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Unconjugated and total estriol in human amniotic fluid – changes in the ratios between the two estriol levels with advancing gestational age

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Advances in methodology such as transabdominal amniocentesis for the withdrawal of amniotic fluid and radioimmunoassays for very sensitive and specific measurement of hormones and other physiological compounds have made amniotic fluid examinations a new tool in the biochemical monitoring of fetal well-being. Several studies on the composition of amniotic fluid cover the concentrations of total estriol in normal late pregnancy [6, 7, 12, 13]. In a few other studies examinations were undertaken whether and to what extent changes in the total estriol concentration in amniotic fluid accompany specific disorders during pregnancy [2, 8, 9, 14]. As far as our knowledge of pertinent literature goes, representative examinations of the content of unconjugated estriol in amniotic fluid and its ratio to total estriol in amniotic fluid are not in existence.

The present study had two main objectives, to clarify 1) how the concentrations of unconjugated and total estriol in amniotic fluid of normal human pregnancies with advancing gestational duration do change, and 2) whether the ratio of unconjugated to total estriol in amniotic fluid remains constant during the course of pregnancy or not.

1 Material and methods

172 amniotic fluid samples from 115 pregnancies with normal outcome were examined. Amniotic fluid withdrawals by means of ultrasonic-controlled transabdominal amniocentesis were performed in the gestational weeks 15–20 and 29–40. They

were indicated for various clinical reasons, chiefly to exclude chromosomal anomalies of the conceptus in the early second trimester, suspected fetal hypotrophy or retarded fetal lung maturation at term etc. Amniotic fluid withdrawals in the pregnancy period from week 21 to 28 were for obvious reasons so seldom carried out, that the few amniotic fluid estriol values available to us at this stage are not published in this paper. The newborn infants of the pregnancies examined had a birthweight of 3.32 ± 0.35 kg., their placentas weighed 0.63 ± 0.13 kg. The clinical and acidity state of the newborn infants immediately after birth was good, as judged by our modified scoring system (10.2 ± 1.1 points) and pH measurement in umbilical artery blood (median pH 7.31) [10].

The amniotic fluid samples were stored at a temperature of -20°C . until their processing, which took place at the latest within one week after withdrawal.

In each of the 172 samples examined, determinations of unconjugated and total estriol were made, applying the same radioimmunoassay procedure. All of the estriol determinations were performed in duplicate. The analyses of unconjugated and total estriol were carried out in the same RIA run on each patient.

1.1 Preparation of the amniotic fluid samples

For unconjugated estriol assay, 0.1 ml. of the sample were extracted with 3 ml. ether in stoppered

glass tubes by Vortex mixing for 3 minutes. Subsequently the tubes were centrifuged at 2,000 g and a temperature of 2°C. for 10 minutes. 1 ml of the supernatant solvent containing the extracted unconjugated estriol was transferred to the RIA tube, evaporated at 50°C. in a vacuum, and the estriol remainder was thoroughly dried.

For total estriol assay, the amniotic fluid samples underwent an enzyme hydrolysis which led to a cleavage of the estriol conjugates (sulphates and glucuronides) and made the liberated estriol ether-extractable. For hydrolysis a commercially available mixture of beta-glucuronidase/arylsulphatase (5.2 and 2.6 U./ml. respectively) from *Helix pomatia* was used (BOEHRINGER, Inc., Mannheim, Germany), for producing the optimal hydrolysis medium a 0.1 N acetate buffer solution of pH 4.6.

0.02 ml. amniotic fluid, 0.02 ml. enzyme mixture and 0.2 ml. acetate buffer were mixed in glass tubes and kept at 37°C. for 240 minutes, or at 53°C. for 90 minutes. In this way, complete hydrolysis of the estriol conjugates was achieved, as was shown in repeated tests with pure estriol conjugates in aqueous solution or in body fluids with known estriol content. The estriol recovery rate from the enzyme-hydrolyzed estriol conjugates was 95–100%.

