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Studies on Urea Cycle Enzyme Levels in the Human Fetal Liver at Different Gestational Ages

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ABSTRACT. Urea cycle enzymes involved in the detoxification of ammonia were studied in liver tissues of 57 male and 49 female fetuses of different age groups ranging from 13 to 36 wk of gestation. Surgical wedge biopsies of liver from 18 male and 12 female adults were used as controls. Significant enzyme activity was found to be present as early as the 13th wk of gestation. As gestational age advanced, enzyme activity gradually increased, reaching about 90% of the adult activity by the 36th wk of gestation. (*Pediatr Res* 31: 143–145, 1992)

Abbreviations

CPS, carbamyl phosphate synthetase
OCT, ornithine carbamyl transferase
AS, argininosuccinate synthetase
AL, argininosuccinate lyase
TCA, trichloroacetic acid

In mammals, urea is the main end product of nitrogen metabolism. Approximately 90% of the total nitrogen excreted by ureotelic animals takes the form of urea (1). The main site of urea metabolism is liver. There are very few reports on urea cycle enzyme estimations in human fetal liver. Because the sample size in humans is very small, meaningful statistical data cannot be derived (2, 3). Slemons and Moris (4) reported the presence of urea cycle enzymes in maternal and fetal serum, but not in tissues. Hammet (5) demonstrated high levels of urea cycle enzymes in placenta of toxemic patients. Mandersheid (6) was the first to measure urea from fetal liver tissue using the tissue slicing technique of Krebs and Henseleit (7). Subsequent workers reported urea cycle enzymes and urea levels in fetal tissue, blood, and amniotic fluid of varying gestational age groups (8–13). The difference between urea cycle enzyme levels in rat fetal liver and in human fetal liver was first reported by Raiha and Kretchmer (9). They observed that rat fetal liver has negligible urea cycle enzyme activity. Kennan and Cohen (3) demonstrated urea cycle enzyme activity in human fetal liver at 12 wk of gestation.

Hepatocyte transplantation is emerging as an alternative to orthotopic liver transplantation in the treatment of acute liver failure (14, 15). Studies are being carried out to assess the efficacy of hepatocyte transplantation in acute and chronic liver failure. Transplantation of fetal hepatocytes has already been used in the treatment of Fabry's and Gaucher's disease, with results that have stimulated continued research (16). The biology of hepatocytes is also being studied in great depth. The data from these experiments justify the consideration of fetal hepatocyte transplantation in acute liver failure (17). In the present study, urea cycle enzymes were measured in human fetal livers (n = 106) starting at 13 wks of gestation. Fetal values for urea cycle enzymes are compared with those of adult human liver tissues used as controls.

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MATERIALS AND METHODS

Fetuses included in the study were collected within 2 h of abortion after consent was obtained from the parents or guardians. The fetuses were obtained from different maternity hospitals surrounding this research center (Intensive Liver Care Unit, Osmania General Hospital). Fetal samples were derived from spontaneous abortions, still births, and, occasionally, medical termination of pregnancies due to maternal causes. Premature infants near full term who died in the sick nursery were also used for the study after consent was obtained. The adult human liver tissue was obtained from patients undergoing laparotomy for nonliver diseases and was included in the study after consent was obtained. The guardians of the fetuses were informed about the use of fetal livers in the treatment of human patients suffering from acute hepatic failures.

This therapeutic research project was sponsored by the Indian Council of Medical Research, New Delhi. The ethical committee consists of prominent researchers and legal advisers who deliberate about the use of the fetal liver tissue for the treatment of human patients with fulminant hepatic failure.

