Concentrations of Copper and Zinc in Liver and Serum Samples in Biliary Atresia Patients at Different Stages of Traditional Surgeries

CHIE SATO,[1] HIROSHI KOYAMA,[2,4] HIROSHI SATOH,[2] YUTAKA HAYASHI,[1] TSUNEO CHIBA[3] and RYOJI OHI[1,5]

[1]*Department of Pediatric Surgery,* [2]*Environmental Health Sciences, Tohoku University Graduate School of Medicine, Sendai, Japan,*
[3]*Department of Pediatric Surgery, National Hospital Organization Sendai Medical Center, Sendai, Japan,*
[4]*Department of Public Health, Gunma University Graduate School of Medicine, Maebashi, Japan, and*
[5]*Miyagi Children's Hospital, Sendai, Japan*

SATO, C., KOYAMA, H., SATOH, H., HAYASHI, Y., CHIBA, T. and OHI, R. *Concentrations of Copper and Zinc in Liver and Serum Samples in Biliary Atresia Patients at Different Stages of Traditional Surgeries.* Tohoku J. Exp. Med., 2005, **207** (4), 271-277 —— Many patients with biliary atresia (BA) have impaired metabolism of copper (Cu) and zinc (Zn) because of the obstruction of bile ducts. An excessive Cu accumulation is cytotoxic and results in fibrosis in hepatic tissues. Since Zn works antagonistically to Cu, lower Zn concentrations may deteriorate liver damage. In the 1980's, we performed a series of surgeries on BA patients for the construction and alteration of the bile flow route, which is the major excretion route for Cu. We obtained liver and serum samples at each surgery, and measured Cu and Zn concentrations by inductively coupled plasma atomic emission spectrometry. Hepatic Cu concentration decreased with the improvement of cholestasis after the establishment of bile excretion. Conversely, when cholestasis persisted or recurred, increases in hepatic and serum Cu concentrations were noted. Hepatic Zn concentration was lower than previously reported normal values. High hepatic and serum Cu concentrations due to persistent or recurrent cholestasis and low hepatic Zn concentration may deteriorate hepatic fibrosis and liver cirrhosis. —— biliary atresia; copper; zinc; bile excretion; fibrosis
© 2005 Tohoku University Medical Press

Copper (Cu) is a trace element essential for the growth and differentiation of cells. Physiologically, Cu is accumulated at a high level in the liver of neonates (Widdowson et al. 1972). Until 2-3 months after birth, the liver Cu concen- tration rapidly decreases. In contrast to the liver, serum concentration of Cu was low at birth around 31 μg/100 ml (0.29 μg/g; calculated by the present authors from serum specific gravity) and it increased to the adulthood level of 98

μg/100 ml (0.95 μg/g) by 7-12 months (Hatano et al. 1982).

In pathological conditions, such as Wilson disease, liver cirrhosis (LC) and primary biliary cirrhosis (PBC), an excessive amount of Cu is accumulated in the liver, which promotes hepatic fibrosis (Arakawa and Suzuki 1993). In biliary atresia (BA), since bile ducts are blocked and cholestasis persists, it is expected that Cu concentration in the liver is elevated. Indeed the Cu concentration was reported to be high in liver samples obtained from infants with BA at autopsy (Reed et al. 1972; Goksu and Ozsoylu 1986). Ohi and Lilly (1980) observed an excessive Cu accumulation in the liver at the time of the radical surgery of BA patients, and demonstrated a possibility of improving Cu excretion into bile following the surgery. Bayliss et al. (1995) also reported that Cu is high in liver specimens obtained at the time of the radical surgery of BA patients, though the concentrations of zinc (Zn) and manganese are low.

From the 1980s to the early 1990s, liver transplantation was not as prevalent as today. The common therapy for BA was a series of surgeries carefully performed to maintain the maximum quality of life retaining the patients' own liver as much as possible. The first surgery, a radical surgery, was hepatic portoenterostomy with an external fistula. After confirming the release of bile and improvement of liver function, in particular, the total bilirubin (TB) level returning to normal, the second surgery was performed. It converted the external fistula so that bile flowed into the jejunum instead of the external fistula. After confirming the stability of liver function, the third surgery was carried out to close the external fistula.

In the present study, concentrations of Cu in liver and serum samples of BA patients at different stages of the surgical therapies were measured. Since the bile flow was drastically changed during the surgical therapies, it is of interest to analyze the changes of the concentrations of Cu as well as Zn.

