of the total incubation time (days 14–21); however, catalase activity did not increase.

SOD is present in organs that are highly vulnerable to ROS (Munim, Asayama, Dobashi, Suzuki, Kawaoi & Kato, 1992). Interestingly, we found that AOE levels were strikingly higher in the liver than in the other organs. This might be explained by the extensive metabolizing and detoxifying capacity of this organ, for example, the rate of extra-mitochondrial release of H_2O_2 is approximately two orders of magnitude greater in the liver than in the brain (Wilson *et al.* 1992). In this study, hyperoxia induced a 2- to 10-fold increase in SOD enzyme activity in all organs, with the exception of the brain. This observed increase in AOE activity was higher when hyperoxia was applied later in fetal development. A possible explanation for the lack of AOE activity induction in the brain tissue might be the decrease in cerebral blood flow caused by hyperoxia. A decrease in cerebral blood flow due to cerebral vaso-constriction, caused by hyperoxia, has been described for premature infants exposed to 80% oxygen during initial stabilization in the delivery room (Leahy, Cates, MacCallum & Rigatto, 1980; Lunstrøm, Pryds & Greisen, 1995). However, no data are available to support the hypothesis that cerebral vasoconstriction occurs during the perinatal period. Another explanation for the lack of induction of brain SOD activity by hyperoxia could be the inhibition or scavenging of superoxide, e.g. its reaction with nitric oxide (NO) (Radi, Beckman, Bush & Freeman, 1991). This reaction is more than 3 times faster than the enzymatic dismutation of superoxide radicals catalysed by SOD ($k_{SOD} = 2 \times 10^9$ M^{-1} s^{-1}; Radi *et al.* 1991). Moreover, the reaction product ONOO· (peroxynitrite) has also been found to inactivate SOD activity (MacMillan-Crow, Crow, Kerby, Beckman & Thompson, 1996). In contrast, administration of lead acetate (1·25 and 2·5 μmol (kg egg weight)$^{-1}$) to 14-day-old chick embryos increased AOE activity significantly in all organs, including the brain (Somashekaraiah, Padmaja & Prasad, 1992). This might be explained by the high doses of lead acetate and the depletion of glutathione in this study.

Induction of GPx and catalase activity was observed only in chick embryos exposed to hyperoxia on days 10 and 11 of the incubation period (test group 1). Interestingly, in test groups 2 and 3, the levels of GPx and catalase enzyme activity decreased compared with the control group. Different changes in enzyme activity between the three AOEs in the developing chick embryo might be explained by differences in regulation of gene expression during the incubation period (Clerch & Massaro, 1992; Chen & Frank, 1993). CuZn-SOD and catalase are regulated at a pretranslational level and their increase in activity is associated with an increase in mRNA concentration (Clerch & Massaro, 1992, 1995; Chen & Frank, 1993). In contrast, GPx is regulated at a translational or post-translational level and changes in GPx concentration are caused by an increase in stability of mRNA. However, all studies on gene expression of AOEs have been performed during the final 20% of the incubation period and postnatally (Clerch & Massaro, 1992, 1995; Chen & Frank, 1993). The regulation of differential AOE gene expression during early perinatal development, in which we observed important differences compared with the more developed chick embryo, has never been studied.

SOD might be particularly important as protection against early acute oxygen toxicity of premature animals and therefore is induced earlier than GPx or catalase (Crapo & Tierney, 1974; Yam, Frank & Roberts, 1987). However, an increase in SOD enzyme activity without induction of GPx or catalase could result in an increase in hydrogen peroxide and hydroxyl radicals, due to the Haber–Weiss reaction (Ditelberg, Sheldon, Epstein & Ferriero, 1996). An imbalance in the ratio of SOD to GPx and catalase may, therefore, result in an increase in lipid peroxidation (de Haan, Cristiano, Ianello & Kola, 1995). Hyperoxia applied to adult and prematurely delivered rats led to the induction of SOD enzymes in lung tissue (White, Ghezzi, McMahon,

Dinarrello & Repine, 1989; Chen, Whitney & Frank, 1994). Our study corroborated this finding. However, levels of GPx and catalase also increased in prematurely delivered rats, a finding that was not observed in chick embryos. In another study, exposure of chick embryos to hyperoxia (60 %) for 72 h on days 16–18 of the incubation period did not result in increased levels of lipid peroxidation (Stock, Silvernail & Metcalfe, 1990). This absence of lipid peroxidation might have been the result of an increase in the AOE activity, as observed in our study. This is supported by the presence of lung injury and the absence of an increase in AOE activity in premature rabbits exposed to hyperoxia 3 days before the end of gestation (Frank & Sosenko, 1991).

We have demonstrated that hyperoxia in the developing chick embryo induces a temporary increase in AOE activity. This increase depends on the AOE, organ, incubation time and time point of exposure to hyperoxia. In further studies, it remains to be established whether this increase in AOE activity is sufficient to circumvent cell damage. Furthermore, studies of the regulation of differential gene expression would provide more insight into the development of the AOE defence system during perinatal development. Although the results obtained using this animal model are interesting, more studies are warranted to determine whether our observations are relevant for the premature human.

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Corresponding author

J. C. van Golde: Department of Pediatrics – Neonatology, University Hospital Maastricht, PO Box 5800, 6202 AZ Maastricht, The Netherlands.

Email: JVG@SKIN.AZM.NL