The ether extraction of total estriol from the hydrolyzed amniotic fluid samples was then performed in the same way as that of unconjugated estriol. To each radioimmunoassay tube 0.1 ml. of the estriol-containing extract was given.

The extraction yield amounted to $98.3 \pm 1.4\%$ as measured by the recovery of added quantities of tritium-labelled estriol.

In order to obtain consistently reliable estriol results, we found the following steps of sample preparation essential:

- 1) Usage of highly purified ether from freshly opened containers (for example diethylether "Uvasol" for spectroscopy, E. MERCK, Inc., Darmstadt, Germany);
- 2) precooling of the ether to 0°C or below for all pipetting operations to avoid evaporative losses;
- 3) evaporation of the ether under subtle conditions and thorough desiccation of the hormone remainder.

1.2 Radioimmunoassay

To determine unconjugated and total estriol the same radioimmunoassay was performed which followed in essence the procedure described by TULCHINSKY and ABRAHAM [15]. The estriol extracts were incubated with estriol antiserum and labelled estriol under the following conditions: Incubation medium: 0.04 M phosphate-buffer pH 7.4 with 0.1% bovine serum albumin added; incubation temperature: 0° to 4°C.; incubation period: 2 hours. Following incubation free estriol was separated from antiserum bound estriol by addition of a charcoal/dextran suspension (1 mg. charcoal per tube). After centrifugation the radioactivity of the supernatant fluid was measured in a Liquid scintillation counter ("Isocap 300", NUCLEAR CHICAGO). The estriol concentrations of the samples assayed were calculated using a special computer program.

The estriol antiserum used was raised in white New Zealand rabbits through repeated injections of a conjugate of estriol-6-(O-carboxymethyl)-oxim with bovine serum albumin as immunogen (SORIN BIOMEDICA, Saluggia, Italy). This antiserum had a cross-reaction of 0.01% with estradiol and 0.001% with estrone at 50% displacement. The final working dilution of the antiserum had an estriol binding ability of approximately 50%.

As tracer 2,4,6,7-³H-(N) estriol in ethanol solution was used up to 0.5 µCi for 100 samples.

In the range between 10–100 pg. estriol per RIA-tube the intraassay variation coefficient was 5.4%, the interassay variation coefficient was 8%. In the RIA system applied, the lowest detectable amount of estriol was 6 ± 2 pg. The recovery of added known estriol amounts was nearly quantitative at 98%.

2 Results

Table I gives a comprehensive picture of the increase of unconjugated and total estriol in amniotic fluid during the early second trimester and the third trimester, as well as of the ratios between unconjugated and total estriol.

Gestational weeks	Number of samples assayed	Unconjugated estriol (ng./ml.) (mean $\pm$ s.d.)	Total estriol (ng./ml.) (mean $\pm$ s.d.)	Ratio of unconjugated estriol to total estriol (per cent; mean $\pm$ s.d.)
15-18	67	1.22 $\pm$ 0.52	27.51 $\pm$ 11.82	4.43 $\pm$ 1.79
19-20	19	1.81 $\pm$ 0.78	42.65 $\pm$ 16.54	4.24 $\pm$ 1.67
29-32	10	3.85 $\pm$ 1.87	215.37 $\pm$ 124.74	1.79 $\pm$ 1.07
33-36	39	3.84 $\pm$ 3.38	364.77 $\pm$ 215.34	1.05 $\pm$ 0.70
37-40	37	7.82 $\pm$ 6.89	777.21 $\pm$ 412.42	1.01 $\pm$ 0.58

Tab. I. Concentrations of unconjugated estriol and total estriol in amniotic fluid at early and late human pregnancy; 172 amniotic fluid samples from 115 pregnancies with normal outcome assayed. Note the significant decline in the ratios between the unconjugated fraction and total estriol with advancing gestational age.

Fig. 1. Individual concentrations of unconjugated estriol in amniotic fluid related to gestational weeks.

