Relationship between asymptomatic hypercholanaemia of pregnancy and progesterone metabolism

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ABSTRACT

The aim of this study was to identify a subgroup of pregnant women with asymptomatic hypercholanaemia of pregnancy (AHP), in which the relationship between alterations in the level and pattern of serum bile acids (BAs) and of progesterone plus progesterone metabolites could be investigated in the absence of overt impairment of hepatobiliary function. Cholanaemia and serum concentrations of progesterone were assayed by an enzymic technique and by ELISA respectively, while BA molecular species and progesterone metabolites were measured by GC-MS, in the serum of 411 healthy pregnant women. Samples were collected after an overnight fast in the final week of each trimester of gestation. Two pregnant women were excluded because of the suspicion of intrahepatic cholestasis of pregnancy (ICP). Cholanaemia was found to increase progressively throughout pregnancy, but with normal mean values lower than 3.0 μM. Thus in our series AHP was defined arbitrarily as the presence of serum total BA concentrations 2-fold higher than this value, i.e. 6 μM, in the absence of hepatobiliary disease or symptoms of ICP. The prevalence of this condition was observed to increase with gestational age. Changes in the pattern of serum BAs in AHP were also found. These were reflected in a marked increase in the proportion of cholic acid together with a decrease in that of deoxycholic acid, while the proportions of chenodeoxycholic acid and lithocholic acid changed only moderately. When groups at the same gestational age were compared, serum progesterone levels were always significantly lower, while those of progesterone metabolites were higher, in women with AHP. Our results suggest that AHP is a relatively common condition in our geographical location, where ICP is rarely diagnosed. Changes in the serum BA pattern in hypercholanaemia resemble those described in ICP. The simultaneous finding of lower serum total progesterone levels along with an increase in its metabolites supports the hypothesis that a primary defect in progesterone metabolism may be involved in the aetiology of ICP.

INTRODUCTION

Intrahepatic cholestasis of pregnancy (ICP) is a liver disease that occurs in the second or third trimester of gestation and disappears spontaneously shortly after delivery. Since it was first described in 1954 by Svanborg [1], many studies have been devoted to elucidating different aspects of this condition. However, the aetiology, pathogenesis and regional prevalence of ICP are not yet well understood. Maternal manifestations are...
commonly benign, such as pruritus (known as pruritus gravidarum) and jaundice (known previously as idiopathic cholestatic jaundice of pregnancy). Although serious complications for the mother can occur [2–4], maternal symptoms are generally merely uncomfortable [5]. However, this disease has more frequent serious consequences for the foetus. ICP is usually accompanied by increased foetal distress and a higher risk of premature delivery, as well as enhanced perinatal morbidity and mortality [6]. ICP also impairs normal placental functions, such as excretion towards the mother of foetal bile acids (BAs) [7].

With regard to the aetiology of ICP, some evidence has suggested that this condition represents a multifactorial alteration in hepatobiliary function due to an abnormal reaction of the maternal liver to endogenous sex steroids or their metabolites [8,9]. Nevertheless, non-genetic factors, together with a metabolic disorder that can be transmitted genetically by individuals of either sex and which determines a predisposition to develop ICP, have also been proposed as factors that may govern the onset of the disease and modulate its clinical and biochemical abnormalities. More recently, a role for decreased selenium levels in the serum of patients with ICP has been suggested to be involved in the peculiar regional distribution of high-prevalence areas [10].

ICP can be classified among the various different types of hepatocellular cholestasis. These are caused by hepatocyte alterations at the basolateral, intracellular or canalicular level and, in many instances, at more than one of these locations at the same time, accounting for the impairment of bile formation [11]. One characteristic that is common to all varieties of hepatocellular cholestasis, including ICP, is an enhanced regurgitation of BAs from hepatocytes back to the blood, together with a lowered output of these compounds in the bile [12]. Thus hypercholanaemia is a sensitive marker of the impaired hepatobiliary function present in patients with ICP that can be detected in these patients by functional tests such as the clearance of sulphobromophthalein from blood; this is altered in normal pregnancies [13], but is decreased more profoundly in patients with ICP [14].

As regards pathogenic mechanisms, BA-induced toxicity is probably involved in the clinical complications usually reported to be associated with ICP [15,16]. This may explain why, as an alternative to previously employed drugs [17–20] that have been only partially successful as palliative therapy for ICP symptoms, administration of ursodeoxycholic acid, similar to its effect in other types of cholestasis associated with several liver diseases [21], has shown beneficial effects for pregnant women with ICP [22–26], with no deleterious effects on the newborn [23].

