INFLUENCE OF MATERNAL ALLOXAN DIABETES OR INSULIN INJECTIONS ON FETAL GLYCOGEN RESERVES, MUSCLE AND LIVER DEVELOPMENT OF PIGS (SUS DOMESTICUS)\textsuperscript{1,2}

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SUMMARY

Pregnant Yorkshire gilts were allotted to three treatment groups during the third trimester of gestation. One group was alloxanized at 70 days of gestation; the second group was injected daily with protamine zinc insulin beginning at 80 days and the third group receiving saline injections served as controls. At 112 days of gestation, the fetuses were delivered alive by hysterectomy, cleaned and weighed.

The results showed that intravenous injections of 50 mg of alloxan/kg body weight was enough to cause diabetes in pigs and demonstrated that pigs could withstand severe diabetes during pregnancy. The body weight of the fetal pigs was not significantly altered. There was no impairment of the prenatal muscle development as evidenced by comparable gastrocnemius muscle weight, muscle DNA, RNA, protein, and semitendinosus muscle glycogen concentrations in the three treatment groups. The liver weight and total liver glycogen content were significantly (\(P<.01\)) elevated in the progeny of diabetic gilts at 112 days of gestation. The increased liver weight was accompanied by a significant increase in cellularity (DNA), RNA (\(P<.01\)) and protein (\(P<.05\)), but reduced cell size (protein/DNA) (\(P<.01\)) when compared to the control progeny. The liver RNA/DNA ratio was unaffected by the treatments. The results indicate that the extra glucose available to the fetuses was utilized for liver cell hyperplasia (DNA multiplication), glycogen, and protein synthesis. Insulin injections during pregnancy did not appear to influence fetal parameters in a significant way; body weight, muscle and liver weight were unaffected by insulin injections. Maternal serum glucose levels were not significantly altered by insulin treatment. Pigs may need a higher insulin injection to affect fetal growth and development. The experiment provided a mechanism for increasing fetal glycogen stores vital for the survival of pigs at birth, and also demonstrated that maternal diabetes did not alter fetal capacity for muscle and body growth.

(Key Words: Gestation, Diabetes, Fetal Development, Liver Glycogen, Pig.)

INTRODUCTION

The problems of newborn pigs are well known to both researchers and swine producers. The high mortality rate observed in neonatal pigs is common throughout the world and is often taken for granted by swine producers. The neonatal pig is vulnerable as it passes from the sheltered fetal life to the more hostile extra-uterine environment. Losses in swine industry result from production of underweight pigs (runts), overlaying of the sows, infections, nutritional and physiological inadequacies of piglets at birth. The insufficient insulation provided by sparse pelage and limited body fat predisposes the piglet to a metabolic demand which can hardly be maintained. The lack of brown adipose tissue (Bruck, 1970) and low level of fatty acid oxidizing enzyme at birth (Mersmann and Phinney, 1973) means that the newborn pig would depend on glycogen stores and blood glucose to subsist until sufficient energy supply is derived from the sow’s milk. Liver and muscle glycogen stores have been studied in pigs before and after birth (McCance...
and Widdowson, 1959; Mersmann et al., 1972; Hakkarainen, 1975; Herbein, 1976). Rapid accumulation occurred during the last few weeks preceding birth, followed by a less rapid loss in the muscle after birth when compared to the liver. Reports have shown that liver glycogen stores were rapidly depleted within the first 18 hr of postnatal life (Swiatek et al., 1968; Hakkarainen, 1975; Elliot and Lodge, 1977). Other studies showed that dietary treatment of the sow did not significantly increase the glycogen content of the liver (Elliot and Lodge, 1977). The rapid fall in liver glycogen stores after birth illustrated the importance of energy yielding reserves to the survival of the newborn pig.

Maternal-fetal glucose relationships studied in swine showed a negative correlation between glucose tolerance of the sow and the mean-body weight of the offspring (Anderson et al., 1971.) This would indicate that the fetal pig made use of elevated glucose to increase growth. On the other hand, limiting glucose availability to the fetus is expected to decrease fetal growth since glucose is the main metabolic fuel utilized by the fetus. Girard et al. (1974) have shown that maternal insulin injections decreased the level of fetal insulin and fetal blood glucose in rats. The present studies were designed to investigate the effect of maternal alloxan diabetes and insulin injection during the third trimester of gestation on fetal body weight, muscle and liver development. Furthermore, the experiment would determine whether the alteration in maternal glucose supply had any influence on the fetal liver and muscle glycogen reserves.

