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ORIGINAL ARTICLE

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Development of haemoglobin subtypes and extramedullary haematopoiesis in young rats. Effects of hypercapnic and hypoxic environment

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Abstract The influence of repeated hypoxia on the development of haemoglobin (Hb) subtypes and on extramedullary haematopoiesis (EMH) was investigated in young Wistar rats of different ages. The rats were exposed to hypercapnic/hypoxic and to "simple" hypoxic conditions. The results obtained were compared to those of an untreated age-matched control group. Different globin chains were measured using HPLC and time-of-flight (TOF) mass analysis. The number of EMH cells was evaluated by cell counting. By determining the proportions of α- and β-chains, fetal, neonatal and mature types of globin chain composition could be differentiated. The β-2 chain levels were significantly higher in hypercapnic/hypoxic environments than in the controls and simple hypoxic environments. The numbers of EMH cells in the two groups subjected to hypercapnia/hypoxia decreased significantly more slowly compared to the controls and simple hypoxia groups. Therefore, the development of Hb subtypes and the EMH activity in rats were influenced by both repeated hypercapnia and hypoxia.

Key words Sudden infant death syndrome (SIDS) · Fetal haemoglobin · Rat globin · High performance liquid chromatography (HPLC) · Extramedullary haematopoiesis (EMH)

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Introduction

Cases of sudden and unexpected infant death, most of which have been classified under the sudden infant death syndrome (SIDS), constitute a significant socio-medical problem. From some pathological findings, e.g. extramedullary haematopoiesis (EMH) [1, 2] and fatty changes in the liver [3], elevated levels of fetal haemoglobin (Hb) [4, 5] and from epidemiological data, e.g. significance of the prone sleeping position [6, 7] it can be hypothesised that hypercapnia and hypoxia could have significant effects especially in conjunction with other risk factors [8, 9, 10, 11, 12, 13, 14, 15, 16, 17] but the causal mechanisms are mainly unknown.

We have therefore employed an animal model using Wistar rats to investigate the age-dependent changes of Hb subtypes and the changes caused by several types of chronic hypoxia.

Material and methods

Animal experiments

New-born Wistar rats (n = 137, SLC, Tokyo, Japan) were divided into three groups and each group was treated for 3 h in an atmosphere-controlled chamber 2, 3, 9, 10, 16, 23, and 24 days after birth using different gas compositions (Table 1) to simulate hyper-

Table 1 Different gas compositions used for the treatment of the three groups

Group	Air volume	Additional gas volume	Oxygen amount
Hypercapnic/hypoxic $n = 24$	1800 ml/min	300 ml CO_2/min	13.9–15.2%
Simple hypoxia $n = 26$	1800 ml/min	300 ml N_2/min	13.9–15.1%
Control group $n = 87$	2100 ml/min	0	20.9%

Table 2 Average number of EMH cells per field of view at different ages are given for the three groups (* significant differences to the hypoxia related groups ** significant differences between the CO_2 hypercapnic/hypoxic and the N_2 simple hypoxic groups)

Age (days)	Controls	CO_2 rats	N_2 rats
12-day fetus	55 (1/4)		
20-day fetus	834		
1	897		
7	268		
14	198	227	205
21	28*	183	187
28	0*	135**	28
Adult rat	0		

capnic/hypoxic, simple hypoxic and "normal" atmospheric conditions (controls). The gas flow and composition was monitored by using a WE-1B flow volume meter (Sanwa, Tokyo Japan) and an OX-51 flow oxygen concentration counter with a E7068CM galvanic cell (Iuchi, Osaka Japan).

From the three groups five or six rats were sacrificed at different ages (Table 2) in a 100% carbon dioxide atmosphere after exposure times ranging between 30 s to 3 min. Blood samples and liver specimens were taken immediately after death.

Liver specimens were fixed in formalin (10% buffered) and embedded in paraffin before haematoxylin-eosin staining (H&E) and 3–4 μm thick sections were examined microscopically (× 100–400) to determine EMH levels (as the number of haematopoietic cells per defined field of view, nine randomly selected fields per section).

The H&E sections were also screened for pathological changes.

Hb determination

Determination of total Hb concentration

An aliquot of the blood samples was centrifuged at 3000 g for 10 min, the erythrocytes were washed 3 times with physiological saline and lysed with 2 vols of Milli Q water. An aliquot of the clear supernatant was diluted to a Hb concentration of 1 mg/ml (fetal rats) or 3 mg/ml (young and adult rats). The Hb concentration was determined by a haemaglobincyanide method with modified Van Kampen-Zijlstra's reagent [18, 19].

Apparatus preparation for liquid chromatography

Trifluoroacetic acid (TFA), acetonitrile (HPLC grade, both Wako Pure Chemical Industries, Osaka, Japan), and Milli Q purified water (Millipore, Bedford, MD) were used for analysis.