Materials and Methods

Subjects

Subjects of this study were BA patients of the Department of Pediatric Surgery of Tohoku University Hospital and the National Hospital of Sendai Medical Center. The sampling period was from October 1989 to May 1992. This period was selected because surgeries performed in series were the common procedure used; therefore, it is possible to evaluate the release of Cu from the liver simultaneously with bile flow in detail. Thirty-three liver and 55 serum samples were obtained. The patients themselves or their guardians gave their consent for analyzing trace elements in the samples.

Analytical methods

Liver samples collected by wedge biopsy during the surgeries were stored at −80°C until analysis. Blood samples were obtained carefully using a syringe so as not to cause hemolysis and transferred to zinc-free test tubes. The samples were allowed to stand at room temperature for 30 min. Then, they were centrifuged at 2,000 rpm for 10 min and the supernatants were stored at −80°C until measurements.

The analysis was carried out according to that of Haraguchi et al. (1988). The samples were weighed and placed in Pyrex test tubes. They were added with nitric acid (1 ml) and perchloric acid (0.25 ml) and heated at the maximum of 120°C for 12 h using a block heater and fully digested. They were diluted with 5% nitric acid to a final volume of 5 ml.

After wet digestion, Cu and Zn concentrations in the liver and serum samples were measured by inductively coupled plasma atomic emission spectrometry (ICP-AES). The ICP-AES equipment used in this study was a multiprogram UOP-2 system (Kyoto-Koken Inc., Kyoto), and the wavelengths used for the measurements of Cu and Zn concentrations were 324.8 nm and 213.9 nm, respectively. The equipment was calibrated using the standard solution for atomic absorption analysis (Wako Pure Chemical Industries, Osaka) for each metal. The standard solution and distilled water were measured before and after the measurements of Cu and Zn to confirm the accuracy and for background subtraction. We used yttrium as an internal standard. Furthermore, bovine liver standard reference materials (SRM1577, National Bureau of Standards, Washington D.C., USA) were used for the confirmation of measurement accuracy.

Statistical Analysis

We used JMP package software for the statistical analysis (SAS institute 2000). The least significant difference method was used after the one-way analysis of variance (ANOVA) for the test among groups. The level of significance was set at $p < 0.05$.

RESULTS

Sampling at different stages of surgeries and the follow-up period

Liver specimens were collected at a surgical operation and serum samples were obtained at routine or preoperative examinations. Hence, our sampling time was divided into six stages (Fig. 1). Sampling Times 1, 2 and 3 corresponds to the first, second and third surgery, respectively. Sampling Time 4 corresponds to the follow-up period after the third surgery, and thus no liver specimens were collected. When there was no improvement or a recurrence of jaundice after the surgeries, liver transplantation was indicated and this corresponds to Sampling Time 5. When jaun-

dice developed or LC worsened after a period of the stable condition, liver transplantation was also indicated and this corresponds to Sampling Time 6. The ages of the patients were younger than 2 years old at Sampling Time 5 and older than 5 years old at Sampling Time 6. The numbers of patients and their characteristics at each Sampling Time were also indicated. Since only several patients repeatedly gave specimens, no longitudinal study was conducted.

Liver function

Fig. 2 shows the data for liver function at each Sampling Time including TB, alkaline phosphatase (ALP), alanine aminotransferase (ALT), cholinesterase (Ch-E), albumin (Alb) and total cholesterol (TC) levels. With the establishment of bile flow, TB and Alb levels tended to return to normal, in particular, the decrease in TB level was noted. The TB levels were elevated again at Sampling Times 5 and 6. The Alb level at Sampling Time 5 decreased significantly. No

| Procedure or period	Sampling time	Patients		
		Age (year)	(No.)	Remarks
The first surgery (hepatic portoenterostomy)	1	0.03–0.21	(18)	All jaundice positive
The second surgery (conversion of the external fistula to an intestinal conduit)	2	0.38–1.63	(8)	Jaundice free except one
The third surgery (closure of external fistula)	3	0.42–2.83	(11)	Jaundice free except one
Follow-up	4	4.13–13.5	(15)	All jaundice free (no liver specimen)
No improvement or recurrence of jaundice Liver transplantation	5	0.58–1.66	(16)	All jaundice positive except one
Development of jaundice or liver cirrhosis Liver transplantation	6	5.26–17.77	(8)	All jaundice positive except one

