

## The effect of maternal smoking and ethanol on fatty acid transport by the human placenta

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(Received 10 July 2000 – Revised 20 September 2001 – Accepted 20 November 2001)

The role of the placenta in controlling the supply of fatty acids to the fetus was investigated in term placentas from non-smokers ( $n$  5), smokers ( $>$ ten cigarettes/d;  $n$  5) and after addition of ethanol at 2 mg/ml ( $n$  4). The maternal side of the placenta was perfused *ex vivo* for 90 min with a physiological mixture of fatty acids and fatty acid:human albumin ratio. There was no effect of smoking on the transfer of linoleic (LA, 18:2  $n$ -6),  $\alpha$ -linolenic ( $\alpha$ LN, 18:3  $n$ -3), arachidonic (AA, 20:4  $n$ -6) or docosahexaenoic acid (DHA, 22:6  $n$ -3), expressed per perfused area (calculated from  $H_2^{18}O$  exchange). However, the presence of ethanol in the perfusate at a concentration of 2 mg/ml significantly reduced ( $P<0.01$ ) the absolute rate of transfer of the two  $n$ -3 polyunsaturated fatty acids,  $\alpha$ LN and DHA. This specific effect of ethanol on  $\alpha$ LN and DHA also resulted in an altered selectivity for transfer of individual fatty acids. In the non-smoking control group the placenta selectively transferred polyunsaturated fatty acids to the fetus in the order DHA  $>$  AA  $>$   $\alpha$ LN  $>$  LA. The order of selectivity was unaltered in placentas from smokers, but the addition of ethanol to the perfusion medium altered the order of selectivity to AA  $>$   $\alpha$ LN  $>$  LA  $>$  DHA. The presence of ethanol in the perfusate was also associated with a significant reduction ( $P<0.05$ ) in the clearance of  $H_2^{18}O$ . These results suggest that the presence of ethanol at a concentration of 2 mg/ml may reduce the availability of polyunsaturated fatty acids to the developing fetus.

**Placenta: Smoking: Alcohol: Transport: Polyunsaturated fatty acids:  
Docosahexaenoic acid: Pregnancy**

All of the  $n$ -3 and  $n$ -6 fatty acid structure acquired by the fetus has to cross the placenta, either in the form of the two essential fatty acids, linoleic (LA, 18:2  $n$ -6) and  $\alpha$ -linolenic ( $\alpha$ LN, 18:3  $n$ -3), or their long-chain polyunsaturated fatty acid (PUFA) derivatives, of which arachidonic acid (AA, 20:4  $n$ -6), eicosapentaenoic acid (EPA, 20:5  $n$ -3) and docosahexaenoic acid (DHA, 22:6  $n$ -3) are metabolically the most important. Apart from their role in maintaining the fluidity, permeability and conformation of membranes, these fatty acids also play a critical role in metabolic control as precursors of the prostacyclins, prostaglandins, thromboxanes and leukotrienes. The ability of the placenta to extract  $n$ -3 and  $n$ -6 fatty acids from the maternal circulation and deliver them to the fetal circulation is therefore important for normal fetal development (Kuhn *et al.* 1985; Kuhn & Crawford, 1986). Selectivity by the human placenta for the transport

of individual fatty acids from the maternal to fetal circulation has been suggested as one mechanism that might explain the fact that the concentrations of some long-chain PUFA are greater in the fetal than maternal circulation (Crawford *et al.* 1976; Hornstra *et al.* 1995; Crawford, 2000), a concept known as 'biomagnification' (Kuhn *et al.* 1985; Kuhn & Crawford, 1986). In addition, it has been shown that the human placenta has the capacity to preferentially transfer the essential fatty acids and long-chain PUFA (particularly DHA) from the maternal to fetal circulation (Haggarty *et al.* 1997, 1999).

