Supplementation of a restricted maternal diet with protein or carbohydrate alone prevents a reduction in fetal muscle fibre number in the guinea-pig

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A 60% reduction in maternal feed intake is known to cause a reduction of approximately 20% in *biceps brachii* fibre number in the guinea-pig fetus. This investigation was designed to isolate the dietary component responsible by reducing all dietary components to 60% of the *ad lib.* level and supplementing the protein, carbohydrate or fat component to the level of the *ad lib.* intake. Fetal muscles were examined at 50 d gestation to determine numbers of primary and secondary fibres, and at term to determine total fibre number. Fetal and neonatal weights were reduced in all restricted groups ($P < 0.05$) when compared with *ad lib.* controls. At term this reduction was significantly less ($P < 0.05$) in the protein-supplemented group (20%) than in the 60%-restricted and fat-supplemented groups (43%) and the carbohydrate-supplemented group (34%). *Biceps brachii* fibre numbers were reduced in the 60%-restricted and fat-supplemented groups by 14–16%, but fibre numbers were similar in control, protein-supplemented and carbohydrate-supplemented groups. Any reduction in fibre number was in the secondary fibre component of total fibre number. Therefore, *biceps brachii* fibre numbers were reduced only when maternal diets were deficient in both protein and carbohydrate.

Protein: Carbohydrate: Malnutrition: Fetal myofibre development: Guinea-pig

A 60% reduction in maternal feed intake is known to cause a reduction of approximately 20% in muscle fibre number in fast muscles (Ward & Stickland, 1991; Dwyer & Stickland, 1992). This reduction is in the secondary fibre component of total fibre number (Ward & Stickland, 1991). Secondary myotubes form as the second generation of muscle fibres, using the primary fibres as a framework on which to develop, and are considered more labile to nutritional influence than primaries (Handel & Stickland, 1987). The purpose of the present study was to determine which component of the maternal diet is most important in affecting the development of muscle fibre numbers.

Severe restriction of the protein component of the maternal diet (to 5 g/kg) in pigs results in a reduction of fetal weights, piglet birth weights, weaning weights and postnatal weight gains (Pond *et al.* 1968, 1987, 1991; Atinmo *et al.* 1974) and this stunting seems to be permanent (Pond & Wu, 1981; Pond *et al.* 1990). In the rat severe protein restriction caused smaller carcase and organ weights in late gestation fetuses (Zeman & Stanbrough, 1969). Mild protein restriction, however, has no effect (Pond, 1973). Energy restriction alone, with normal levels of protein, causes a greater reduction in fetal rat body weight than protein restriction alone (Anugwa & Pond, 1989), although this is not consistently the case in the pig (Pond *et al.* 1987). When non-protein energy restriction causes a reduction in piglet birth weight, this is associated with a low postnatal growth rate (Pond *et al.* 1987).

These findings, however, do not provide much information about the effect of these diets on the muscle cellularity of the developing fetuses. The effects of protein or carbohydrate

restriction in postnatal rats and children suggest that a low-protein diet leads to a reduced protein synthetic capability (Young & Alexis, 1968), therefore affecting cellular hypertrophy. A low-carbohydrate diet, however, causes reduced DNA levels but almost normal protein:DNA values (Cheek & Hill, 1970; Winick, 1970), therefore affecting hyperplasia of cells.

The present hypothesis was that the carbohydrate portion of the diet would affect the hyperplastic phase and, hence, myofibre number; the protein portion would affect only the hypertrophic phase, and not myofibre number. In the present experiment the diets were designed to supplement the individual component under study to *ad lib.* levels whilst all other nutritional components were reduced to 60% of an *ad lib.* intake. It should be emphasized, therefore, that supplemented dietary components were high only in relation to the other restricted components and did not exceed the levels eaten by the control animals.

MATERIALS AND METHODS

This project involved thirty-one pregnant Dunkin-Hartley guinea-pigs obtained from Bantam and Kingman Ltd (Aldbrough, Humb.). Guinea-pigs were in their second parity and weighed between 700 and 900 g at the start of the experiment. On day 2 of gestation females were housed individually in plastic cages and assigned to a nutritional treatment. Diets were based on SG1 pellets (Grain Harvesters, Wingham, Kent) which provide (g/kg): protein 190, starch and sugars 232·5, oil 42·5, fibre 112·5, digestible energy 9·60 MJ. Three test diets were formulated which were designed to provide control levels of the component under study and 60% of *ad lib.* (negative control) levels of all other components (Table 1). The compositions of these diets were as follows (g/kg): R+P, protein 273, starch and sugars 232·5, oil 42·5; R+C, protein 190, starch and sugars 335, oil 42·5; R+F, protein 190, starch and sugars 232·5, oil 91.