Fig. 1. Sampling time and patients' characteristic.
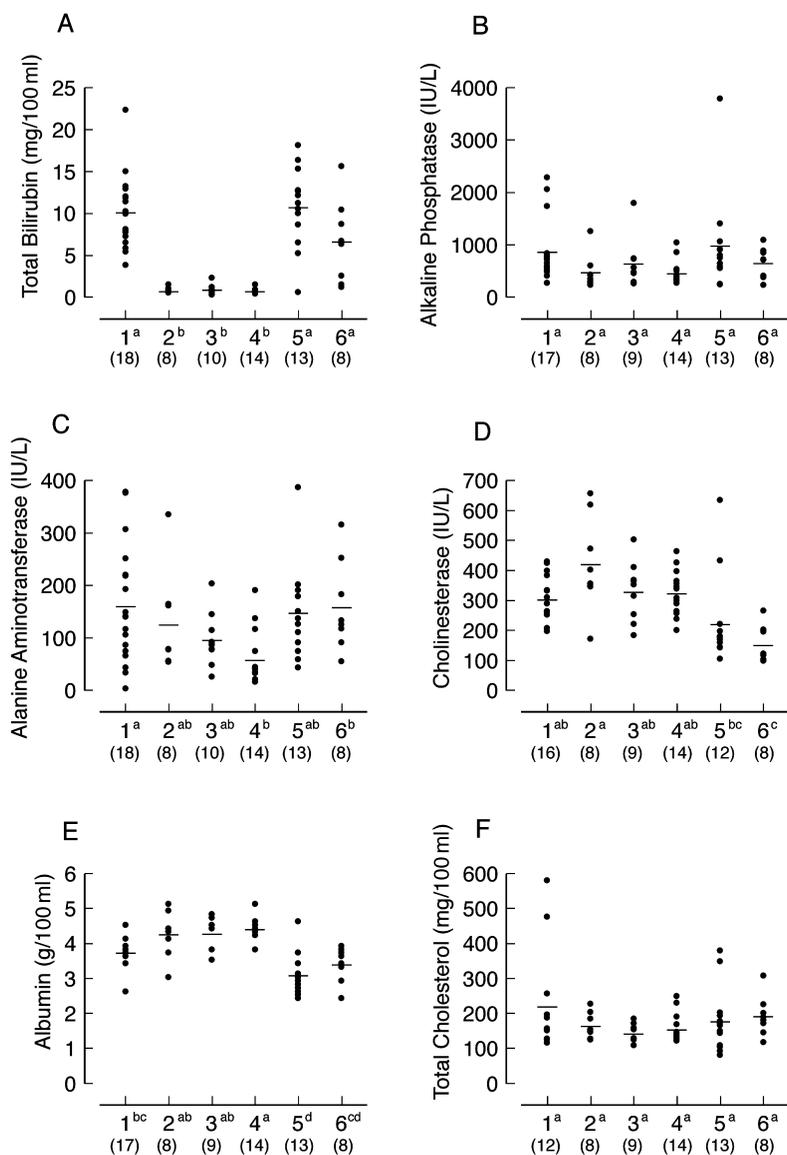
Fig. 2. Liver function at different Sampling Time and follow-up period.
Numerals under the horizontal axis indicate Sampling Time and those in parentheses numbers of samples. The same superscripts on the numerals indicate no significant difference by the least significant difference method.

other indicators showed such drastic changes.

Copper and zinc concentrations

Fig. 3 shows changes in Cu and Zn concentrations during sampling times. The liver Cu (HCu) concentration (Fig. 3A) decreased in the order of Sampling Times 1, 2, and 3. There was a significant difference between Sampling Times 1 and 3. At Sampling Time 5, the HCu concentration increased again, and it was higher than that at

Sampling Time 3, but there was no significant difference. Although there was only a single sample at Sampling Time 6, HCu concentration is remarkably elevated. The serum Cu (SCu) concentration was higher at Sampling Time 5 (Fig. 3C). It tended to increase at Sampling Times 4 and 6, although there was no significant difference from Sampling Time 1.