Smoking during pregnancy has been reported to affect the morphology of the placenta and is associated with a reduction in birth weight (Ellard *et al.* 1996; Lindsay *et al.* 1997). Ethanol consumption during pregnancy has similarly been reported to reduce birth weight and placental weight (Larroque *et al.* 1993), although the

**Abbreviations:** AA, arachidonic acid; DHA, docosahexaenoic acid; LA, linoleic acid;  $\alpha$ LN,  $\alpha$ -linolenic acid; PUFA, polyunsaturated fatty acids.

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effect of ethanol on placental transport appears to differ for different nutrients and species. Hu *et al.* (1994) have shown that biotin uptake by basolateral membrane vesicles derived from human placenta is impaired by 10 h exposure to ethanol at concentrations of 2 and 3 mg/ml but not 1 mg/ml. Studies in rats indicate that acute and chronic maternal ethanol consumption can depress the placental uptake of a variety of amino acids with the acute effect acting via a Na<sup>+</sup>-dependent system involved in amino acid influx into placental cells (Patwardhan *et al.* 1981; Henderson *et al.* 1982). The addition of ethanol to the perfused human placenta apparently has no effect on the *in vitro* transport of two amino acid analogues;  $\alpha$ -aminoisobutyric acid and cycloleucine; (Schenker *et al.* 1989), thiamine (Schenker *et al.* 1990), or Zn (Beer *et al.* 1992), but does affect lipid metabolism by increasing the endogenous production of triacylglycerol, non-esterified fatty acid and phospholipid by the perfused placenta when added at a concentration of 3 mg/ml (Rice *et al.* 1986).

Approximately 50% of the DM of the human brain consists of lipid and about half of this is made up of long-chain PUFA (Gurr, 1993). Deposition of the long-chain PUFA in the fetus is rapid during the period of maximum brain growth in the last trimester (Clandinin *et al.* 1980) and it has been proposed that failure to accomplish a specific component of brain growth due to inadequacy of critical membrane lipids may result in irrevocable damage which cannot be fully restored at some later time and much attention has been given to the role of one long-chain PUFA in particular, DHA, in visual and intellectual development in children; see for example, Birch *et al.* (1992), Innis *et al.* (1996), Lucas *et al.* (1999). Ethanol consumption is known to affect the PUFA status of the brain, retina and liver (Salem & Karanian, 1988; Pawlosky & Salem, 1995) whilst fetal alcohol syndrome is the leading known cause of mental retardation (Bearer *et al.* 1992).

The purpose of the present study was to determine the effect of ethanol in the circulation or smoking during pregnancy on the capacity of the placenta to transport PUFA to the fetal circulation. There is no evidence that the placenta is intrinsically different after moderate alcohol exposure during pregnancy, but there is evidence of acute effects of moderate levels of alcohol on placental function and PUFA metabolism. Conversely, the reported effects of cigarette smoking on the placenta are largely in relation to morphological changes. Thus, the study was designed to investigate the acute effect of alcohol and the chronic effect of smoking since these are likely to be the most physiologically relevant. Specifically, we set out to determine the effect of smoking and ethanol on the order of selectivity and absolute placental transfer rates from the maternal to fetal circulation of the important PUFA (DHA,  $\alpha$ LN, LA and AA) when the maternal face of the placenta was perfused with a physiological mixture of fatty acids and albumin (Haggarty *et al.* 1999).

### Materials and methods

Ethical permission for the study was obtained from the Joint Ethical Committee of the Grampian Health Board and the University of Aberdeen and mothers taking part in the

study gave informed consent. The fatty acid composition of red cells was measured, as described by Lakin *et al.* (1998), in twenty-four mothers (eleven smokers, thirteen non-smokers) with uncomplicated full-term pregnancies, and perfusion carried out in a subset of these (*n* 14). To assess smoking frequency, a short multiple-choice questionnaire was used as this improves disclosure (Mullen *et al.* 1991). Only mothers who smoked ten or more cigarettes per d were included in the smoking group and the non-smokers did not smoke throughout pregnancy.

