Aust. J. Biol. Sci., 1982, **35**, 517–25

Effect of Streptozotocin on Foetal Lambs in Mid-pregnancy

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Abstract

Streptozotocin was administered to 11 foetal lambs *in utero* at 70–85 days of gestation. Four of the foetuses survived and, when delivered at 134–142 days, exhibited significant growth retardation of the trunk and delayed osseous maturation in limb bones. The foetal kidneys and livers were most affected, but in three of the four foetuses, the weight of the brain was appropriate for gestational age. Likewise head size, measured by length or biparietal diameter, was normal. The insulin content of the foetal pancreas was less after streptozotocin-treatment than in normal animals of a similar gestation. Two streptozotocin-treated foetuses, catheterized at 120 days gestation, had higher glucose concentrations and lower insulin responses than in controls when infused with glucose. Plasma concentrations of ovine placental lactogen were lower in streptozotocin-treated foetuses than in controls, but serum somatomedin-like activity measured by receptor assay was greater than in controls. When the foetal serum was chromatographed on Sephadex G150 at acid pH, the major increase in somatomedin-like activity was found in the smaller molecular weight fraction (molecular size about 10000). The foetal growth retardation associated with streptozotocin administration in mid-pregnancy may be due to insulin deficiency, but the normal brain weight which occurred suggests that some other factor (possibly a somatomedin) regulates the growth of this organ.

Introduction

There is a variety of clinical and experimental evidence which suggests that insulin is an important growth hormone in foetal life. Infants of diabetic mothers are often large-for-dates and this is attributed to prolonged foetal hyperinsulinaemia (Pederson *et al.* 1954). Detailed autopsy of these infants indicates that there is an increased mass of muscle, bone, adipose tissue, subcutaneous tissue, and an enlargement of all visceral organs with the exception of brain and lungs (reviewed by Hill 1978). Conversely, the endocrine pancreas is small in small-for-dates infants (Van Assche *et al.* 1977). Infants who are born with pancreatic agensis or transient neonatal diabetes mellitus are profoundly growth-retarded (reviewed by Hill *et al.* 1980). Animal experiments have complemented these clinical observations. Diabetes mellitus in pregnant rhesus monkeys results in foetal macrosomia (Mintz *et al.* 1972; Cheek and Hill 1975), and such foetuses are both hyperglycaemic and hyperinsulinaemic (Mintz *et al.* 1972). The daily subcutaneous injection of insulin to near-term foetuses of pregnant rats increases the birth weight of the pups (Picon 1967). Chronic insulin administration to foetal rhesus monkeys accelerates foetal growth and results in enlargement of the foetal liver, placenta, heart and spleen (Susa *et al.* 1979).

However, there have been few attempts to ablate the foetal endocrine pancreas in order to study the effect of insulin deficiency in foetal life. In the only documented

study, attempted ablation of pancreatic beta cells of foetal rhesus monkeys at 110 days of gestation resulted in a variable disturbance to foetal growth (Cheek and Hill 1975). Streptozotocin, which is widely used to induce experimental diabetes (Rerup 1970), is capable of reducing the pancreatic secretion of insulin in foetal lambs (Shelley 1975). The aim of the present study was to examine the morphological and endocrine changes which follow the administration of streptozotocin to foetal lambs in mid-pregnancy. Particular attention was devoted to the measurement of serum somatomedin-like peptides (Daughaday *et al.* 1972), because these growth-promoting proteins have been implicated in normal foetal growth (Brinsmead and Liggins 1979*a*; Sara and Hall 1980), and because insulin has been implicated in regulation of the somatomedins (Chochinov and Daughaday 1976).