The columns used were a SynChropak RP-4 (SynChrom, Lafayette, Ind.) and a μBondasphere 5 μC 18–300 A (Nihon Waters, Tokyo, Japan). Neither pre-columns nor guard columns were used.

For separation on the column, solvent A (80:20 mixture of 0.1% TFA in water and 0.1% TFA in acetonitrile) and solvent B (40:60 mixture of the same solutions) were used [20]. The gradient of 38–55% B in 80 min and 55% B for 90 min was used for the identification and quantification. The flow rate for the column was 1.0 ml/min [5].

Determination of globin peaks by HPLC analysis

The determination of each globin peak was performed by time-of-flight (TOF) mass spectrometry analysis using a Kompact MALDI II attached to a programmed computer analysing system (Kratos, Sweden) [5] and confirmed by comparison with previously reported data [21, 22, 23, 24, 25, 26, 27, 28].

Quantitative determination of globin chains

The amount of the different globin chains was determined by measuring the relative peak areas of α- and β-chains on the chromatograms. The amount of fetal Hb was calculated as:

Hb-f(%) = β2-chain ratio/β1-chain + β2-chain ratio

The statistical significance was estimated by a paired t-test ($p < 0.05$).

All experiments using Wistar rats were carried out in accordance with the "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) and had been approved by the ethical committee of Nagoya City University School of Medicine.

Results

Globin composition

The determination of molecular weight of globin peaks by TOF mass analysis allowed the differentiation of the globin fractions (Fig. 1). The proportion of β-1 chains was the lowest 1 day before birth and increased during the first 3 weeks of life while the levels of β-2 chains were the re-

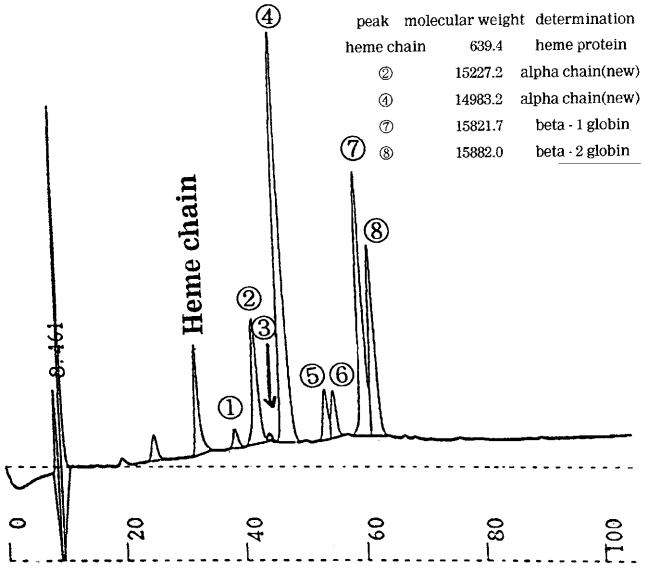

Fig. 1 HPLC peak pattern and molecular weights of seven different globin chains and the haem protein of Wistar rats

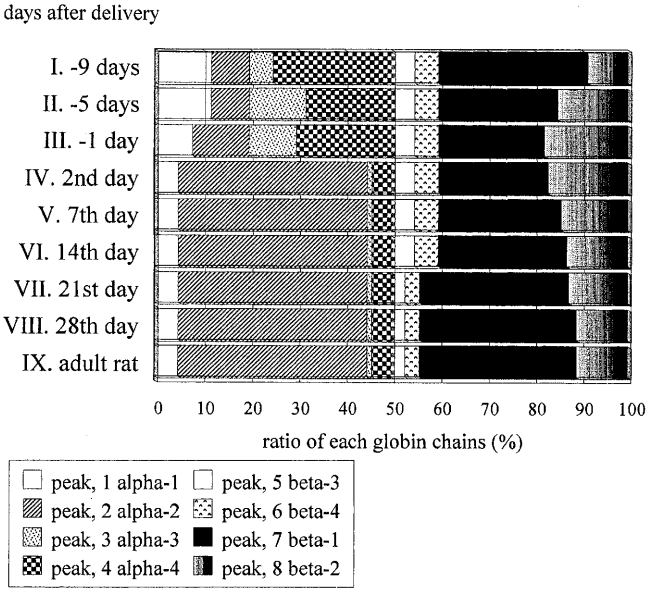

Fig. 2 Normal average globin ratios of rats at different ages determined in each of six rats of the control group. The first to third patterns are considered to be fetal types, the fourth to sixth patterns are typical neonatal patterns and the other three are mature types