In a study that used RIAs to measure the postprandial levels of cholyglycinate in serum samples from a group of pregnant Australian women, the existence of a subgroup of asymptomatic individuals with higher than normal serum concentrations of this particular conjugated BA was reported [27]. Although in certain countries, such as Spain, the prevalence of ICP is considered to be low [28], as far as we are aware no studies have been conducted to establish the prevalence of subclinical ICP or of asymptomatic hypercholanaemia of pregnancy (AHP) in the general population. The aim of the present study was to identify a subgroup of pregnant women with AHP in which the relationship between alterations in the levels and pattern of serum BAs and progesterone plus progesterone metabolites could be investigated in the absence of a marked impairment in hepatobiliary function.

MATERIALS AND METHODS

Materials

BAs, Tris, 3α-hydroxysteroid dehydrogenase, diaphorase and resazurin were purchased from Sigma-Aldrich Quimica S.A. (Madrid, Spain). Heps was purchased from Roche (Barcelona, Spain). Steroids used as standards for GC-MS were purchased from Steraloids Inc. (Newport, RI, U.S.A.). All other chemicals were obtained from Merck (Barcelona, Spain) or Sigma-Aldrich Quimica S.A.

Subjects

This study was carried out on 411 healthy pregnant women (27±5 years old) whose pregnancies were followed at the Salamanca University Hospital between 1996 and 1999. Exclusion criteria were: presence or history of gallstones, cholecystopathy, pre-eclampsia or any other known disease that could cause liver malfunction, as well as the presence of antigenicity for viral hepatitis (HAV, HBV, HCV), HIV, cytomegalovirus, toxoplasma or Epstein–Barr virus. Two pregnant women with elevated serum biochemical markers for cholestasis and pruritus were excluded because ICP was suspected. One of them was later diagnosed as being at high risk of stillbirth, and labour was induced. In contrast, three women with pruritus but with serum biochemical markers within the normal range were included. Results from these three patients were qualitatively and quantitatively very similar to those for the rest of the women with AHP without pruritus. Informed consent was obtained from each patient. The Investigational Review Board of the University of Salamanca, Salamanca, Spain, approved the study protocol, which conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

In previous studies by our group (e.g. see [29]) we have found normal serum total BA concentrations after an overnight fasting period to be lower than 6 μM when assays were carried out using enzymic techniques, and lower than 10 μM when using more sensitive GC-MS...
measurements were carried out. After redissolving the diluted (1:4, v/v) with 0.4 M NH$_4$HCO$_3$, incubated at 64 °C for 2 h and passed gently through the cartridge. This was washed with water (10 ml), 10% (v/v) acetone (2 ml) and water (10 ml). BAs were then recovered with methanol (4 ml), which was then divided into two aliquots, which were dried and stored at −20 °C until measurements were carried out. After redissolving the BAs in one of the dry aliquots in methanol (200 μl), total serum BAs were measured by an enzymic fluorimetric assay based on the stoichiometric conversion of one molecule of 3α-hydroxy-BA into one molecule of 3-oxo-BA, together with the generation of one fluorescent molecule of resorfin from one molecule of resazurin through the sequential action of 3α-hydroxysteroid dehydrogenase and diaphorase in the presence of NAD$^+$ as cofactor [31]. Norchenodeoxycholic acid was added to the other aliquot as the first internal standard, after being subjected to enzymic desulphation, deglucuronation and deamidation using sulphatase, glucuronidase and cholyglycine hydrolase respectively [32,33]. Unconjugated products were extracted from the hydrolysate by liquid–solid extraction in C18 cartridges. Methyl ester derivatives were prepared by reaction with ethereal diazomethane. Trimethylsilyl ether derivatives were prepared in pyridine/hexamethyldisilazane/trimethylchlorosilane (3:2:1, by vol.) for 45 min at 55 °C [34]. Before injection into the gas chromatograph, 5β-cholestane was added to the samples as a second internal standard.

GC-MS analyses were carried out using a modification of the method described by Malavolti et al. [32] on a gas chromatograph (HP 5890 series II; Hewlett-Packard, Madrid, Spain) connected to a mass spectrometer (HP 5972; Hewlett-Packard), as described previously [35]. Using commercially available standards, five major progesterone metabolites in the serum of pregnant women [36], i.e. 5α-pregn-3β,20α-diol, 5α-pregn-3α,20α-diol, 5β-pregn-3α,20α-diol, 5α-pregn-3α-ol-20-one and 5α-pregn-3β-ol-20-one, were identified and their serum concentrations measured by GC-MS.

**Data analysis**

Values are given as means ± S.E.M. Comparisons between the normocholanaemic and hypercholanaemic groups at the same gestational age were carried out by Student’s $t$-test. Comparison in the same group at different gestational ages was carried out by a paired $t$-test. The least-squares method was used for linear regression analysis.