Materials and Methods

Animal Experiments

Merino sheep of the same strain were used throughout the study. Eleven pregnant ewes were subjected to laparotomy at 70–85 days of gestation as previously described (Brinsmead *et al.* 1980). The foetal head was delivered through a uterine incision without loss of amniotic fluid. Streptozotocin (Lot No. 60, 273–2 generously provided by Upjohn Pty. Ltd.), was dissolved in 2 ml of sterile citrate buffer, pH 4·0, at 4°C and injected into a foetal jugular vein. Five foetuses of 70 days gestational age were given 37·5–75 mg of streptozotocin (estimated to represent 200–400 mg/kg), and a further six foetuses were given 70–100 mg at 80–85 days gestation (250–330 mg/kg). Tetracycline (250 mg) was placed into the amniotic fluid, the foetal head returned to the uterus and the incisions repaired. Foetal life was checked at regular intervals thereafter by detection of foetal heart motion with ultrasound. The ewes were offered 1000 g daily of dried rumen-pelleted ration containing 14% protein and supplemented with essential minerals.

In two of the four pregnancies in which the foetus survived after the streptozotocin administration, laparotomy of the ewe was performed again at 120 days of gestation. Indwelling catheters were placed into a foetal carotid artery and a jugular vein as previously described (Brinsmead *et al.* 1980). Seven foetal lambs were catheterized at a similar gestation to act as controls. The catheterized animals, were left undisturbed for 6 days following surgery. At 127 days of gestation (streptozotocin-treated) and 110–126 days gestation (controls), two samples of blood were withdrawn from the foetal carotid artery within 30 min. A sterile solution of 50% (w/v) glucose in water was then infused into the foetal jugular vein at a rate of 16·9 mmol/h for 3 h. Blood samples of 6 ml were withdrawn from the foetal carotid artery at 60-min intervals from the commencement of the glucose infusion and placed into duplicate tubes containing heparin or flouride plus oxalate. The plasma was separated by immediate centrifugation and stored frozen at − 20°C. Foetal arterial blood pH and gas tensions were measured using a Corning 165/2 pH blood gas analyser at the beginning and end of each infusion.

The ewes with surviving pregnancies were killed between 134 and 142 days of gestation and foetal autopsy performed as previously described (Brinsmead *et al.* 1980). Foetal blood was collected by cardiac puncture and allowed to clot at 4°C for 24 h. The serum, separated by centrifugation, was stored at − 20°C. The pancreas from seven foetal lambs (two streptozotocin-treated and five controls) was dissected free of surrounding tissues and immediately frozen in sealed plastic bags. After 9–13 weeks the frozen portions were weighed, thawed, homogenized and extracted with 2 vol. of acid-ethanol (1 part concentrated HCl to 40 parts absolute ethanol). The homogenate was allowed to stand overnight at 4°C, then centrifuged at 10000 *g* for 30 min. The supernatant was neutralized with 1 M NaOH, stood for 60 min at 4°C and then recentrifuged. The resulting supernatant was diluted in buffer and assayed for insulin in content by radioimmunoassay.

Because only 4 of the 11 foetuses survived after streptozotocin administration and because their gestational age varied by 6 days at autopsy, their morphometry was compared to 22 normal foetal lambs killed between 125 and 145 days of gestation. Plasma placental lactogen and somatomedin concentrations were compared with those of catheterized foetuses previously reported (Brinsmead *et al.* 1980). Foetal hind limb ossification was assessed by radiology and compared with the data of Lascelles (1959).

Glucose and Hormone Assays

Plasma glucose concentrations were measured by the glucose oxidase method in a Beckman Analyser Model II. Insulin and ovine placental lactogen (oPL) concentrations were measured by specific radioimmunoassay as previously described (Brinsmead *et al.* 1980). Mean intra-assay variation for insulin and oPL determinations were 6·2 and 7·5% respectively, and mean inter-assay variation for oPL was 17·2%.

Somatomedin-like receptor activity (SmLRA) in foetal serum was measured using the radio-receptor assay described by Brinsmead and Liggins (1978*a*), and modified by the use of foetal liver cell microsomes as receptor (40 μg protein per tube) as previously described (Brinsmead *et al.* 1980). The ligand used was ^{125}I-labelled Multiplication-stimulating activity (^{125}I-MSA) with a potency of 86 milliunits of insulin-like activity per milligram in an insulin receptor assay. Serum was extracted by chromatography over Sephadex G150 at pH 2·5 before measurement of SmLRA. The SmLRA eluted in three regions (K_{av}. 0–0·10, 0·10–0·42 and 0·42–0·80), and these are designated I–III. In these assays SmLRA is expressed in terms of unlabelled MSA standard (μg/ml). The inter- and intra-assay variation were 13·1% and 11·1% respectively. The binding of ^{125}I-MSA to unextracted foetal serum was performed as previously described (Brinsmead and Liggins 1978*b*). The ligand was first purified by binding to and eluting from foetal liver receptor. In this assay the results are expressed as that percentage of the total added ^{125}I-MSA which did not bind to dextran-coated charcoal.