The perfusion experiments were conducted on term placentas delivered vaginally or by elective Caesarean section from otherwise uncomplicated pregnancies and the placentas were transferred to the perfusion chamber within 20 min of delivery. Both maternal and fetal circulations were perfused using the method described in detail elsewhere (Haggarty *et al.* 1997, 1999). The placental lobule was perfused with modified Krebs Ringer with the maternal perfusate being delivered at 20 ml/min by four glass cannulas inserted into the intervillous space and the outflow collected via a funnel placed under the lobule. The fetal perfusate (7 ml/min) was passed through a fine polythene cannula (i.d. 1.2 mm) inserted into the artery supplying the lobule, the outflow being collected by a similar cannula placed in the lobular vein. The preparation was maintained between 37 and 38°C. The solutions in both fetal and maternal circuits were oxygenated with O<sub>2</sub>-CO<sub>2</sub> (95:5, v/v). Both the fetal and maternal circulation were open throughout the perfusion. The open-circuit design, where both perfusates pass through the placenta only once, was employed because a closed circuit design could potentially result in back transfer of labelled fatty acids from the fetal to maternal circulation and non-steady state with respect to the maternal fatty acid concentration and possibly specific activity, thereby complicating the interpretation of the isotopic data.

The lobule was initially perfused for 30 min with Krebs Ringer containing no fatty acid or albumin. Both circuits were then switched to Ringer containing fatty-acid-free human albumin (5 g/l; Sigma, Poole, Dorset, UK); in the ethanol studies this and all subsequent perfusates also contained ethanol at a concentration of 2 mg/ml. After a further 30 min, the fetal circuit was changed to Krebs Ringer containing 5 g fatty-acid-free human albumin/l and the maternal circuit to Krebs Ringer containing 5 g fatty-acid-free human albumin/l and a mixture of radiolabelled fatty acids (Sigma) and H<sub>2</sub><sup>18</sup>O as a flow-limited marker. The albumin: free fatty acid molar ratio (0.78) was kept the same as it was *in vivo* in human pregnancy (Friedman *et al.* 1978; Olufemi *et al.* 1991); concentration of albumin 5 g/l, fatty acid 87  $\mu$ M; the proportionate fatty acid composition was designed to mimic the average fatty acid composition of the non-esterified fatty acid fraction from the fed and fasted blood samples collected from women in the last trimester of pregnancy (g/100 g total fatty acids): palmitic 59.9, oleic 23.5, LA 6.9,  $\alpha$ LN 1.3, dihomo- $\gamma$ -linolenic 1.0, AA 1.9, EPA 3.1, DHA 2.3 (Haggarty *et al.* 1999). The specific activity of the labelled fatty acids in the medium were (kBq/ $\mu$ mol): [<sup>14</sup>C]LA 28, [<sup>14</sup>C] $\alpha$ LN 162, [<sup>14</sup>C]AA 8, [<sup>14</sup>C]DHA 26. The fatty-acid-free albumin was dissolved

in the maternal and fetal perfusates. The labelled fatty acid mixture was taken up in a small volume of ethanol and added to the maternal perfusate and the mixture allowed to equilibrate overnight to allow the fatty acids to bind to albumin. The final concentration of ethanol in the maternal perfusates after addition of fatty acids was 0.1–0.2 mg/ml. In the perfusions designed to determine the effect of ethanol on placental fatty acid transport ethanol was added to increase the concentration by 2 mg/ml.