The liver Zn (HZn) concentrations were lower than the previously reported normal values
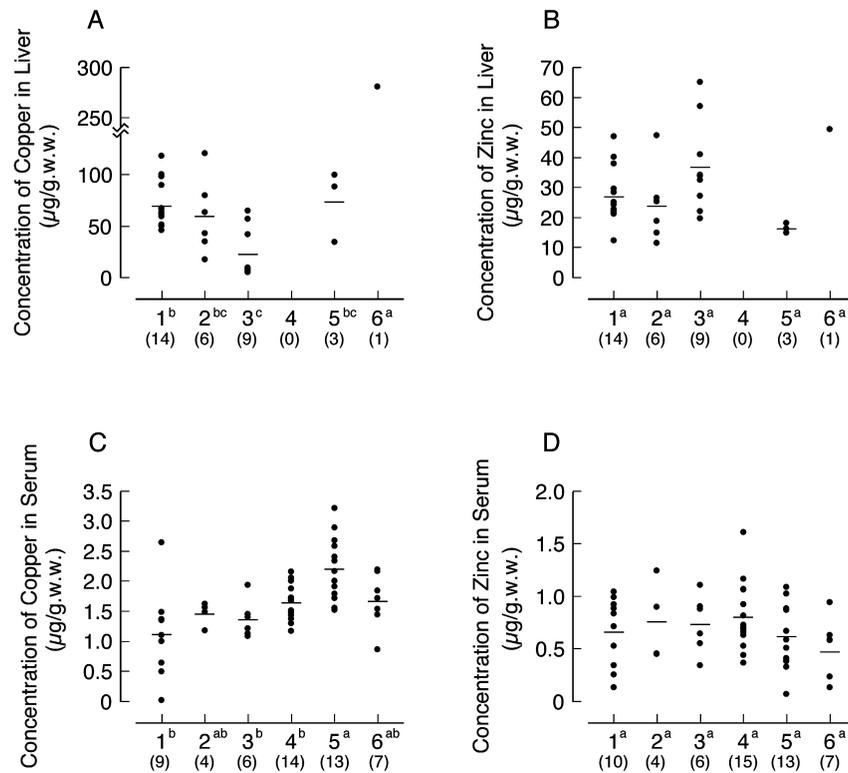
Fig. 3. Concentrations of copper and zinc in liver and serum at different Sampling Time and follow-up period.
Numerals under the horizontal axis indicate Sampling Time and those in parentheses numbers of samples. The same superscripts on the numerals indicate no significant difference by the least significant difference method.

for the infant (61 μg/g w.w.; Dorea et al. 1987) at all the Sampling Times (Fig. 3B). It tended to decrease from Sampling Times 1 to 2, but increased at Sampling Time 3. It was lower at Sampling Time 5. No significant difference was, however, observed among all the Sampling Times.

The serum Zn (SZn) concentration did not show remarkable changes at different Sampling Times (Fig. 3D). The averages at all Sampling Times, except for Sampling Time 6, were within normal range (0.61-1.07 μg/g: Endo 1995).

DISCUSSION

Cu is an essential trace element. It is absorbed in the duodenum, binds to albumin and amino acids in serum, and is then taken up by hepatocytes. It becomes free in hepatocytes, and is incorporated into ceruloplasmin (CP) and other

Cu-binding proteins and enzymes in microsomes. The major portion of Cu, however, is released into bile (Arakawa and Suzuki 1993). Accordingly, if the bile flow is disturbed such as in the case of cholestasis, Cu is accumulated in the liver and Cu metabolism is impaired. If an excessive amount of Cu is accumulated in hepatic tissues, cytotoxic reactions occur (Sternlieb 1980). Furthermore, Cu is a cofactor of lysyl oxidase, which is involved in the formation of molecular bridges in collagen. Therefore, an excessive accumulation of Cu in the liver and an increase in the serum copper concentration promote hepatic fibrosis (Arakawa and Suzuki 1993).

Physiologically, Cu accumulates at a high level in the liver of neonates (Widdowson et al. 1972). The HCu concentration is 10-fold higher than that of adult levels (Dorea et al. 1987; Bem et al. 1988). About 50% of Cu in the body of neo-

nates are stored in the liver (Widdowson 1974) and bind to metallothionein (MT) (Klein et al. 1991). MT is considered to be a Cu-storing protein, which protects hepatocytes from Cu cytotoxicity. Until 2-3 months after birth, the liver Cu concentration rapidly decreases.

It is speculated that in BA patients HCu concentration is high because of physiological conditions at neonatal period and pathological conditions of the disease. In the present study, HCu concentration decreased after establishment of the bile flow route and a significant decrease was noted between Sampling Times 1 and 3. But drastic changes in Cu metabolism physiologically observed were not observed. This is probably because the period of marked changes was within Sampling Time 1 (0.03-0.21 years old). Alternatively, the pathophysiology of the disease might underlie the disturbance of marked changes.