All results are expressed as the mean±s.d. Statistical comparisons were made with the paired or unpaired Student's *t*-test.

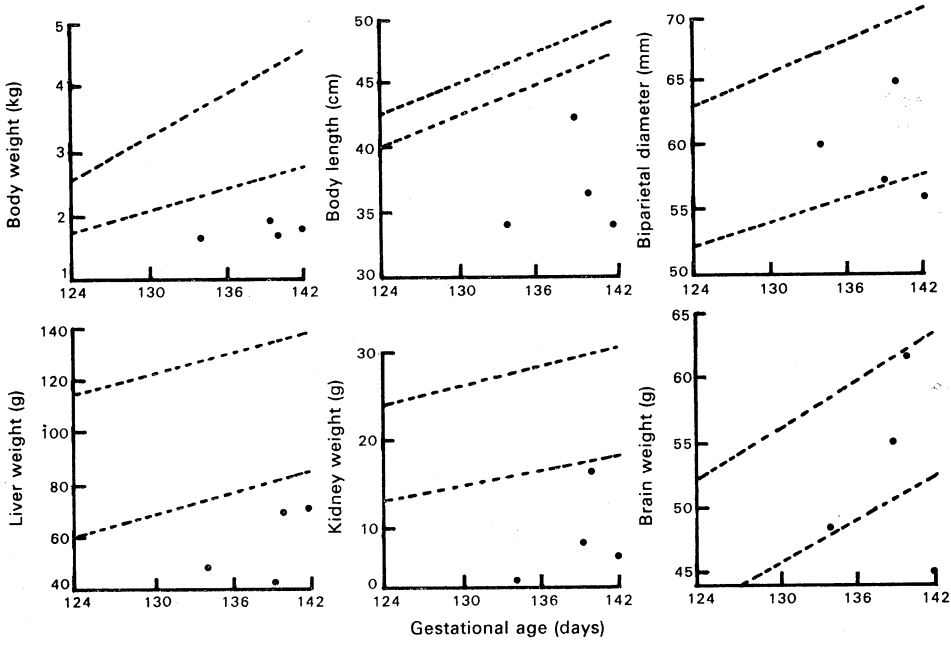

Fig. 1. Some morphometric characteristics of four foetal lambs treated with streptozotocin in mid-pregnancy. The area enclosed by the interrupted lines represents the range (mean±2 s.d.) of these characteristics in a group of 22 normal sheep of the same breed, killed in this laboratory.

Results

All foetuses given streptozotocin at 70 days of gestation died within 10 days of the treatment. These foetuses were not aborted per vaginum and the pregnancies were apparently resorbed. Four of the six foetuses treated after 80 days of gestation survived. In this group two foetuses were aborted and they had received the greatest doses of streptozotocin (>300 mg/kg).

At the time of delivery the four surviving streptozotocin-treated foetal lambs were significantly small for their gestational age, i.e. total body weight and crown rump length were greater than two standard deviations below the mean of normal foetuses (Fig. 1). Their limb lengths were also significantly less than normal (data not shown). However, their biparietal diameter (Fig. 1) and head length measured from the tip of the nose to the back of the occiput (data not shown) were within normal limits in three of the four lambs. Osseous maturity, as assessed radiologically by the appearance of ossification centres in the hind limb, was delayed by streptozotocin administration. The mean gestational age of the treated lambs at the time of autopsy was 139 ± 4 days, but the ossification of the hind limbs was consistent with only 129 ± 7 days of gestation. This difference is significant ($P < 0{\cdot}05$).