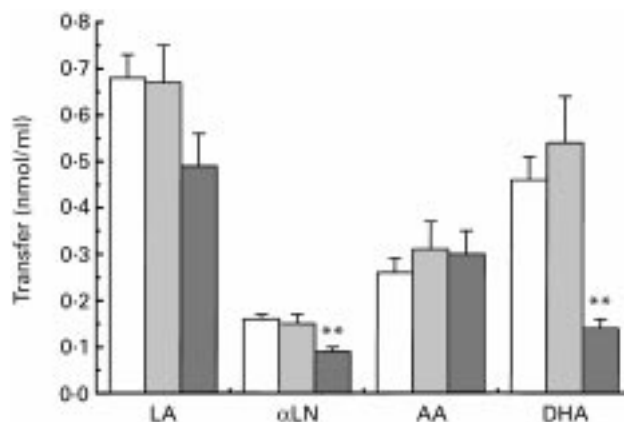
All the measurements were carried out under steady state as previously described (Haggarty *et al.* 1997). The perfusate was collected from the maternal and fetal circuits over 15 min periods and stored frozen until analysed. The pooled maternal and fetal perfusates were acidified to pH 3.0 and lipids extracted (Bligh & Dyer, 1959). After methylation the fatty acids were isolated by reverse-phase HPLC on a Rainin Dynamax<sup>®</sup> Microsorb<sup>™</sup> 4.6 × 250 mm column packed with 5 µm particle size C<sub>18</sub> (Rainin Instrument Co. Inc., Woburn, MA, USA) and protected by a C<sub>18</sub> guard column (Upchurch Scientific, Oak Harbor, WA, USA). Peaks were detected using a Waters 490E multiwavelength UV-Vis detector set (Waters, Elstree, Hertford, UK) at 198 nm and samples collected by a Gilson model 201 fraction collector (Gilson Medical Electronics S.A., Villiers-le-Bel, France). The solvents used were HPLC grade 'Far UV' acetonitrile (BDH Merck Ltd, Poole, UK) and 30 mM orthophosphoric acid (BDH Merck Ltd), both filtered through 0.45 µm nylon filters (Watman International, Maidstone, Kent, UK) and degassed with CP grade He (BOC Ltd, Manchester, UK). Fractions were collected every 2 min and the radioactivity determined by liquid scintillation counting on a Canberra-Packard 1900CA liquid scintillation counter (Packard Instruments B.V., Groningen, The Netherlands). The H<sub>2</sub><sup>18</sup>O concentration in the maternal and fetal perfusates was determined after equilibration with CO<sub>2</sub> (Midwood *et al.* 1992) on a SIRA-12 isotope ratio MS (VG, Middlewich, Ches., UK) relative to a series of laboratory reference waters previously calibrated against international standards.

An index of the perfused area in each experiment was derived from the clearance of H<sub>2</sub><sup>18</sup>O (ml/min) added to the maternal circulation, and the absolute rates of fatty acid transfer expressed relative to H<sub>2</sub><sup>18</sup>O clearance (nmol/ml). Clearance (ml/min) of the H<sub>2</sub><sup>18</sup>O from the maternal to fetal circulation was calculated from the arterial and venous fetal concentration difference, the maternal concentration and the fetal flow rate (ml/min):

$$\text{Clearance} = \frac{(\text{fetal arterial concentration} - \text{fetal venous concentration}) \times \text{fetal flow rate}}{\text{maternal concentration}}$$

Clearance of the individual fatty acids was similarly calculated using the arterial and venous fetal concentration difference, the maternal concentration and the fetal flow rate. The selectivity for individual fatty acids was assessed by expressing the clearance rate of αLN, AA and DHA relative to LA (Haggarty *et al.* 1997).

The significance of differences between the means was

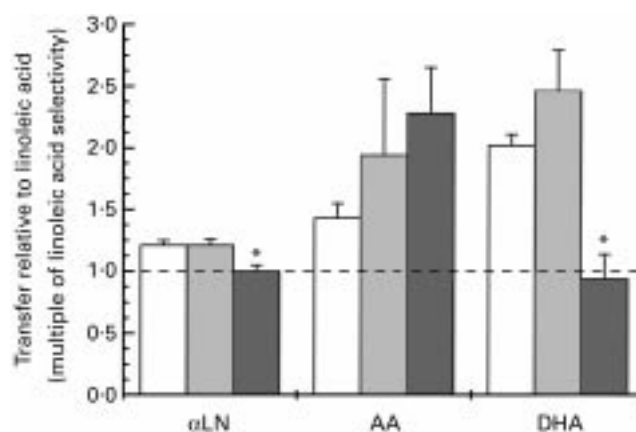


**Fig. 1.** Comparison of absolute placental transfer rates, normalised for perfused areas in exchange, (nmol/ml) of linoleic acid (LA), α-linolenic (αLN), arachidonic (AA) and docosahexaenoic acid (DHA), in placentas from non-smokers (*n* 5), smokers (*n* 5) and in control placentas (*n* 4) after addition of ethanol to the perfusate at a concentration of 2 mg/ml. For details of procedures, see p. 248. □, Control; ▨, smoking; ■, alcohol. Values are means with standard errors shown by vertical bars. Mean values were significantly different from those of the control group: \*\**P*<0.01.