All diets were enriched with vitamin C. Pregnant guinea-pigs were randomly assigned to treatments as follows: (1) Controls, *ad lib.* access to SG1 pellets, *n* 6; (2) 40R, pair-fed with SG1 pellets at 60% of *ad lib.* intake, *n* 6; (3) R+P, protein supplemented, *n* 7; (4) R+C, carbohydrate supplemented, *n* 6; (5) R+F, fat supplemented, *n* 6.

All animals were given unlimited access to clean, fresh drinking water containing vitamin E (30 mg/l; Roche Products Ltd, Welwyn Garden City, Herts.) since they were not allowed access to hay for the duration of the experiment.

Three randomly selected pregnant animals from each nutritional group were killed at 50 d gestation by an intraperitoneal injection of sodium pentabarbitone (1 ml/kg body weight; Euthesate; Willows Francis Veterinary, a division of A. H. Robins, Crawley, West Sussex), and the remainder were allowed to litter and neonates were killed at term. Stillborn animals or pups which died within 24 h of birth were counted and weighed. Muscles were not always available from these animals, therefore, for consistency, muscles were analysed only from those animals which were alive at the time of sampling. *Biceps brachii* muscles were removed from all fetuses and neonates (*n* 99), mounted on cork chucks and rapidly frozen in hexane cooled in liquid N_2. Whole muscle transverse sections (10 μm) were cut at $-25°$ in a cryostat and stained for myosin ATPase (*EC* 3.6.1.32) activity after the method of Guth & Samaha (1970). At 50 d gestation all biceps muscle fibres are present but it is still possible to determine which fibres developed as primary and secondary fibres on the basis of their ATPase staining pattern (Ward & Stickland, 1991). Muscles from fetuses (*n* 57) were used to determine values for secondary:primary (S:P) fibres, and total fibre numbers. Neonates provided information about growth rate throughout gestation and muscles from neonates (*n* 42) were used to determine total myofibre numbers only, since cell lineages could no longer be distinguished at this age. All fibres were counted blind to

Table 1. *Example of the composition of diets eaten by different dietary groups, matched to a control animal eating 50 g feed* ad lib.*

Dietary group	Total feed (g/d)	Protein (g)	Carbohydrate (g)	Fat (g)	Total ME (KJ)
Control (*n* 6)	50	9·5	11·63	2·13	477·5
40R (*n* 6)	30	5·7	6·98	1·28	286·5
R+P (*n* 7)	33·8	9·23	6·98	1·28	363·2
R+C (*n* 6)	34·7	5·7	11·52	1·28	358·8
R+F (*n* 6)	30·9	5·7	6·98	2·90	345·4

ME, metabolizable energy; Control, *ad. lib.* access to SG1 pellets (Grain Harvesters, Wingham, Kent).
40R, pair-fed with SG1 pellets at 60% of *ad lib.* intake; R+P, protein-supplemented; R+C, carbohydrate-supplemented; R+F, fat-supplemented.
* For details of diets, see p. 174.

diet groups using a Seescan Image Analysis system (Seescan Plc, Cambridge). Nutritional groups were compared using Newman-Keul ANOVA statistics.

RESULTS

Maternal and fetal weight

The effect of nutritional treatments on maternal and fetal weight gain is shown in Table 2. At both 50 d gestation and term, control dams tended to gain more weight than any restricted group. Variations between individuals were, however, quite large. By term only the 40R group and the R+C groups showed a mean net weight loss when the influence of conceptus weight was removed (−23 and −3 g respectively). This was significantly different from controls only in the 40R group. Fetal and placental weights were reduced by undernutrition in all restricted groups. The greatest reductions were seen in the 40R and R+F groups (33%) and the least in the R+P group (16%). By birth the R+P group was intermediate between the control group and the remaining restricted groups. Neonatal mortalities also followed the same pattern and were restricted to the smallest neonates in each group; mean body weight of neonatal mortalities was 45·28 g (range 29·7–52·5 g, *n* 16).