The frozen hind-quarter was later thawed and gastrocnemius muscle dissected out, weighed, and a representative sample taken from the entire length of the muscle for DNA, RNA and protein analysis. The thawed liver tissue was homogenized in .25 M sucrose media containing 1 mM dithiothreitol and 10 mM Tris (pH 7.4) with glass Teflon Potter Elvejhem homogenizer. An aliquot of the homogenized liver was used for DNA, RNA and protein DNA and RNA was determined by a modified Schmidt Thannhouser Method (1945) as previously outlined (Ezekwe and Martin, 1975). Protein was determined by the method of Lowry et al. (1951). Deproteinized serum was used for glucose assay. Glucose was determined by the glucose oxidase method (Sigma Chemical Co., St. Louis, MO).

Hormone Assay. Porcine immunoreactive insulin was assayed by the double antibody radioimmunoassay (RIA) of Hales and Randle (1963). Porcine insulin (Lilly Research Lab #615–D63–10) was used for radioiodination according to Greenwood et al. (1963) and Niswender et al. (1969). Antisera to porcine insulin prepared in guinea pigs (Miles Lab #64–104–16) was used. Second antibody was produced in sheep. The lower range of sensitivity for the conditions of our assay was 3.7 μU/ml of serum. All samples were done in a single assay.

Statistical Analysis. Statistical analysis was one by one-way analysis of variance. Differences between means were compared by Duncan’s new multiple range test and computed according to Steel and Torrie (1960).

MATERIALS AND METHODS

Eighteen Yorkshire gilts with known breeding dates were utilized. The animals were randomly allotted to three treatment groups. Six gilts each were assigned to controls (saline-injected), insulin-injected, and diabetic groups. Four days before initiation of treatments, gilts were removed from pasture and placed in a sheltered concrete pen. They were fed ad libitum on a diet containing 15.8% protein. Maternal diabetes was induced by intravenous (ear vein) injection of alloxan at 70 days of gestation according to methods of Romsos and Leveille (1971). A full dose of 200 mg/kg body weight of alloxan was to be injected in four divided doses of 50 mg/kg body weight. This design was adopted in order to monitor the severity of diabetes after each dose. However, a single injection of 50 mg/kg body weight, 25% of the level used by Romsos et al. (1971) and Phillips et al. (1976), was enough to cause severe diabetes. Diabetic condition was tested after 24 hr by the use of a commercial Test Tape and later by maternal blood sample (jugular vein) on day 3 and 8 after injection. Blood glucose of at least 300 mg percent was regarded as a positive indication of diabetes. All the gilts were diabetic. Two gilts died of peritonitis towards the end of gestation.

A daily injection of commercial protamine zinc insulin (.5 U/kg body weight) or daily injection of saline was used for the insulin and control group, respectively. Weekly blood samples from the jugular vein were taken from all the treatment groups (4 hr after injection of
Samples were centrifuged at 3,000 g for 20 min, the serum was collected and frozen at -20 C for insulin and glucose determinations.

At 112 days of gestation, pigs were delivered by hysterectomy from gilts made unconscious from carbon dioxide. The piglets were removed alive, cleaned and weighed. Fetuses (3 to 5) from each litter were utilized for all the tissue determinations. Fetal pigs were killed by decapitation. The liver tissue was quickly removed, blotted dry, and weighed; a piece was cut out and triplicate samples were immediately digested in 30% KOH saturated with Na2SO4 for glycogen determination according to methods of Lo et al. (1970). Samples of semitendinosus muscle were treated similarly for glycogen assay. The hind-quarter of the fetal pig was cut out and frozen at -20 C for later biochemical determinations. Samples of the liver tissue were frozen and used later for nucleic acid (DNA, RNA) and protein assays.