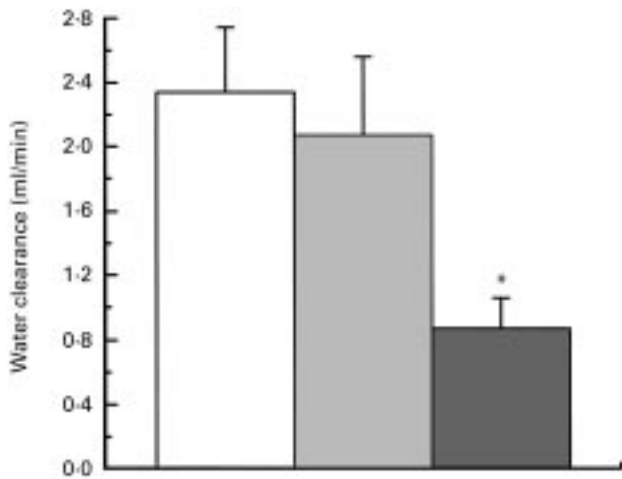
calculated by two-tailed *t* test (Minitab Inc., Philadelphia, PA, USA).

## Results

The rates of transfer of the *n*-6 fatty acids, LA and AA, per perfused area (calculated from H<sub>2</sub><sup>18</sup>O exchange) were not different between the control, smokers and ethanol group (Fig. 1). Neither was the rate of placental transfer of αLN and DHA affected by smoking during pregnancy. However, the presence of ethanol in the perfusate at a



**Fig. 2.** Comparison of selectivity for placental transfer of α-linolenic (αLN), arachidonic (AA) and docosahexaenoic acid (DHA), expressed as a proportion of that for linoleic acid (LA), taken as unity (— — —), in placentas from non-smokers (*n* 5), smokers (*n* 5) and in control placentas (*n* 4) after addition of alcohol to the perfusate at a concentration of 2 mg/ml. For details of procedures, see p. 248. □, Control; ▨, smoking; ■, alcohol. Values are means with standard errors shown by vertical bars. Mean values were significantly different from those of control group: \**P*<0.05.



**Fig. 3.** Comparison of water clearance rates as measured with  $H_2^{18}O$  in placentas from non-smokers ( $n$  5), smokers ( $n$  5) and after the addition of alcohol to the perfusate at a concentration of 2 mg/ml ( $n$  4). For details of procedures, see p. 248. □, Control; ■, smoking; ■, alcohol. Values are means with standard errors shown by vertical bars. Mean values was significantly different from those of the control group: \* $P$ <0.05.

concentration of 2 mg/ml significantly reduced ( $P$ <0.01) the absolute rate of transfer of the two  $n$ -3 PUFA,  $\alpha$ LN and DHA. The addition of ethanol resulted in an increase in the absolute rate of transfer of AA and a 60% increase in selectivity for this fatty acid over LA (Fig. 2), but this effect was not significant. However, the specific effect of ethanol on the  $n$ -3 fatty acids resulted in a significantly altered selectivity for transfer ( $P$ <0.05). When the placenta was perfused with fatty acids in the ratios found in maternal circulating non-esterified fatty acids, the placenta selectively transferred PUFA to the fetus in the order DHA > AA >  $\alpha$ LN > LA. The order of selectivity was unaltered in placentas from smokers but the addition of ethanol to the perfusion medium had a large enough effect on DHA to alter the order of selectivity to AA >  $\alpha$ LN > LA > DHA. Apart from an effect on transfer which was apparently specific to the  $n$ -3 fatty acids the presence of ethanol in the perfusate was associated with a significant reduction ( $P$ <0.05) in the clearance of  $H_2^{18}O$  (Fig. 3).