**RESULTS**

In the present work, both confirmatory and interesting new results were obtained. The total BA concentrations in the serum of pregnant women were found to increase progressively during pregnancy, but with normal mean values lower than 3.0 μM (Table 1). If a cut-off limit for normality is established at twice this value, i.e. at 6.0 μM, some healthy pregnant women could be included in a category that could be defined as moderate subclinical hypercholanaemia or AHP. No statistically significant association between this condition and maternal age or number of previous pregnancies was found. Once
Table 1  Serum biochemical analysis

Values are means ± S.E.M from serum samples collected from overnight-fasted patients at the end (final week) of each trimester of gestation. The numbers and proportions of women included in each group are given in Figure 1. Comparisons of biochemical parameters, except serum BA concentrations, between the normocholanaemic (serum BA concn < 6 μM) and hypercholanaemic (serum BA concn > 6 μM) groups at the same gestational age were carried out by Student’s t-tests.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>First trimester</th>
<th>Second trimester</th>
<th>Third trimester</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actual BA concentration (μM)</td>
<td>2.1 ± 0.1</td>
<td>8.9 ± 0.4</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>Total bilirubin (mg/dl)</td>
<td>4.0 ± 0.2</td>
<td>1.8 ± 0.7</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>176 ± 5</td>
<td>184 ± 5</td>
<td>213 ± 4</td>
</tr>
<tr>
<td>Triacylglycerols (mg/dl)</td>
<td>80 ± 4</td>
<td>130 ± 8</td>
<td>118 ± 3</td>
</tr>
<tr>
<td>Aspartate aminotransferase (units/l)</td>
<td>8.6 ± 0.4</td>
<td>9.0 ± 2.0</td>
<td>9.9 ± 0.4</td>
</tr>
<tr>
<td>Alanine aminotransferase (units/l)</td>
<td>8.2 ± 0.5</td>
<td>10.5 ± 1.5</td>
<td>9.9 ± 0.4</td>
</tr>
<tr>
<td>Alkaline phosphatase (units/l)</td>
<td>97 ± 4</td>
<td>100 ± 12</td>
<td>112 ± 3</td>
</tr>
<tr>
<td>γ-Glutamyltranspeptidase (units/l)</td>
<td>10.6 ± 0.6</td>
<td>8.4 ± 2.4</td>
<td>8.4 ± 0.4</td>
</tr>
</tbody>
</table>

Figure 2  Proportions of various BA species in the serum of healthy pregnant women with normocholanaemia or hypercholanaemia

Normocholanaemia (left panel; n = 16) and hypercholanaemia (right panel; n = 14) are defined as serum BA concentrations lower and higher respectively than 6 μM. Values are means ± S.E.M. from samples collected at the end (final week) of the third trimester of pregnancy. Significance of differences: * P < 0.05 compared with normocholanaemic group (Student’s t-test). CA, cholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid.

pregnancies of the same gestational age had been divided into either normocholanaemic or hypercholanaemic groups, the frequency of the latter condition was observed to increase as pregnancy progressed (Figure 1). However, as expected from the values of the parameters used as exclusion criteria for the pregnant women entering this study, no significant alterations above the normal range were found in the biochemical markers used in clinical practice to monitor liver function (Table 1). Moreover, no significant correlation was found between the degree of hypercholanaemia and any of these parameters (results not shown). However, three women belonging to the hypercholanaemic group, but none from the normocholanaemic group, had generalized pruritus.

The observed changes in serum BAs in the hypercholanaemic group were not restricted to the total concentration of BAs, but also affected the proportions of the different molecular species (Figure 2). When the pattern of serum BAs in the last trimester of pregnancy was compared between the normocholanaemic and hypercholanaemic groups, the major differences observed in the latter were a marked increase in the proportion of cholic acid and a decrease in that of the corresponding secondary BA, i.e. deoxycholic acid, while the proportions of chenodeoxycholic acid and its secondary derivative, lithocholic acid, did not differ significantly between groups. Regarding BAs that are less abundant in human serum, an increase in the proportion of urso-
deoxycholic acid and a decrease in that of o xo-BA species were observed in the hypercholanaemic group. Serum samples from six women belonging to each group in the second trimester of gestation were also analysed by GC-MS. The profiles of BA species in these samples (results not shown) were very similar to those described above for the same groups in the third trimester of pregnancy (Figure 2).

Since an increase in the formation of progesterone metabolites in pregnant women suffering from ICP has been described, the serum levels of progesterone and its major metabolites were determined. Serum progesterone concentrations, as measured by a highly specific ELISA kit, increased with gestational age. On comparing groups of the same gestational age, serum progesterone levels were always found to be significantly lower in women with hypercholanaemia (Figure 3). To elucidate whether the decrease in the serum progesterone concentration was accompanied by changes in the levels of progesterone metabolites, these latter species were enzymically deconjugated prior to carrying out GC/MS analysis. The values thus obtained revealed that the serum concentrations of progesterone metabolites were higher in hypercholanaemic than in normocholinaemic women (Figure 4). When measurements were carried out on samples from women in the second trimester of gestation belonging to each group (n = 6 for each group), the profiles of the five progesterone metabolites (results not shown) were very similar to those described for the same groups in the third trimester of pregnancy (see Figure 4).