RESULTS

Pregnant gilts injected with 50 mg of alloxan/kg body weight exhibited a marked polyurea and glucosuria after 24 hr, and were severely hyperglycemic. Figure 1 shows serum glucose and insulin levels before the injection and at subsequent bleeding times. The preinjection serum glucose level for diabetic gilts was 106.2 ± 9.6 mg/100 ml. On day 3 post-injection (73 days' gestation), the level increased to 552.3 ± 95.4 and remained high throughout gestation. All diabetic gilts used for the study maintained a hyperglycemic condition greater than 300 mg/100 ml of serum. The pretreatment glucose level for the insulin and control gilts were 80.6 ± 5.3 and 82.9 ± 6.4 mg/100 ml, respectively. Serum glucose tended to increase in the insulin treated group during gestation. There was a slight elevation towards the end of gestation in the control group. The pretreatment insulin level in diabetic gilts was 26.6 ± 6.3 μU/ml; this was reduced to 6.8 ± 1.6 μU/ml after induction of diabetes. The low level of insulin was maintained through the remainder of pregnancy. The pretreatment insulin levels in the insulin treated and control gilts were 57.7 ± 17.5 and 77.3 ± 24.9 μU/ml, respectively. The expected rise in insulin level in the insulin treated gilts was observed and it was higher than the control level.

The body weight, muscle weight, and muscle cellular constituents of the fetal pigs were not significantly altered by the treatments (table I). The effects of treatments on fetal liver weight and other cellular constituents (table II) were the most significant findings of this experiment. The liver weight was significantly increased (P<.01) in the diabetic progeny as compared to the insulin treated and control progeny. Both liver glycogen concentrations and total liver glycogen content were elevated (P<.01) in the diabetic progeny. The liver weight and glycogen content were not different between control and insulin treated progeny. Total DNA and RNA content were significantly greater (P<.01) in the diabetic progeny when compared to control or insulin treated. No differences were noted between the insulin treated and control groups. Total liver protein showed a marked elevation in the diabetic group (P>.05) while insulin treated progeny and control group did not differ in this parameter. RNA/DNA ratios were similar in all the treatment groups while protein/DNA ratio was reduced in the diabetic progeny (P<.01) when compared to control progeny. The control and insulin treated progeny did not show differences in protein/DNA ratio.

Discussion

O'Hea and Leveille (1970) reported that pigs injected with 50 mg of alloxan/kg body weight exhibited a marked polyurea and glucosuria after 24 hr, and were severely hyperglycemic. Figure 1 shows serum glucose and insulin levels before the injection and at subsequent bleeding times. The preinjection serum glucose level for diabetic gilts was 106.2 ± 9.6 mg/100 ml. On day 3 post-injection (73 days' gestation), the level increased to 552.3 ± 95.4 and remained high throughout gestation. All diabetic gilts used for the study maintained a hyperglycemic condition greater than 300 mg/100 ml of serum. The pretreatment glucose level for the insulin and control gilts were 80.6 ± 5.3 and 82.9 ± 6.4 mg/100 ml, respectively. Serum glucose tended to increase in the insulin treated group during gestation. There was a slight elevation towards the end of gestation in the control group. The pretreatment insulin level in diabetic gilts was 26.6 ± 6.3 μU/ml; this was reduced to 6.8 ± 1.6 μU/ml after induction of diabetes. The low level of insulin was maintained through the remainder of pregnancy. The pretreatment insulin levels in the insulin treated and control gilts were 57.7 ± 17.5 and 77.3 ± 24.9 μU/ml, respectively. The expected rise in insulin level in the insulin treated gilts was observed and it was higher than the control level.

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Discussion

O'Hea and Leveille (1970) reported that pigs injected with 50 mg of alloxan/kg body weight were resistant to the drug. Since then, efforts have been made to produce diabetes in the pig. A successful method of alloxan administration using a higher dose level was reported by Romsos et al. (1971) and O'Hea et al. (1971). Other workers have adopted a dose level of 200 mg/kg body weight of alloxan as an effective diabetogenic dose for pigs (Phillips et al., 1976). The result of the present investigation showed that 50 mg/kg body weight of alloxan was diabetogenic in pregnant pigs. Maternal serum glucose level was maintained between 447 and 550 mg/100 ml (see figure 1) after the injection. This was similar to the values reported by Phillips et al. (1976) for miniature pigs treated with alloxan at 200 mg/kg body weight. It was worthy of note that in a preliminary experiment, two pregnant gilts alloxanized with 200 mg/kg body weight died within 24 hours.