The SCu concentration of a neonate is low because CP is not yet synthesized. Then with the synthesis of CP in the liver, SCu concentration increased and HCu concentration markedly decreased (Widdowson 1974; Hatano et al. 1982). No appreciable changes were observed in SCu concentration at Sampling Times 1-3. This is probably because the pathophysiology of the disease might disturbed protein syntheses. It is desirable to examine the syntheses of CP and other Cu binding proteins in future studies.

In Sampling Time 5 (0.71-1.12 years old) the patients showed high HCu concentrations possibly due to cholestasis and high SCu was also noted. The high concentrations of HCu and SCu might induce the synthesis of proteins, such as, CP, MT and superoxide dismutase (SOD) to protect hepatocytes from Cu toxicity. The patients at Sampling Time 5 deteriorated to LC rapidly, because their ability to synthesize proteins was insufficient, or the amount of accumulated Cu might exceed the binding capacity of CP and MT to Cu as speculated by Arakawa and Suzuki (1993) in the case of PBC. Thus, Cu was released into the blood, resulting in an increased SCu concentration.

These findings with an extremely high HCu

at Sampling Time 6 indicates that although TB level decreased and kept low during the Sampling Times 2-4, it is plausible Cu was not excreted well from or even accumulated in the liver.

Hanamatsu et al. (1982) in our laboratory examined histological changes in the liver occurring between the radical surgery and fistula closure. They observed that, while bile stasis in the liver completely disappeared, hepatic fibrosis progressed in most of the patients. Kimura et al. (1980) reported that the recovery of the liver was not homogeneous in patients whose prognosis was excellent after the radical surgery. Their livers contained regions that were almost histologically normal and regions with prominent scar formation, postnecrotic and biliary cirrhosis caused by poor bile excretion and frequent cholangitis. They concluded that the nodal pathological changes described above were latent and were not reflected in liver function. Therefore, decrease of TB level does not indicate a good prognosis.

In contrast to the changes in Cu concentration, only slight and statistically not significant changes in Zn concentration were observed accompanying bile flow change. The HZn concentration was lower than the previously reported normal values at all Sampling Times. The mean HZn concentration of an infant (< 16 w) was reported to be 61 μg/g w.w. (Dorea et al. 1987). During the infancy period, the concentration gradually approached that of an adult; 80.2 ± 6.7 μg/g w.w. (Suita et al. 1988). The mean SZn concentration was within the normal range except that at Sampling Time 6. The normal SZn concentration range is 63-110 μg/100 ml (Endo et al. 1995) or 0.61-1.07 μg/g w.w. (calculated by the present authors from serum specific gravity). In general SZn concentrations were rather low, particularly in patients with severe cholestasis (Sampling Times 5 and 6), though no clear clinical symptoms with hypozincemia were observed in our patients. Our results agreed with those previously reported for BA patients (Suita 1986, 1987, 1988; Cywess and Miller 1990; Endo 1991; Bayliss et al. 1995).

Zn enzymes such as DNA and RNA poly-

merases exert strong effects on liver regeneration. Moreover, in the case of hepatic fibrosis, Zn works antagonistically to Cu (Arakawa and Suzuki 1993). Zn inhibits the cross-linking of covalent bonds in collagen through lysyl oxidase. Further, the demand for Zn increases at the time of stress such as invasive surgeries. Repeated surgeries and complications such as cholangitis may increase the demand for Zn. However, reduced liver protein synthesis decreases Zn bio-availability. The low HZn concentration might aggravate latent and local liver damage.

Considering the poor excretion of Cu from the liver and accumulation in the liver and possible interaction between Cu and Zn, even when the liver function of patients seems good, the conditions of a high HCu concentration and a low HZn concentration may aggravate latent hepatic fibrosis and LC. Therefore, it is necessary to develop an indicator to assess HCu accumulation. A possible candidate is SCu concentration, because the concentration increased at Sampling Time 5.

Acknowledgements

References

Arakawa, Y. & Suzuki, I. (1993) Hepatic disease and trace elements. *The Journal of Therapy,* **75**, 894-905. (in Japanese)

Bayliss, E.A., Hambidge, K.M., Sokol, R.J., Stewart, B. & Lilly, J.R. (1995) Hepatic concentrations of zinc, copper and manganese in infants with extrahepatic biliary atresia. *J. Trace Elements Med. Biol.*, **9**, 40-43.