The weight of a number of visceral organs was also significantly less than normal. The kidneys and liver were affected the most (Fig. 1). In contrast the wieght of the brain (cerebrum and cerebellum together) was within normal limits in three of the four surviving foetuses (Fig. 1). The foetal adrenals were of normal weight and, like those of the foetal brain, relatively large for the body weights.

Table 1. Plasma glucose concentrations in two streptozotocin-treated foetal lambs and seven controls during infusion with glucose

Sheep No.	Plasma glucose concn (mmol/l) after infusion for:				
	− 30 min	0 min	60 min	120 min	180 min
Controls[A]	$0{\cdot}52 \pm 0{\cdot}25$	$0{\cdot}55 \pm 0{\cdot}44$	$2{\cdot}73 \pm 0{\cdot}70$	$3{\cdot}31 \pm 0{\cdot}81$	$3{\cdot}94 \pm 0{\cdot}23$
83F	1·1	1·1	7·0	7·1	3·8
76F	0·9	0·8	6·5	5·6	5·7

[A] Results are means ± s.d.

Random blood glucose concentrations were greater in the two catheterized and streptozotocin-treated foetuses ($0{\cdot}70 \pm 0{\cdot}27$ and $0{\cdot}96 \pm 0{\cdot}60$ mmol/l) than in the catheterized control animals ($0{\cdot}55 \pm 0{\cdot}44$ mmol/l) but this difference is not significant ($P > 0{\cdot}05$). Foetal arterial blood pH and pO_2 tensions in streptozotocin-treated animals were normal both before (pH = 7·426 and 7·420, pO_2 = 25·2 and 22·7 kPa) and after (pH = 7·300 and 7·410, pO_2 = 27·0 and 18·5 kPa) glucose infusion into the foetus for 3 hours. These values were not significantly different from those in the control animals before (pH = $7{\cdot}369 \pm 0{\cdot}017$, pO_2 = $2{\cdot}83 \pm 0{\cdot}39$ kPa) and after (pH = $7{\cdot}350 \pm 0{\cdot}042$, pO_2 = $2{\cdot}59 \pm 0{\cdot}19$) the glucose infusion. Glucose concentrations rose higher in streptozotocin-treated feotuses than in the control animals during glucose infusion (Table 1).

At the time of delivery, insulin concentrations in the plasma from the streptozotocin-treated foetal lambs varied considerably (0·1, 0·25, 0·35 and 1·25 ng/ml). However, the insulin response to glucose infusion in the two catheterized, treated foetuses was less than that observed in the control animals (Table 2). Likewise the insulin content of the pancreas from these two animals (each 0·5 ng/mg wet weight) was considerably less than that present in five normal animals of a similar gestation ($11{\cdot}4 \pm 2{\cdot}7$ ng/mg wet weight).

The results of assay of serum somatomedin activity and plasma oPL concentrations are summarized in Table 3. After acid extraction and gel chromatography of serum taken at the time of foetal death, peak III somatomedin-like activity (K_{av}, 0·42–0·80)

was found to be greater in streptozotocin-treated foetal lambs (15·4±4·9 μg/ml) than that in normal animals (3·7±1·9 μg/ml; $P < 0·005$). This fraction is thought to represent somatomedin-like peptides dissociated from their carrier proteins (Zapf *et al.* 1978). The percentage of binding of ^{125}I-MSA to whole serum from the streptozotocin-treated foetuses (19·0±2·8) was significantly less than that percentage of

Table 2. Plasma insulin concentrations in two streptozotocin-treated foetal lambs and seven controls during infusion with glucose

Sheep No.	Plasma insulin concn (ng/ml) after infusion for:				
	− 30 min	0 min	60 min	120 min	180 min
Controls[A]	0·683±0·307	0·914±0·462	1·20±0·580	1·516±0·760	1·485±0·383
83F	0·1	0·2	0·6	0·5	0·6
76F	0·3	0·2	0·45	0·45	0·85

[A] Results are means ± s.d.