Fibre numbers

Biceps brachii primary fibre number and mean S:P ratio were determined from 50 d fetal muscles and are shown in Fig. 1(*a* and *b*). Total muscle fibre numbers were counted in both 50 d fetuses and neonates. Results from fetuses and neonates could be combined since ANOVA tests revealed no significant difference in myofibre number between the two ages for any nutritional groups.

Primary fibre number did not differ between groups ($P > 0{\cdot}05$). However, mean S:P ratio was similar for control, R+P and R+C groups and significantly greater than those for 40R and R+F groups ($P < 0{\cdot}05$) by approximately 13·5%. Combined total fibre number data (Fig. 1(*c*)) demonstrate a similar pattern to that of mean S:P ratio, with total fibre number being reduced by 14–16% in the 40R and R+F groups ($P < 0{\cdot}05$).

DISCUSSION

In the present study it has been shown that a restricted maternal diet supplemented with either protein or carbohydrate was sufficient to maintain levels of secondary fibre hyperplasia (Fig. 1(*b*)) in the fetus at those of *ad lib.* controls, resulting in no reduction in *biceps brachii* fibre number (Fig. 1(*c*)). Primary fibre number is unaffected in any group

Table 2. *Effect of nutritional treatments on maternal weight gain, and on fetal, placental and neonatal weights in guinea-pigs**

(Values are means with their standard errors)

Dietary group...	Control		40R		R+P		R+C		R+F	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
50 d gestation										
Daily maternal wt gain (g)	5·6	1·3	0·2	1·8	3·4	0·8	1·8	1·4	−0·7	4·1
No. of dams	3		3		3		3		3	
No. of fetuses	9		13		13		10		12	
Term										
Daily maternal wt gain (g)	7.5[a]	1·1	0.7[b]	0·9	3.9[ab]	1·9	3·3[ab]	0·1	2·6[ab]	0·5
No. of dams	3		3		4		3		3	
No. of young	12		13		11		13		14	
Litter size: Median	3·5		4·5		3		4		4	
Range	2–5		3–5		2–5		2–6		2–6	
Fetal wt at 50 d (g)	36·6[a]	2·8	24·2[b]	1·8	30·6[b]	1·4	29·0[b]	1·5	24·6[b]	1·5
Percentage reduction	0·0		33·8		16·4		20·7		32·9	
Placental wt (g)	5·7[a]	0·4	3·6[b]	0·2	4·3[b]	0·2	4·5[b]	0·3	3·6[b]	0·2
Percentage reduction	0·0		37·6		24·9		21·3		36·5	
Birth wt (all) (g)	90·4[a]	4·6	51·1[c]	3·1	71·8[b]	3·7	59·4[c]	4·9	52·1[c]	3·6
Birth wt (live) (g)	90·4[a]	4·6	56·2[c]	3·4	73·3[b]	3·8	66·6[c]	5·1	67·5[c]	5·6
Percentage reduction	0·0		37·8		18·9		26·3		25·4	
Percentage mortality	0·0		46·15		9·10		30·86		46·15	

[a,b,c] Mean values with unlike superscript letters were significantly different ($P < 0·05$).

Control, *ad lib.* access to SG1 pellets (Grain Harvesters, Wingham, Kent); 40R, pair-fed with SG1 pellets at 60% of *ad lib.* intake; R+P, protein supplemented; R+C, carbohydrate supplemented; R+F, fat supplemented.

* For details of diets and procedures, see p. 174.