2.1 Unconjugated estriol in amniotic fluid

Fig. 1. shows the individual values of unconjugated estriol related to the gestational week at amniotic fluid withdrawal. An increase in the concentration of unconjugated estriol in the amniotic fluid can be seen with advancing gestational duration. In the gestational weeks 15–18 as well as 19–20, the mean values lie below 2 ng./ml. and in the gestational weeks 29–32 and 33–36 below 4 ng./ml. Towards term a sharper increase of unconjugated estriol in amniotic fluid can be observed; the mean value in the gestational weeks 37–40 is more than double that of the corresponding mean values during the previous 8 gestational weeks.

The scatter of the single values around the appropriate mean values is considerable (Tab. I). Up to the gestational week 32, the relative standard deviations move between 40 to 50% (gestational weeks 15–18: 42.26%, 19–20: 43.03%, 28–32: 48.61%), thereafter they are even higher (gestational weeks 33–36: 88.03%, 37–40: 88.05%).

2.2 Total estriol in amniotic fluid

Fig. 2 shows the single values of total estriol in amniotic fluid in relation to the gestational age. Again, one can observe a continuous increase in the total estriol concentrations towards term;

Fig. 2. Individual concentrations of total estriol in amniotic fluid related to gestational weeks.

taken as a whole, however, it is much sharper than that in the concentrations of unconjugated estriol in amniotic fluid. Whilst the mean values of total estriol in amniotic fluid fluctuate between 20 ng./ml. and 50 ng./ml. during the early second trimester, in the gestational weeks 29–32 and 33–36 they lie very much higher, namely at 200 ng./ml. and 400 ng./ml. respectively. In the last four weeks of pregnancy the mean value shoots up even to 800 ng./ml. approximately.

The relative standard deviations are about 40% in the second trimester (gestational weeks 15–18: 42.95%, 19–20: 38.78%) and are thus similar to those of unconjugated estriol in the corresponding gestational stage. In the third trimester the deviations are greater than those in the second trimester, they lie between 50 and 60% (gestational weeks 29–32: 57.92%, 33–36: 59.03%, 37–40: 53.06%) and are considerably smaller than those of unconjugated estriol in amniotic fluid at the same gestational period.

2.3 Ratios of unconjugated to conjugated estriol in amniotic fluid

As described above, in the course of pregnancy the concentrations of unconjugated estriol in amniotic fluid increase to a lesser extent than those of total estriol. From this discrepancy in the elevation of the two estriol levels there results a decreasing proportion of unconjugated estriol to total estriol with advancing pregnancy. In the second trimester the ratios of unconjugated estriol to total estriol are on the average above 4%. They decline to term to a level of about 1% (Tab. I). The calculation of the regression equation shows a highly significant correlation between the decrease of these ratios and the increase of gestational age.

Fig. 3 shows as fairly large variation in the individual ratios with considerable overlapping of these, related to the stage of pregnancy. Nevertheless, in the amniotic fluid values from the early second trimester no single ratio under 3% was to be found. In contrast, in the third trimester and particularly towards term, ratios over 3% are rather unusual.

3 Comment

Concerning unconjugated estriol in amniotic fluid, relatively high concentrations of this estriol frac-

tion are characteristic of early gestation. Whether and to what extent assays of unconjugated estriol in amniotic fluid are of clinical importance, cannot be assessed at present. Probably estimations of unconjugated estriol in amniotic fluid are inferior to those of total estriol in amniotic fluid for the diagnosis of fetal distress, as, already in the course of normal pregnancy, the concentrational increase of unconjugated estriol in amniotic fluid is much less than that of total estriol in amniotic fluid and, moreover, the scatter of unconjugated estriol values in amniotic fluid in the third trimester is much higher than that of total estriol.

Our values of total estriol in amniotic fluid fluctuate during the third trimester in similar concentration ranges as reported by other authors at this stage of pregnancy. The sharp increase in total estriol in amniotic fluid towards term commencing in normal pregnancies about the gestational weeks 35 or 36 is conspicuous. Characteristically, in the gestational week 33–36 and 37–40 values of below 200 ng./ml. and 400 ng./ml. respectively hardly ever occur in normal pregnancies, a fact which may be useful in the diagnosis of fetal hypotrophy due to placental insufficiency [2].