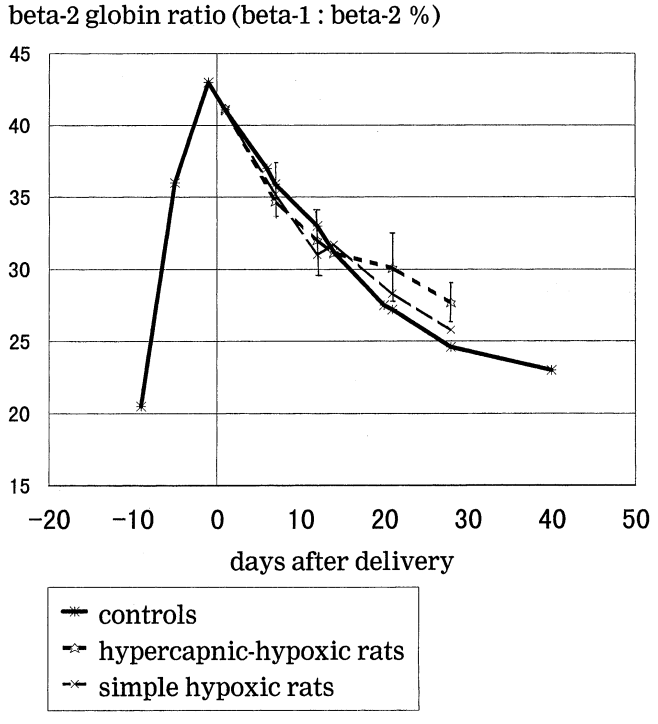

Fig. 3 Age-dependent changes of the average β-2 globin ratios including standard deviations in the three groups investigated

verse (Fig. 2). By determining the proportions of α- and β-chains it was possible to differentiate between fetal types, neonatal patterns and mature types of globin composition (Fig. 2). The average levels of β-2 globin chains increased rapidly during the fetal period and reached a maximum just before birth. The levels then began to decrease until 4 weeks after birth in all groups investigated. The β-2 chain levels of the hypercapnic/hypoxic groups also decreased according to the age but 2 weeks after birth this decrease became relatively slower so that the differences between these groups and the controls were statistically significant 21 days after birth (Fig. 3). The regression analysis of the β-2 chain levels showed a decreasing pattern of the control cases ($y = 37.5 – 0.361x$) compared to the pattern of the hypercapnic/hypoxic group.

EMH

EMH was observed in the liver starting at the 12th day after gestation (Fig. 4a) and the cell numbers increased until the 20th day post gestation (Fig. 4b) and the day just after birth and then decreased with the age (Table 2, Fig. 4a–d). In both groups subjected to hypoxia, this decrease was less steep as in the controls, the hypercapnic/hypoxic group exhibiting the slowest decrease ($p < 0.0125$; Table 2, Fig. 4e).

Other results

At the beginning of the experiments more severe hypoxic or hypercapnic environments with oxygen concentrations less than 8% were set up and all of the 7-days-old rats died within 10 min. Under the environments containing 9–13% oxygen the rats died after the second treatment. The results were therefore not included in this paper.

To estimate the consciousness of the rats during the experiments, the level of activity was observed. Under the simple hypoxic environments the rats showed abnormal face washing and mounting activity. Under the hypercapnic/hypoxic environments the rats showed snap-breathing and pre-coma symptoms during the 3 h of exposure.

Discussion

Many different minor findings have been reported in SIDS cases but the pathophysiological significance of some of these is questionable. For this and other reasons, it has also been suspected that cases classified as SIDS do not form a homogeneous group [29, 30, 31, 32, 33]. It is therefore very important to recognise the mechanisms and processes of death in each individual case. It is also important to elucidate the pathophysiological significance of minor findings.

In the present study an animal model was used to investigate hypoxia-related changes of the haematopoietic system, especially the subclasses of Hb and their relative proportions and the number of EMH cells as an equivalent for the haematopoietic activity.

Congote and Muray [4] and Iwahara et al. [28] described different types of Hb in rats – the embryonic and adult types, but they did not analyse the subtypes quantitatively. In the present study the investigation of “normal” untreated rats (controls) allowed age-dependent standards