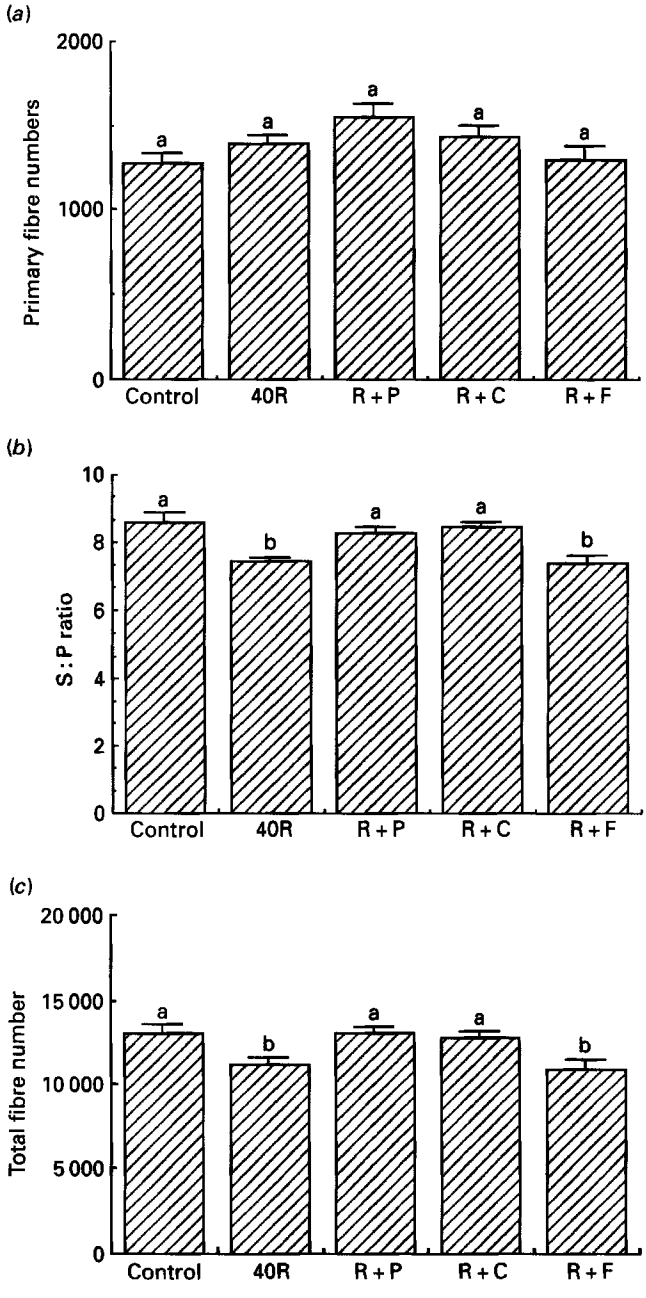

Fig. 1. Effect of nutritional treatments on mean *biceps brachii* myofibre numbers of guinea-pigs. (*a*) Primary fibre number, (*b*) secondary:primary fibre number (S:P) ratio, and (*c*) total myofibre number. Control, *ad lib.* access to SG1 pellets (Grain Harvesters, Wingham, Kent); 40R pair-fed with SG1 pellets at 60% of *ad lib.* intake; R+P, protein supplemented; R+C, carbohydrate supplemented; R+F, fat supplemented; for details of diets and procedures, see p. 174. Values are means with their standard errors represented by vertical bars. a,b, Mean values with unlike superscript letters were significantly different ($P < 0.05$).

(Fig. 1(*a*)). However, none of the restricted diets, despite supplementation, was able to maintain fetal growth at the same rate as that seen in the control *ad lib.*-fed fetuses (Table 2). The birth weights of the R + P protein-supplemented group were intermediate between those of the controls and other restricted groups. A high level of neonatal mortality was seen in the 40R (60 %-restricted) and R + F (fat-supplemented) groups (Table 2), which was restricted to the smallest neonates. In general, it seems that a body weight of less than 50 g severely compromises the survival of the neonatal guinea-pig. A low birth weight tends to be associated with a reduced myofibre number (Handel & Stickland, 1987), therefore, it is likely that these animals possessed a low fibre number. Thus, it is likely that the mean total fibre numbers of the 40R and R + F groups are an overestimation of the true values. This would explain the reduction in fibre number of only 15% in these groups; reductions of 20% have been reported for this level of undernutrition (Ward & Stickland, 1991; Dwyer & Stickland, 1992). However, these groups still had a significant reduction in fibre number (Fig. 1(*c*)). Furthermore, Fig. 1(*b*) demonstrates a significant reduction in secondary fibre proliferation in the 40R and R + F groups only, determined at day 50 when all animals were included. Therefore, despite the 30% neonatal mortality in the R + C group (Table 2), it is likely that the estimate for total myofibre number in this group is accurate and that fibre development in only the 40R and R + F groups was affected by the nutritional treatment. The 9% neonatal mortality in the R + P group is within the normal preweaning rate found in the guinea-pig (Sutherland & Festing, 1986).