The fatty acid composition of red blood cells may be taken as a broad indication of the availability of individual PUFA in the fetal circulation over a period of weeks. Apart from a small difference between the smokers and non-smokers in the maternal red blood cell fatty acid concentration of 16:1 (0.50 (SD 0.14) *v.* 0.67 (SD 0.11) % respectively), there were no significant differences between the two groups in either the maternal composition, the cord blood composition or the difference between the maternal and cord blood composition (results not shown).

### Discussion

During most of pregnancy the fetus does not have a significant store of fatty acids such as DHA and is therefore dependent on a continuous supply of these fatty acids from the placenta to support development. Anything which

interrupts this supply during the relatively narrow time frames within which the key structures in the brain and retina are formed may have significant functional consequences. The purpose of the present study was to determine whether smoking and alcohol consumption during pregnancy could alter the capacity of the placenta to transport important PUFA to the fetal circulation. The perfusion results suggest that smoking during pregnancy has no effect on either the total placental transfer per perfused area or the selectivity for individual fatty acids and therefore that the previously described alterations in placental morphology in smokers appear to have no effect on fatty acid transport function. It is still possible that placental fatty acid transport may be impaired *in vivo* due to the effect of nicotine and the other components of cigarette smoke on placental blood flow for example. However, there were no significant differences between smokers and non-smokers in the fatty acid composition of maternal and cord red blood cells or in the percent difference between maternal and cord blood fatty acids. These results support the conclusions from the perfusion data that the placental supply of fatty acid to the fetus is likely to be unaffected in mothers who smoke during pregnancy.

The alcohol concentration of 2 mg/ml chosen for these present studies represents a compromise: it is the lowest level at which effects on placental transport had previously been reported for other nutrients (Hu *et al.* 1994) and lower than that previously shown to produce changes in placental export of lipid classes (Rice *et al.* 1986), and whilst it would represent a significant intake of alcohol (2.5-fold the legal limit for driving in the UK) people do drink to such levels of intoxication. When placentas were perfused with ethanol at this concentration, there was no significant alteration in AA transfer to the fetal circulation per perfused area but there was a reduction in the transfer of LA,  $\alpha$ LN and DHA and this effect was significant for the two  $n$ -3 fatty acids with the transfer of DHA in particular being reduced to a third of that found in control placentas. The selective alteration in the transfer of individual fatty acids resulted in a change in the order of selectivity from DHA > AA >  $\alpha$ LN > LA in the control placentas to AA >  $\alpha$ LN > LA > DHA in the presence of ethanol. The observed hierarchy of fatty acid transfer to the fetal circulation may arise for several reasons: (1) selective uptake by the syncytiotrophoblast; (2) different metabolic fates for individual fatty acids within the cell; and (3) differential release into the fetal circulation of fatty acids available within the cell. The available evidence indicates that selectivity for individual fatty acids operates both at the level of placental uptake (Campbell *et al.* 1996; Haggarty *et al.* 1999) and placental metabolism (Haggarty *et al.* 1997) and one or both of these may be affected by the presence of ethanol. Campbell *et al.* (1996) have reported preferential binding of fatty acids by isolated human placental membranes in the order AA > LA >  $\alpha$ LN > oleic and have proposed that such differential binding is mediated by a specific placental membrane fatty acid binding protein. It is worth noting that the order of selective transfer in the control placentas was similar to the order of double bond number (in parentheses) in these

molecules: DHA (6) > AA (4) >  $\alpha$ LN (3) > LA (2); therefore, the physico-chemical properties conferred by the number of double bonds may influence the preference for fatty acid transfer via the physical interaction of fatty acids with cell membranes and/or binding proteins. Ethanol may have a direct effect on the binding properties of fatty acid binding proteins, perhaps related to the *n*-terminus double-bond position, since the effect of ethanol was only significant for the two *n*-3 PUFA.