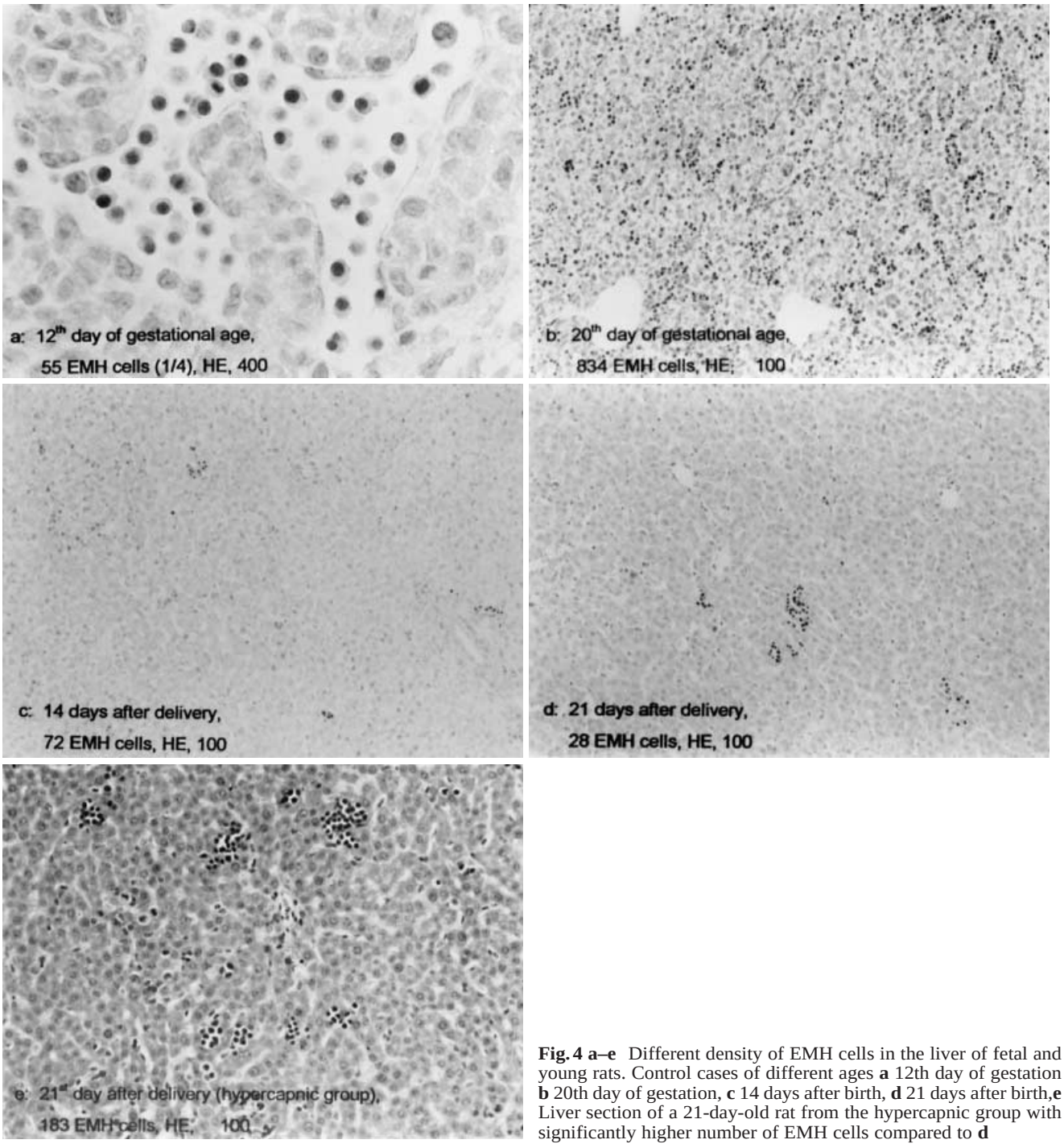

Fig. 4 a–e Different density of EMH cells in the liver of fetal and young rats. Control cases of different ages **a** 12th day of gestation **b** 20th day of gestation, **c** 14 days after birth, **d** 21 days after birth,**e** Liver section of a 21-day-old rat from the hypercapnic group with significantly higher number of EMH cells compared to **d**

of α- and β-chain ratios to be defined in rats and also for EMH activity in the liver. The comparison of these standards with the results obtained in the hypercapnic/ hypoxic groups showed that the concentration of β-2 globin chains is a very convenient indicator of preceding chronic hypoxia in rats but it is not suitable to indicate single or short hypoxic episodes.

Under hypercapnic/hypoxic conditions the Hb structure and EMH do not fully convert from the fetal to the adult subtype patterns. But even in the two different types of hypoxia (simple hypoxia and hypercapnic hypoxia) rather different patterns could be observed indicating a much stronger effect of the hypercapnic type of stimulation [34, 35].

These results suggest that the levels of oxygen and carbon dioxide have significant influence on the Hb structure and EMH. The regulation of respiration is controlled by many factors such as blood pH, pCO_2, pO_2, the nervous regulatory system, the action regulatory system [36, 37, 38, 39] and each of these systems is activated independently.

In particular, respiratory activity is strongly activated by increasing CO_2 concentrations and less strongly by decreasing O_2 but the combined action of these factors is a very strong trigger [36, 39].

The difference in activity between the two groups of exposure to hypoxia can be considered as a symptom either of a predominant sympathetic condition in the hypercapnic group or of parasympathetic nerve predominance in the simple hypoxic group.

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