There are very few reports concerning total estriol concentrations in amniotic fluid in the second trimester. In four publications such values about 13 cases have been given [1, 3, 4, 5]. In 7 cases examined by DICZFALUSY [3] and DICZFALUSY and MAGNUSSON [1] mean total estriol concentrations in amniotic fluid of 25.7 ng./ml. and 30.8 ng./ml. in the gestational weeks 12–20 were measured, values of the same order of magnitude as ours. In the 86 amniotic fluid samples of gestational weeks 15–20 examined by us, there was no value of total estriol over 80 ng./ml. and none below 10 ng./ml. Which clinical predictive value total estriol values in amniotic fluid in the second trimester might have, cannot be answered at this time.

The continuous decrease of the ratios between unconjugated and total estriol in amniotic fluid in dependence on the advance of pregnancy was unexpected for us. One may hypothesize that for the realization of this interesting phenomenon two events are essential. 1. It is conceivable that the large amounts of estriol, circulating in the feto-placental unit and being excreted with fetal

Fig. 3. Individual estriol ratios (unconjugated E3: total E3) in amniotic fluid related to gestational weeks, and their regression line. Equation of regression: $y = 7.81 - 0.18x$; $r = 0.81$, $2\alpha < 0.001$; $n = 172$, $\nu = 170$.

urine into amniotic fluid after onset of the fetal kidney function, in late pregnancy — with advanced biochemical maturation of the conceptus — reach the amniotic cavity to a greater extent as estriol conjugates than in previous stages of pregnancy, with the result that the proportion of unconjugated estriol in amniotic fluid decreases more and more towards delivery. As a matter of fact fetuses and freshly newborn infants do have obvious difficulties in conjugating other endogenous compounds such as bilirubin for urinary excretion, as exemplified by icterus neonatorum. Therefore it does not appear impossible that

similar transitory metabolic limitations are of influence to the fetal excretory pattern of estriol, however with another timing than in the case of bilirubin. 2. As in the early second trimester and previously, amniotic fluid is predominantly being produced by transudation from maternal plasma, and to a minor extent by fetal excretion [11], one may suggest that the unconjugated estriol fraction in amniotic fluid is in this phase of pregnancy essentially of maternal origin arriving directly to the amniotic cavity across the fetal membranes. Unconjugated steroids from maternal plasma penetrate via vascularized chorionic tissue and amniotic

epithelium into the amniotic cavity with ease. So it seems possible that the relative decrease of unconjugated estriol in amniotic fluid with advancing

pregnancy is a partial consequence of a relative diminution of this transudational area and an augmentation of the amniotic fluid volume.

Summary

In 172 amniotic fluid samples of the early second and the third trimester from 115 normal pregnancies the concentrations of unconjugated and total estriol were measured by means of radioimmunoassay. The mean concentrations of unconjugated estriol in amniotic fluid rose from 1.22 ng./ml. in the gestational weeks 15–18 to 7.82 ng./ml. in the gestational weeks 37–40, that is 6.4 times the former value. In contrast, the mean concentrations of total estriol in amniotic fluid increased more strongly, namely from 27.51 ng./ml. in the gestational weeks 15–18 to 777.15 ng./ml. in the gestational weeks 37–40,

that is 28.3 times the former value. With advancing pregnancy the proportion of unconjugated estriol to total estriol in amniotic fluid fell, obeying a significant correlation, from 4.43% in the gestational weeks 15–18 to 1.01% in the gestational weeks 37–40. The presumable reasons for this are the rising conjugation rate of estriol excreted with fetal urine, concomitant to fetal maturation, and the relative transfer diminution of unconjugated estriol from maternal plasma into amniotic fluid with the advance of gestation.

Keywords: Amniotic fluid, estriol concentrations, estriol radioimmunoassay, fetus, fetal maturation, gestational age, ratios between the two estriol levels, total estriol, unconjugated estriol.