A total of 106 human fetuses consisting of 57 males and 49 females was included in the study. They were arranged into six gestational age groups: Group I, 13–16 wk; group II, 17–20 wk; group III, 21–24 wk; group IV, 25–28 wk; group V, 29–32 wk; and group VI, 33–36 wk. Liver perfusion was done within 2 h of abortion by the method of Habibullah *et al.* (18). Thirty adult patients undergoing laparotomy were used as controls, and wedge biopsies of liver were obtained. The biopsy tissue was immediately chilled in a sterile, dry bottle of 5 mL capacity kept in an ice box. This tissue was not perfused but was thoroughly washed with the Hank's buffer used in fetal perfusion to remove blood elements. The biopsy tissue was minced before being used for the enzyme estimation. The enzyme estimations were done by the procedures described below.

CPS. CPS was assayed by the method of Levin (19). The liver was homogenized in a solution that contained 0.01 M ATP and 0.01 M $MgSO_4 \cdot 7H_2O$, adjusted to pH 6.7 at 37°C with solid $KHCO_3$. This homogenate was diluted 1:1 with 0.1% cetyl ammonium bromide until a final liver concentration of 1:10 was achieved. The preparation was kept cold until use.

Several solutions were required to form the substrate mixture. Solution A contained 0.03 M ATP and 0.03 M $MgSO_4 \cdot 7H_2O$, adjusted to pH 6.7 with solid $KHCO_3$. Solution B contained 0.04 M *l*-ornithine monohydrochloride and 0.04 M N-acetyl glutamic

acid and was adjusted to pH 6.0 with approximately 1 N KOH solution, made 0.4 M with a calculated amount of solid ammonium bicarbonate, and adjusted to pH 7.5 with solid $KHCO_3$. The substrate was prepared by mixing two parts solution A with one part solution B, resulting in a pH of ~7.0. The substrate mixture was gassed with CO_2 just before use until the pH was ~6.8 at 37°C. For the assay, 0.1 mL of homogenate was mixed with 0.3 mL of substrate solution in a small tube and incubated at 38°C for 20 min. To stop the reaction, 0.2 mL of 7% perchloric acid was added, the tube was centrifuged, and 0.25 mL of 1% dimethyl glyoxime in 96% ethanol was added to 0.25 mL of the supernatant, followed by the addition of 2.5 mL of acid mixture (4 g of phenazone in a mixture of 76 mL of concentrated H_2SO_4, 11 mL of concentrated H_3PO_4, and 163 mL of water). After thorough mixing, the tubes were covered with vaccine caps pierced by syringe needles and heated in a bath at 100°C in darkness for 20 min. The tubes were then cooled and read at 447 nm. The activity was expressed in μmol of citrulline formed per h per g tissue.

OCT. OCT was assayed according to the method described by Vaclav and Vera (20). In this method, 0.1 mL of 1.1% tissue homogenate was incubated with 0.2 mL of ornithine glycylglycine buffer (80 mg of glycylglycine base and 100 mg of *l*-ornithine monohydrochloride, dissolved in ~8 mL of water, pH adjusted to 8.0 by 1 N NaOH), along with 0.1 mL of carbamyl phosphate solution (12 mg/mL dissolved in cold, double-distilled water). The tubes were incubated at 37°C for 20 min. The control set was deproteinized before incubation by adding 0.1 mL of 0.45% phenyl mercuric borate and 0.15 mL of 10% TCA. Test reaction was stopped only after incubation. Both control and test samples were centrifuged at 4000 rpm. To 0.25 mL of the supernatant, 0.25 mL of dimethyl glyoxime and 2.5 mL of antipyrine in acid mixture were added. The samples were heated for 20 min in a boiling water bath. After cooling, the enzyme test was read against a blank at 447 nm. The activity was expressed in μmol of citrulline formed per h per g tissue.