^{125}I-MSA bound to serum from normal animals (24·2±0·2, $P < 0·005$). Likewise peak II somatomedin-like activity (K_{av} 0·10–0·42), which may represent the activity of somatomedin-binding proteins (Zapf *et al.*, 1978; Brinsmead *et al.* 1980) was lower in streptozotocin-treated foetuses (12·9±4·1 μg/ml) than that in serum from normal animals (17·5±1·1 μg/ml; $P < 0·05$). Plasma concentrations of oPL were lower than normal in the streptozotocin-treated foetal lambs.

Table 3. Serum somatomedin activity and plasma placental lactogen concentrations in foetal sheep after streptozotocin treatment

Results are mean ± s.d. Difference between streptozotocin-treated and control foetuses significant at $P < 0·05$

Treatment	Number of foetuses	Extracted somatomedin-like receptor activity (μg/ml)[A]		Binding of ^{125}I-MSA (%)	Placental lactogen (ng/ml)
		Peak II	Peak III		
Controls	5	17·5±1·1	3·7±1·9	24·2±0·2	101±29
Streptozotocin-treated	4	12·9±4·1	15·4±4·9	19·0±2·8	54±12

[A] Peaks II and III refer to elution fractions on Sephadex G150 and correspond to K_{av}. 0·1–0·42 and 0·42–0·8 respectively.

Discussion

Streptozotocin is widely used to produce experimental diabetes mellitus because of its specific cytotoxic action on beta cells of the pancreas (Rerup 1970). Streptozotocin consists of 1-methyl-nitrosourea linked to position C_2 of D-glucose and not surprisingly therefore it can be toxic to glucose-sensitive organs including the kidney and liver. In species other than sheep (e.g. rats, mice) its diabetogenic and lethal dose levels vary widely and little is known about these effects in the foetus. The high foetal mortality which followed streptozotocin administration in this study may indicate that its effects were due to some non-specific toxic action. In surviving foetuses the organ-weight disturbances may therefore reflect only that organ's sensitivity to the agent or the timing of the insult in relation to the organ's development. However, there is evidence from this and other studies that the effect of the streptozotocin on

foetal growth may have been due to pancreatic beta cell damage and subsequent insulin deficiency.

Shelly (1975), administered streptozotocin to foetal lambs in late gestation at a similar dose level to that used in this study and documented subsequent foetal hyperglycaemia and an impaired insulin response to a glucose challenge. The results of the present study also suggest that streptozotocin administered to foetal lambs in mid-pregancy is capable of inducing long-standing insulin deficiency because, in treated foetuses, the insulin content of the pancreas was low. Although basal plasma insulin concentrations varied widely within the normal range expected for this hormone (Bassett and Thorburn 1971), there was an impaired insulin response to a glucose challenge.

Cheek and Hill (1975) administered streptozotocin to foetal rhesus monkeys at about 110 days of gestation and killed the animals near term (160 days). As in the present study, there was considerable foetal mortality subsequent to the streptozotocin injection, but, in contrast to the present findings, most of the surviving foetuses were not growth-retarded but large for their gestational age. Significant islet hyperplasia was noted in the pancreas of the streptozotocin-treated monkeys, and it is possible that this may have been due to regeneration of the beta cells. Such cell regeneration has been noted after streptozotocin administration to adults of some species (Rerup 1970). Adrenal gland hyperplasia was noted in the streptozotocin-treated foetal monkeys (Cheek and Hill 1975) and in the present study relative adrenal enlargement was found in streptozotocin-treated lambs. While there is some evidence that adrenalectomy enhances the growth of foetal lambs (Barnes *et al.* 1977), Cheek and Hill postulated that the adrenal enlargement which followed streptozotocin administration to foetal monkeys may have been responsible for their greater than normal birth size.

The morphological changes which follow streptozotocin administration to foetal lambs are of interest when contrasted to those organ growth changes observed with chronic hyperinsulinaemia of the foetus. Women who are diabetic during pregnancy may deliver infants who are both hyperglycaemic and hyperinsulinaemic, and such infants exhibit a characteristic macrosomia with enlargement of all body organs with the exception of brain and lungs (Hill 1978). Similar changes are also seen in foetuses delivered to rhesus monkeys which have been rendered diabetic during pregnancy (Mintz *et al.* 1972; Cheek and Hill 1975). The low brain weight in such animals is due to fewer cells in both the cerebrum and cerebellum (Cheek and Hill 1975). Likewise, whilst chronic administration of insulin directly to foetal monkeys results in increased body and organ weight, the brains of these animals do not share in this growth enhancement (Susa *et al.* 1979). In contrast, the present study indicates that after streptozotocin administration to foetal lambs in mid pregnancy, there is a profound reduction in body weight and length, reduced liver and kidney weight, delayed long bone ossification, but normal cranial size and brain weight.