Figure 1 showed that preinjection insulin level (26.6 ± 6.3 μU/ml) decreased to 6.8 ± 1.6 μU/ml and remained fairly steady through the
TABLE 1. BODY WEIGHT, MUSCLE WEIGHT, AND MUSCLE CELLULAR CONSTITUENTS IN FETAL PIGS AT 112 DAYS OF GESTATION

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Gilt treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diabetic (12)</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>1.06 ± .03</td>
</tr>
<tr>
<td>Muscle weight (g)</td>
<td>2.90 ± .04</td>
</tr>
<tr>
<td>Muscle glycogen (mg/gm)</td>
<td>75.74 ± 6.14</td>
</tr>
<tr>
<td>Total muscle DNA (mg)</td>
<td>5.61 ± .20</td>
</tr>
<tr>
<td>Total muscle RNA (mg)</td>
<td>4.30 ± .43</td>
</tr>
<tr>
<td>Total muscle protein (mg)</td>
<td>235.93 ± 20.71</td>
</tr>
<tr>
<td>RNA/DNA</td>
<td>.72 ± .04</td>
</tr>
<tr>
<td>Protein/DNA</td>
<td>41.70 ± 2.50</td>
</tr>
</tbody>
</table>

*Number of animals utilized per group.
\[b\] Means ± SEM.

No significant (P>.05) differences among treatments for any of the parameter.

Experimental period. The pretreatment insulin level was in general agreement with those of other investigators (Romso et al., 1971; O'Hea and Leveille, 1970); however, the present studies showed a greater depression of insulin levels indicating a more effective response. Pregnancy may have contributed to increased sensitivity to alloxan observed among the gilts. Reduced to 25% of control values of insulin in the diabetic gilts was accompanied by a five-fold elevation in serum glucose (see figure 1).

The tendency for the insulin and glucose levels to increase during gestation in the control gilts indicated a development in insulin resistance observed in pigs (Atinmo et al., 1974) and other species (Metzger et al., 1977). The expected rise in serum insulin level in the insulin injected gilts was observed (figure 1); however, this was not followed by a fall in serum glucose. The reason for this was not clear. The insulin injected gilts may be synthesizing more liver glycogen in response to exogenous insulin. On being stressed by venipuncture, stored glycogen may have been discharged into the blood stream causing a temporary hyperglycemic condition. Shah et al. (1977) showed that mild stress has a significant effect on carbohydrate metabolism of the rat. Therefore, the observed higher serum glucose in insulin

TABLE 2. FETAL LIVER WEIGHT AND LIVER CELLULAR CONSTITUENTS AT 112 DAYS OF GESTATION

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Gilt treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diabetic (12)</td>
</tr>
<tr>
<td>Liver weight (gm)</td>
<td>42.51 ± 3.24**</td>
</tr>
<tr>
<td>Liver glycogen (mg/gm)</td>
<td>156.12 ± 4.10**</td>
</tr>
<tr>
<td>Total liver glycogen (mg)</td>
<td>6700 ± 600**</td>
</tr>
<tr>
<td>Total liver DNA (mg)</td>
<td>105.27 ± 2.49**</td>
</tr>
<tr>
<td>Total liver RNA (mg)</td>
<td>182.50 ± 10.72**</td>
</tr>
<tr>
<td>Total liver protein (mg)</td>
<td>3400 ± 200*</td>
</tr>
<tr>
<td>RNA/DNA</td>
<td>1.75 ± .10</td>
</tr>
<tr>
<td>Protein/DNA</td>
<td>32.32 ± 1.25</td>
</tr>
</tbody>
</table>

\[a\] Number of animals utilized per group.
\[b\] Mean ± SEM.
\[*\], \[**\] Diabetic progeny differs significantly from control and insulin group at P<.05 and P<.01, respectively.
\[c, d, e\] Means with different superscripts are significantly different at P<.05 and P<.01, respectively.
Figure 1. Maternal serum glucose (mg/100 ml) and immunoreactive insulin (μU/ml) levels in gilts before and after alloxan, insulin and saline treatment.

injected gilts when compared to the control may have been due to blunted response and/or sampling stress response.

Experimental maternal diabetes has been shown to increase (Sylbuski and Manghan, 1971) or decrease (Angervall et al., 1965) fetal body weight in rats. More severe hyperglycemia resulting from maternal alloxan treatment produced fetal growth retardation in rats (Pitkin and Van Orden, 1974). Increased body weight in infants of diabetic mothers (IDM) has been reported in humans (Osler, 1960). The lack of enlarged body size in the present studies may have been due to severe hyperglycemia noted in the pregnant gilts, or possibly by some limiting nutrient other than glucose (i.e., amino acids, vitamins, etc.)