AS. AS was estimated by the method described by Levin (19) and modified by Sadasivudu and Indira Rao (21). In the estimation of AS, the incubation mixture contained 0.8 mL of substrate mixture in 0.05 M phosphate buffer (pH 7.3), which contained 0.01 M each of citrulline, aspartic acid, ATP, $MgCl_2$, and arginase (21 U). Reaction started with the addition of 0.5 mL of 20% (wt/vol) homogenate (in 0.25 M sucrose). At the end of 1 h incubation at 37°C, 1.5 mL of acid mixture (one part concentrated H_2SO_4 and three parts concentrated H_3PO_4) and 0.1 mL of isonitrosopropiophenone (5% in absolute alcohol) were added. It was kept in a boiling water bath for 30 min. After cooling the tubes, absorbance was read at 540 nm. Simultaneously, a urea standard was set up by adding to the standard 0.5 mL of substrate mixture and 0.5 mL of water. The color that developed was read at 540 nm. A reagent blank was also set up. Enzyme activity was expressed as μmol of urea formed per g wet weight of tissue per h.

AL. AL was assayed by the method of Levin (19) as described by Sadasivudu and Indira Rao (21). The assay system for AL consisted of 0.3 mL of 1 M phosphate buffer (pH 7.3), 0.6 mL of argininosuccinate (6.0 mM), 0.2 mL of 10% (wt/vol) homogenate in 0.25 M sucrose, and 0.1 mL of arginase (10.5 U). At the end of 1 h of incubation at 37°C, the reaction was stopped by the addition of 1.2 mL of 10% TCA. Controls were identical, except that TCA was added before incubation. In the supernatant, the urea was estimated by the method of Wybenga *et al.* (22). The enzyme activity was expressed as μmol of urea formed per g wet weight of tissue per h.

Arginase. Arginase was assayed by the method of Herzfeld and Raper (23). Tissue was homogenized in a medium consisting of 56 mM imidazole and 56 mM $MnCl_2$ (pH 7.4). The homogenate was incubated for 10 min at 50°C. The activated preparations were centrifuged, and the supernatants were used for measurement of enzyme activity. Enzyme assay was carried out in a total of 0.8 mL of incubation mixture consisting of 100 μmol of *l*-arginine, 60 μmol of glycine buffer (both adjusted to pH 9.5 with 1.0 N NaOH), and 0.1 mL of 0.1% (wt/vol) homogenate. After incubation at 37°C for 10 min, the reaction was stopped by the addition of 0.5 mL of 50% (wt/vol) TCA before incubation. The mixture was centrifuged, and 0.5 mL of the supernatant was taken for estimation of urea by the method of Wybenga *et al.* (22). The enzyme activity was expressed as μmol of urea formed per g wet weight of tissue per h.

RESULTS

Table 1 shows the urea cycle enzyme activities in fetal liver tissues of different gestational age groups and in adult liver tissue. All of the urea cycle enzymes were detectable in the human fetus as early as 13 wk of gestational age. OCT, CPS, AS, and AL levels increased as gestational age advanced, reaching 80–90% of adult activity by 36 wk (Table 2). Arginase also displayed the same trend, but with gradual increase, and reached about 66% of adult activity by 36 wk. No difference in the enzyme activity was observed between males and females.

DISCUSSION

The direct measurement of urea production in human fetal liver at 3–4 mo of gestation was reported by Mandersheid (6).

Table 1. *Urea cycle enzyme activities in human fetal liver tissues in different gestational age groups compared with human adult liver tissues*