Sparing of brain growth is a characteristic of human and sheep foetuses delivered after chronic intrauterine oxygen deprivation (Robinson 1979). However, the streptozotocin-treated foetuses in the present study had normal blood gas pH and oxygen tensions when studied in later gestation. Chronic foetal hypoxaemia is associated with low plasma insulin concentrations and a low pancreatic content of insulin (Van Assche *et al.* 1977). Infants born with pancreatic agenesis are profoundly retarded, but there is relative brain sparing. Together, these observations

provide substance for our hypothesis that whereas pancreatic insulin is necessary for normal body growth, brain growth is regulated differently (Brinsmead and Thorburn 1981). It is possible that the major stimulus for foetal brain growth is a somatomedin as proposed by Sara and Hall (1979).

Serum somatomedin activity is lowered after induction of diabetes in adult rats (Phillips and Young 1976; Baxter *et al.* 1979), or pancreatectomy in dogs (Eingenmann *et al.* 1977). However, whilst insulin may be important for somatomedin generation in adult life, the results of the present study suggest that serum somatomedin-like activity is increased rather than reduced by insulin deficiency in the foetus. By way of corollary we have previously reported that serum somatomedin-like activity is low in human neonates with hyperinsulinaemia (Brinsmead and Liggins 1979*b*).

There is increasing evidence that serum binding proteins are of considerable importance in the activity of the somatomedins. Studies by Meuli *et al.* (1978) and Daughaday *et al.* (1980) have shown that these high affinity proteins specifically bind somatomedin peptides and inhibit their *in vivo* effects. Most of the presently available somatomedin assays are variously affected by these binding proteins and some form of extraction of serum is necessary prior to assay. Sephadex gel chromatography of serum samples at acid pH, whilst tedious in its performance, is valuable as it permits some estimation of the concentration of binding proteins. These are eluted in the early column fractions and are active in the assay (Brinsmead and Liggins 1978*b*). Alternatively, some estimate of the binding capacity of sera for somatomedins can be made by addition of radioactive ligand to unextracted samples, incubation and separation with activated charcoal. In a previous study (Brinsmead *et al.* 1980) we found that nephrectomy of the foetus results in increased concentrations of both small molecular weight somatomedin peptides and large molecular weight activity (binding proteins). In the present study whilst small molecular weight activity was increased to a similar degree to that found in serum from nephrectomized foetuses, large molecular weight activity (and the sera's capacity to bind the MSA ligand) was less than normal and significantly less than that which is measured after nephrectomy. The ratio of small to large molecular weight somatomedin activity is significantly greater after streptozotocin-treatment (12:1) than after nephrectomy (1:6). It is possible that placental lactogen may have a role in this distribution of activity since foetal oPL concentrations are elevated after nephrectomy (Brinsmead *et al.* 1980) and low after streptozotocin treatment (the present study). In extrauterine life growth hormone influences the concentrations of both free somatomedin peptides and their binding proteins (Zapf *et al.* 1978). The biological significance of the large molecular weight somatomedin-like activity is unknown. However, exploration of its possible role in the regulation of foetal brain growth may prove fruitful since, in contrast to the changes observed after streptozotocin treatment, nephrectomy of the foetal lamb results in considerable disturbance to the growth and development of this organ (Brinsmead *et al.* 1980).

Acknowledgments

This study was supported by The National Health and Medical Research Council of Australia. The authors are grateful to Garry Smith, Lincoln, Nebraska for providing Dowex 50 MSA; B. J. Bancroft, M. Norton, R. P. Young and K. Harrison for technical assistance and to Margaret Bell for typing the manuscript.

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Manuscript received 2 July 1981, accepted 20 July 1982