The lack of fetal response to maternal insulin injection might be due to insulin insensitivity during pregnancy (Hyttten and Leitch, 1964). The reason for this is still unclear. Burt (1960) suggested that increased glucocorticoids in late pregnancy might be involved. In humans, a proteolytic enzyme capable of inactivating insulin has been identified in placental membrane (Frienkel and Goodner, 1960). These factors in combination may contribute to the insulin resistance and lack of response observed in the fetal parameters of the present experiment.

Fetal muscle growth was mainly achieved by hyperplasia (Winick and Noble, 1965). In the fetal pig, muscle DNA has been shown to increase rapidly between 90 and 100 days of gestation, then remained constant until birth (Herbein, 1976). The values reported for muscle weight and muscle cellular constituents in the present studies were in agreement with those of others (Robinson, 1969; Widdowson, 1971; Herbein, 1976). Though gastrocnemius muscle weight, DNA and RNA content were not stimulated by maternal diabetes, there was no depression in muscle development following the treatment. Maternal diabetes produced an increase in whole body DNA in rat fetuses (Pitkin et al., 1971). The present result showed that the rate of muscle protein synthesis (RNA/DNA), muscle cell size (protein/DNA) or total muscle protein content were not altered.

Muscle glycogen concentration has been studied in the pig (McCance and Widdowson, 1959; Mersmann et al., 1972; Hakkarainen, 1975). Hakkarainen (1975) showed that mobilization of muscle glycogen was slower when compared to the liver, indicating decreased importance of muscle glycogen for neonatal piglets in glucose homeostasis. However, muscle glycogen served as a substrate for the muscle when piglets sought and found their way to the teat (Dawes, 1968).

In contrast to muscle, the liver responded remarkably to maternal glucose supply (see table II). Porcine liver cell growth starts about 6 weeks of fetal age (Hakkarainen, 1975). Hyperplasia is the principal mechanism involved in liver cell growth during the prenatal and early postnatal life in the pig (Hakkarainen, 1975; Herbein, 1976). Polyploidy is often associated with liver cell growth. Although no information is available on polyploidy in porcine liver, studies in mice (Epstein, 1967) showed that these nuclear changes first appeared when the species has passed the age of weaning. Therefore, the DNA content in the liver was regarded as an index of liver cell growth (hyperplasia). The values reported in this study for total liver DNA are in agreement with those of Robinson (1969) and Herbein (1976). The increased liver weight noted in the progeny of diabetic gilts indicated that the increased hyperplastic
growth resulted in larger liver size. The reduced cell size (protein/DNA) also suggested that liver cell growth response was hyperplastic rather than hypertrophy (cell size). Similar response to selection in mice has been reported (Robinson and Bradford, 1969). It would appear that cellular response to experimental treatment depended on the cellular growth phase during the treatment. Similar RNA/DNA ratio noted in the present study would suggest comparable rate of protein synthesis in the liver.

The increase in DNA and RNA was reflected on the protein content of the liver (see table II) in the diabetic progeny. The remarkable increase in the liver glycogen concentration as well as total glycogen content in the diabetic progeny may be of significance to the newborn pigs. Since carbohydrate is the major metabolic substrate in newborn pigs, the observed increase in liver glycogen in diabetic progeny may be of fundamental importance in baby pig survival. Since increased dietary energy during gestation did not appear to stimulate fetal glycogen deposition beyond the control level (Elliot and Lodge, 1977), the results of the present study provided a potential mechanism for increasing the availability of this important source of energy to newborn pigs. The twofold increase in liver glycogen observed in this experiment illustrated the tremendous capacity of the fetal liver for glycogen storage, and its ability to respond to appropriate stimulation. The normal presence of glucokinase enzyme in the liver may be responsible for this ability of the liver to handle increased glucose supply.

The lack of response by the fetal liver from insulin treated gilts might mean that the dose level of injected insulin was too low to influence carbohydrate metabolism in pigs. In rats, maternal insulin injection resulted in offspring with decreased body weight, decreased insulin, and glucose level (Sodoyez-Goffaux and Sodo- yez, 1976). It would be pertinent to investigate the influence of higher dose levels of insulin in pregnant pigs.

The results of this study showed that liver glycogen stores and liver cell growth were significantly increased by elevated maternal glucose resulting from maternal diabetes. The study also showed that muscle development was not affected adversely by the treatment. The increase in liver glycogen in the fetal pigs may be a means of improving the survival rate of baby pigs which appear metabolically immature at birth.

**LITERATURE CITED**


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