Zusammenfassung

Unkonjugiertes Östriol und Gesamtöstriol im menschlichen Fruchtwasser – Veränderungen der Verhältniswerte zwischen den beiden Östriolspiegeln mit fortschreitender Schwangerschaftsdauer.

In 172 Fruchtwasserproben des frühen zweiten und des dritten Schwangerschaftsdrittels aus 115 normalen Schwangerschaften wurden die Konzentrationen von unkonjugiertem Östriol und Gesamtöstriol mittels Radioimmunoassay gemessen.

Die mittleren Konzentrationen des unkonjugierten Östriols im Fruchtwasser stiegen von 1,22 ng/ml in den Schwangerschaftswochen 15–18 auf 7,82 ng/ml in den Schwangerschaftswochen 37–40, d.h. das 6,4fache des erstgenannten Wertes.

Im Gegensatz dazu zeigten die mittleren Konzentrationen des Gesamtöstriols im Fruchtwasser einen viel stärkeren

Anstieg, nämlich von 27,51 ng/ml in den Schwangerschaftswochen 15–18 auf 777,15 ng/ml in den Schwangerschaftswochen 37–40, d.h. das 28,3 fache des Wertes aus der Frühschwangerschaft.

Mit fortschreitender Schwangerschaft verminderte sich der Anteil des unkonjugierten Östriols am Gesamtöstriol in einer signifikanten Korrelation von 4,43% in den Schwangerschaftswochen 15–18 auf 1,01% in den Schwangerschaftswochen 37–40.

Vermutliche Gründe dafür sind die mit der Fetalreifeung steigende Konjugationsrate des mit dem fetalen Urin ausgeschiedenen Östriols und die relative Verminderung des Übertrittes von unkonjugiertem Östriol aus dem mütterlichen Plasma ins Fruchtwasser mit Fortschreiten der Schwangerschaft.

Schlüsselwörter: Fet, Fetalreifeung, Fruchtwasser, Gesamtöstriol, Östriolkonzentrationen, Östriol-Radioimmunoassay, Schwangerschaftsdauer, unkonjugiertes Östriol, Verhältniswerte der beiden Östriolspiegel.

Résumé

De l'oestriol non-conjugué et de l'oestriol total dans le liquide amniotique humain – variations des taux respectifs des deux taux d'oestriol avec l'évolution de la grossesse. Les concentrations d'oestriol non-conjugué et d'oestriol total ont été dosées par méthode radioimmunologique sur 172 échantillons de liquide amniotique prélevés au début du deuxième et au cours du troisième trimestre de 115 grossesses normales.

Les concentrations moyennes de l'oestriol libre dans le liquide amniotique montent d'une valeur de 1,22 ng/ml entre les 15e et 18e semaines de grossesse à une valeur de 7,82 ng/ml entre les 37e et 40e semaines de grossesse, c'est-à-dire à une valeur 6,4 fois supérieure à celle observée initialement.

L'on observe par contre une augmentation nettement plus importante des concentrations moyennes de l'oestriol

total du liquide amniotique, soit de 27,51 ng/ml entre les 15e et 18e semaines de grossesse à 777,15 ng/ml entre les 37e et 40e semaines de grossesse, c'est-à-dire une multiplication par 28,3 de la valeur relevée en début de grossesse. La fraction de l'oestriol libre par rapport à l'oestriol total diminue significativement au cours de la grossesse, d'un rapport de 4,43% entre les 15e et 18e semaines de grossesse à un rapport de 1,01% entre les 37e et 40e semaines de grossesse.

Les raisons probables de ce phénomène sont l'augmentation du taux de conjugaison de l'oestriol émis dans l'urine foetale au fur et à mesure de la maturation foetale ainsi que la relative diminution du passage d'oestriol libre du plasma maternel au liquide amniotique avec l'avancement de la grossesse.

Mots-clés: Dosage radioimmunologique d'oestriol, durée de la grossesse, foetus, liquide amniotique, maturation foetale, oestriol libre, oestriol total, relation entre les deux taux d'oestriol, taux d'oestriol.

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