Bem, E.M., Piotrowski, J.K., Sobczak-Kozlowska, M. & Dmuchowski, C. (1988) Cadmium, zinc, copper and metallothionein levels in human liver. *Int. Arch. Occup. Environ. Health*, **60**, 413-417.

Cywes, C. & Millar, A.J. (1990) Assessment of the nutritional status of infants and children with biliary atresia. *S. Afr. Med. J.*, **77**, 131-135.

Dorea, J.G., Brito, M. & Araujo, M.O.G. (1987) Concentration of copper and zinc in liver of fetuses and infants. *J. Am. Coll. Nutr.*, **6**, 491-495.

Endo, M., Fuchimoto, Y., Ukiyama, E., Tamura, T., Hoshino, K., Kuroda, T. & Yokoyama, J. (1991) Evaluation of postoperative zinc and copper dynamics in infants and children with biliary atresia with special reference to progression of liver cirrhosis. In: *Biliary Atresia*, edited by R. Ohi, Proceedings of the 5th International Sendai Symposium on Biliary Atresia, Icom Associates Inc., Tokyo, pp. 210-214.

Endo, M., Masuyama, H., Watanabe, K., Hagane, K., Ikawa, H., Yokoyama, J. & Kitajima, M. (1995) Calculation of biliary atresia prognostic index using a multivariate linear model. *J. Pediatr. Surg.*, **30**, 1575-1579.

Goksu, N. & Ozsoylu, S. (1986) Hepatic and serum levels of zinc, copper, and magnesium in childhood cirrhosis. *J. Pediatr. Gastroenterol. Nutr.*, **5**, 459-462.

Hanamatsu, M., Mochizuki, I., Ohi, R. & Kasai, M. (1982) The histological changes of the liver. *J. Jpn. Soc. Pediatr. Surg.*, **18**, 165-168.

Haraguchi, H., Kubota, M., Morita, M., Miyazaki, A., Fuwa, K. & Furuta, N. (1988) *Icp Emission Spectrometry.* 1st ed., Kyouritsu Syuppan Inc., Tokyo, pp. 1-94.

Hatano, S., Nishi, Y. & Usui, T. (1982) Copper levels in plasma and erythrocytes in healthy Japanese children and adults. *Am. J. Clin. Nutr.*, **35**, 120-126.

Kimura, S., Tomomatsu, T., Araki, S., Touken, H., Inaki, A. & Tomooka, Y. (1980) Studies on the postoperative changes in the liver tissue of long-term survivors after successful surgery for biliary atresia. *Jap. J. Pediat. Surg.*, **12**, 1043-1050.

Klein, D., Schols, P., Drasch, G.A., Muller-Hocker, J. & Summer, K.H. (1991) Metallothionein, copper, and zinc in fetal and neonatal human liver: changes during development. *Toxicol. Lett.*, **56**, 61-67.

Ohi, R. & Lilly, J.R. (1980) Copper kinetics in infantile hepato-biliary disease. *J. Pediatr. Surg.*, **15**, 509-512.

Reed, G.B., Butt. E.M. & Landing, B.H. (1972) Copper in childhood liver disease. *Arch. Path.*, **93**, 249-255.

SAS institute (2000) JMP, SAS Institute Inc., Cary, NC, USA.

Sternlieb, I. (1980) Copper and the liver. *Gastroenterology*, **78**, 1615-1628.

Suita, S., Ikeda, K., Doki, T., Yakabe, S., Matsuo, S., Ohgami, H., Sumitomo, K. & Iwanaga, M. (1986) Vitamin A and zinc status in children with biliary atresia. *Jpn. J. Pediatr. Surg.*, **18**, 1355-1362.

Suita, S., Ikeda, K., Doki, T., Naito, K., Handa, N., Matsuo, S. & Ogami, H. (1987) Zinc status and its relations to growth retardation in children with biliary atresia. *J. Pediatr. Surg.*, **22**, 401-405.

Suita, S., Ikeda, K. & Sakaguchi, T. (1988) Trace metal abnormalities during parenteral nutrition. *Jpn. J. Pediatr. Surg.*, **20**, 721-733.

Widdowson, E.M., Chan, H., Harrison, G. & Miller, R. (1972) Accumulation of Cu, Zn, Mn, Cr and Co in the human liver before birth. *Biol. Neonate*, **20**, 360-367.

Widdowson, E.M. (1974) Trace elements in foetal and early postnatal development. *Proc. Nutr. Soc.*, **33**, 275-284.