There is a report of a significant increase in the concentration of DHA in one of the minor lipid fractions (cholesterol ester) of cord serum from women who consumed alcohol during pregnancy but no difference in the other fractions (Denkins *et al.* 2000). However, such data provide only a broad indication of supply as levels of fatty acids in the blood, and the plasma and/or serum in particular, are affected by many factors in addition to placental transfer. Denkins *et al.* (2000) observed that alcohol intake in their study group was greatly diminished in the weeks before birth and they refer to animal studies showing unchanged or elevated DHA concentrations in the plasma but significant reductions in the DHA content of the brain of fetuses whose mothers were exposed to ethanol during gestation (Burdge & Postle, 1995; Denkins *et al.* 2000). Because of these issues, it is necessary to use perfusion or similar techniques to determine whether the difference in brain lipids is likely to be due to a limitation to transfer or some alteration in fatty acid metabolism within the fetal brain.

Ethanol consumption is known to affect the metabolism of PUFA and their concentrations in tissues such as the brain, retina and liver (Salem & Karanian, 1988; Pawlosky & Salem, 1995) via its effects on chain elongation and desaturation, and conversion to other metabolites such as prostaglandins and fatty acid ethyl esters (Cunnane, 1987; Laposata *et al.* 1987). The placenta is an important site of production of the prostacyclins, prostaglandins, thromboxanes and leukotrienes and it has been shown that ethanol addition to the perfused human placenta results in the production of prostaglandin E (Laposata *et al.* 1987; Randall *et al.* 1996). However, prostaglandin E is produced from AA, the placental transfer of which was actually increased by the addition of ethanol although not significantly so. Conversely, if the specific effect of ethanol on  $\alpha$ LN and DHA were to operate via prostaglandin production these would first have to be converted to eicosapentaenoic acid by chain elongation and desaturation and retroconversion respectively and there is little evidence for the former in the human placenta (Haggarty *et al.* 1997). Thus, although ethanol has been shown to alter prostaglandin production from PUFA within the placenta, the effect of ethanol on  $\alpha$ LN and DHA transfer may operate through some other mechanism, e.g. selective oxidation or esterification within the placenta, although it is possible that these other metabolic transformations may be influenced by the effect of ethanol on prostaglandins production. It is known, for example, that ethanol increases the endogenous production of lactate, triacylglycerol, free fatty acids and phospholipids by the perfused placenta (Rice *et al.* 1986), suggesting a direct effect on intracellular metabolism within the placenta.

The observed reduction in placental exchange area after addition of ethanol is consistent with previous studies showing that alcohol administration *in vivo* reduces placental blood flow in sheep (Falconer, 1990) and induces a dose-dependent vasoconstriction in the concentration range of 0.01–3.00 mg/ml *in vitro* in the perfused human placenta (Taylor *et al.* 1994). The vasoconstriction resulting from ethanol addition to the perfused human placenta may also be linked to fatty acid metabolism via its effect on prostaglandin production since the pressor effect of ethanol is largely removed by the presence of the COX inhibitor indomethacin (Taylor *et al.* 1994). Whatever the mechanism, it appears that ethanol results in a selective reduction in placental transfer to the fetal circulation of both  $\alpha$ LN and DHA. When this effect is combined with the reduction in blood flow to the placenta associated with the presence of ethanol, then it is apparent that there will be a reduction in the fetal availability of all the PUFA at an ethanol concentration of 2 mg/ml. The effect will be most pronounced for DHA, which is important for the normal development of the fetal brain and retina. Fetal alcohol syndrome can occur at lower concentrations of ethanol than the 2 mg/ml used here, therefore the relevance of the selective reduction in DHA transfer in particular to all cases of this syndrome would have to be confirmed in perfusion studies at lower concentrations. However, the previously reported dose-dependent vasoconstriction in the human placenta at much lower alcohol concentrations than 2 mg/ml (Rice *et al.* 1986) would in itself reduce placental transport of all the PUFA, including DHA, via its effect on placental blood flow.

### Acknowledgement

This research was supported by The Scottish Office Rural Affairs Department.

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