Placental weight was reduced also in all restricted fetuses at 50 d gestation (Table 2). This tended to be by a similar amount to fetal weight, except in the R + P group where the decrease in placental weight seemed to exceed the decrease in fetal weight (respectively 25 and 16%). This suggests that the placentas of protein-supplemented animals may be more efficient than those of other groups such that a heavier weight of fetus is supported per unit weight of placenta. Evidence from the pig suggests that maternal protein levels at the time of implantation may be important for the early establishment and growth of the placenta (Pond *et al.* 1969).

The important metabolic fuels in the fetus are glucose, amino acids, lactate and fatty acids (Jones & Rolph, 1985). The fetus has high plasma and tissue levels of amino acids which are actively transported over the placenta from the maternal circulation (Jones & Rolph, 1985). Amino acids may supply as much as 25% of the oxidative needs of the ovine fetus (Gresham *et al.* 1972). Thus, amino acids make an important contribution to fetal oxidative metabolism, and a decrease in glucose supply can cause a shift to increased amino acid degradation (Battaglia & Meschia, 1978). This suggests that under conditions of low maternal plasma glucose there may be an increased fetal dependence on amino acids for oxidative metabolism at the expense of protein accretion. This may explain why R + P fetuses were lighter than controls despite similar maternal protein intakes. R + P fetuses were, however, significantly heavier than those of the other nutritional groups; thus, some of the extra protein must have been available to the fetuses for tissue accretion.

Amino acids may also act as precursors for gluconeogenesis. The placenta is incapable of gluconeogenesis (Bossi & Greenberg, 1972); therefore, any newly synthesized glucose must be of fetal or maternal origin. The gluconeogenic enzymes are present in the guinea-pig liver from day 40 of gestation (Jones & Ashton, 1976), but gluconeogenesis itself does not take place until late gestation (Jones & Ashton, 1976; Faulkener & Jones, 1976). Although there is some evidence that undernutrition may lead to an early initiation of gluconeogenesis (Jones, 1982), endogenous production of glucose by the fetus is unlikely to affect fibre numbers since the very early stages of gestation, the first trimester, appear to be the critical period for determination of fibre numbers (Dwyer *et al.* 1993). However, there may have been some maternal synthesis of glucose.

Proteins may also be involved in the maintenance of fetal fibre numbers in an indirect manner by altering or maintaining the endocrine status of the fetus, particularly via insulin and the insulin-like growth factors (IGF). Plasma insulin is reduced in the porcine fetus when the sow is fed on a diet deficient in protein (Atinmo *et al.* 1976), and in children with kwashiorkor (Soliman *et al.* 1986). Secretion of insulin has been shown to be sensitive to dietary protein (Jepson *et al.* 1988). Protein restriction of pregnant rats causes a decrease in circulating somatomedin levels in both fetal and maternal serum (Pilistine *et al.* 1984). Protein restriction of young postnatal rats also causes a reduction in serum IGF-1 and liver and muscle IGF-1 mRNA (Moats-Staats *et al.* 1989; Yahya *et al.* 1990; Vandehaar *et al.* 1991). IGF-1 appears to be linearly related to protein levels when energy levels are kept constant (Dardevet *et al.* 1991). Therefore, IGF-1 levels appear to be maintained independently by carbohydrate and protein levels. Thus, both the levels of glucose and amino acids may act via an insulin pathway, involving insulin and the IGF, to affect fibre number.

The role of fats in fetal muscle development appear fairly minor since plasma levels of fatty acids in the fetus are usually low until late gestation (Jones & Rolph, 1985). In addition, higher levels of fat in the maternal diet seemed ineffective at preventing low birth weight and postnatal mortality (Table 2).

In conclusion, the hypothesis that the carbohydrate portion of the diet would be sufficient to maintain control numbers of muscle fibres was confirmed. However, the protein portion of the maternal diet was also found to be equally effective. The extra protein in the diet may have been used as a supply of amino acids for oxidative metabolism, as precursors for gluconeogenesis, and/or may have played a role in the maintenance of fetal endocrinology, such that fibre number development was not impaired.

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