Group	Gestational age (wk)	Sex	*n*	CPS*	OCT*	AS†	AL†	Arginase†
I	13–16	M	10	130.8 ± 34.3	1125.6 ± 239.4	47.8 ± 8.2	86.8 ± 14.0	20 138.0 ± 3271.5
		F	7	142.2 ± 18.2	1156.8 ± 219.7	45.5 ± 3.06	81.7 ± 10.9	23 728.5 ± 3825.7
II	17–20	M	13	159.2 ± 34.2	1638.0 ± 225.7	53.7 ± 5.9	99.5 ± 9.40	29 364.7 ± 5714.3
		F	9	188.8 ± 22.4	1676.6 ± 167.2	56.2 ± 10.5	115.2 ± 16.5	34 752.2 ± 3784.8
III	21–24	M	10	184.0 ± 27.2	2306.8 ± 123.4	55.1 ± 4.0	109.4 ± 13.2	31 702.0 ± 3255.14
		F	9	194.8 ± 29.8	2333.3 ± 138.4	57.4 ± 3.9	123.1 ± 15.8	33 400.0 ± 3400.7
IV	25–28	M	10	198.8 ± 19.0	2540.2 ± 229.5	64.8 ± 10.6	144.6 ± 23.2	42 288.0 ± 2994.7
		F	8	197.7 ± 13.6	2565.0 ± 299.8	65.3 ± 7.4	147.2 ± 26.1	43 922.5 ± 4551.2
V	29–32	M	9	222.4 ± 12.8	3295.5 ± 331.0	66.7 ± 8.4	171.1 ± 28.5	47 888.8 ± 3212.0
		F	9	210.4 ± 28.4	3491.3 ± 357.6	75.8 ± 13.8	156.0 ± 32.6	48 601.1 ± 4118.3
VI	33–36	M	5	236.4 ± 12.4	4069.2 ± 415.5	71.4 ± 5.8	181.6 ± 28.3	50 474.0 ± 4476.8
		F	7	212.0 ± 13.3	3957.4 ± 238.6	70.5 ± 8.6	193.1 ± 15.9	53 338.5 ± 4280.0
Adult		M	18	276.6 ± 27.2	5301.3 ± 764.9	88.2 ± 9.1	218.0 ± 24.3	77 926.3 ± 13 398.1
		F	12	234.5 ± 21.0	5035.0 ± 1016.8	85.16 ± 8.97	205.8 ± 17.0	80 330.6 ± 4643

* In μmol of citrulline/g tissue/h.

† In μmol of urea/g tissue/h.

Table 2. *Urea cycle enzyme activities in human fetal liver tissues of different gestational ages expressed as percentage of adult normal human liver activity*

Gestational age group (wk)	Sex	CPS	OCT	AS	AL	Arginase
I (13–16)	M	47	21	53	39	25
	F	60	23	53	39	29
II (19–20)	M	57	30	60	45	37
	F	80	33	65	56	43
III (21–24)	M	66	43	62	57	40
	F	82	46	67	67	41
IV (25–28)	M	71	47	73	66	54
	F	84	50	76	71	54.5
V (29–32)	M	80	62	75	78	61
	F	89	69	89	75	60.5
VI (33–36)	M	85	76	80	83	64.5
	F	90	78.5	82.5	93	66
Adults	M	100	100	100	100	100
	F	100	100	100	100	100

Kennan and Cohen (3) assayed AS in three human fetal livers of 12, 20, and 40 wk gestation and in two adult livers. They observed AS activity in fetuses of 12 wk gestation. Later, the urea cycle enzyme levels in five human fetal livers of 16–20 wk gestation were studied by Raiha and Suinkonen (11). They noted significant enzyme activity even at 12 wk of gestation. They hypothesized that the enzyme levels in fetal and newborn livers may be lower than those in the adult, but the data are insufficient for conclusions about enzyme development and are also incomplete for comparison of enzymes at different gestational ages. The sample size is not large enough to allow definitive conclusions. Finally, these studies did not use fetal liver perfusion, which was used in our study to avoid measurement of specific enzymes in contaminating blood elements.

The results of this study demonstrate the presence of significant activity for all five urea cycle enzymes in the human fetus as early as 13 wk of gestation. Furthermore, these enzymes gradually increase during fetal life to reach about 90% of the adult activity by 36 wk gestation. Sex of the fetus does not make any significant difference in the level of urea cycle enzyme in fetal livers of comparable age. From this study, it is evident that fetal hepatocytes have the ability to synthesize urea and detoxify ammonia from an early period of gestation.

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