The value obtained by adding the serum concentrations of these five metabolites was also significantly higher (P < 0.05) in the hypercholanaemic (3.47 ± 0.17 μM) than in the normocholanaemic (1.93 ± 0.20 μM) group.

DISCUSSION

One of the novel findings of the present work is the observation that AHP is a relatively common condition in Spain, where ICP is rarely diagnosed. The prevalence of ICP is particularly high in certain countries, such as Chile [5], Bolivia [37], Scandinavian countries [38] and Portugal [26]. In contrast, it is low (< 0.5 %) in other countries, such as the United States [39], Canada [40], Switzerland [41], France [42], Italy [43] and Spain [28]. Considering both the values for prevalence of the subclinical condition found in the present work and those of a previous study reporting the prevalence of overt ICP in Spain [28], it is possible to calculate that the proportion of pregnant Spanish women that develop ICP would be close to 2–3 % of those who have hypercholanaemia during the second–third trimesters of pregnancy. In fact, in the present work two pregnant women initially included in the population to be studied on the basis of the absence of primary or secondary liver pathology were excluded during the third trimester of gestation because of the suspicion of ICP. Our results are in agreement with those obtained in a previous study on a group of Australian pregnant women in which a subgroup (approx. 10 %) with reduced hepatobiliary function was identified on the basis of their elevated postprandial serum levels of cholyglycinate [27].

An interesting question arises as to whether the existence of such subgroups, defined by others from results obtained using RIA to measure a single conjugated BA [27] or using enzymic techniques to measure total
concentrations of 3α-hydroxy-BA s, as in the present work, is merely an artifact due to the wrong choice of the cut-off for normal values during pregnancy. However, we have found important changes in the proportions of various BA species in the sera of women included in the subgroup with AHP. Moreover, these changes resemble alterations in the pattern of serum BAs found in patients with ICP in a previous study, in which a methodology similar to that employed in the present one was used [44]. Thus both high levels of serum BAs and an altered serum BA pattern suggest the existence in AHP of a partial impairment in the mechanisms responsible for the hepatobiliary clearance of these molecules from maternal blood, which does not necessarily result in the clinical manifestations seen in women with overt ICP.

Another important aspect of the present study concerns the results indicating that there is a relationship between AHP and changes in the serum levels of progesterone and its metabolites. These findings have implications both for our understanding of the aetiology and pathogenesis of ICP and also for practical aspects. An interesting issue that remains unanswered is whether the increase in the levels of progesterone metabolites described previously in the serum of patients with ICP is the cause or the consequence of hepatobiliary impairment. In this respect, if a decrease in the clearance of progesterone metabolites by the liver were the consequence of an impairment in hepatobiliary excretory function, the serum levels of non-biotransformed progesterone would be expected to be unaltered or even enhanced. In contrast, if an increase in the biotransformation of progesterone to more polar metabolites was the primary event, the serum progesterone concentration would be expected to be lowered, or unaffected if enhanced metabolism were compensated by increased production. Our results show that, in women with AHP, a decrease in the serum progesterone concentration is accompanied by an increase in the concentration of its metabolites. This supports, but does not confirm, the hypothesis proposed by Meng et al. [36] that a primary change in the reductive biotransformation of progesterone, resulting in increased formation of progesterone metabolites, may be involved in the aetiology of the impaired hepatobiliary function that characterizes ICP. The combination of altered progesterone metabolism and the existence of predisposing dietary and genetic factors, such as mutations in the multidrug resistance 3 (MDR3) gene [45], might determine whether pregnant women develop ICP or merely AHP. The alternative hypothesis that both findings (increased hypercholanaemia and elevated serum levels of progesterone metabolites) might only be epiphenomena of a hitherto undefined primary alteration cannot be ruled out. However, several additional arguments support a primary role for altered progesterone metabolism in AHP and ICP. Among these is the fact that orally administered progesterone has been reported to act as an exogenous factor able to trigger ICP in predisposed women [42]. Moreover, the beneficial effect of ursodeoxycholic acid in patients with ICP [22–26] may be due in part to its ability to stimulate the biliary excretion of sulphated derivatives of steroid hormones [46].

In summary, our results suggest that AHP is a relatively common condition in our geographical location, where ICP is rarely diagnosed. Changes in the serum BA pattern in hypercholanaemia resemble these described in ICP. The simultaneous finding of lower total progesterone serum levels with an increase in its metabolites supports the hypothesis that a primary defect in progesterone metabolism may be involved in the aetiology of ICP.

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