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# Effect of Hyperinsulinemia on the Development of Blood Coagulation in the Lamb Fetus

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## ABSTRACT

Infants of diabetic mothers have an increased risk for thrombosis. The etiology of their hypercoagulable state is unknown. To examine the effects of hyperinsulinemia on the development of coagulation during fetal life, 10 sets of chronically catheterized fetal lambs were studied. One twin from each pair of 120-d gestation lamb fetuses was infused with insulin at a rate of 2.5 U/h for 48 h, whereas its twin sibling was infused with an equal volume (20 mL) of dextrose 5% in water. Changes in coagulation factor activities were measured before and after the infusions, and differences were analyzed by paired *t* tests. There was a significant decrease in protein C after insulin treatment in the insulin-treated twins. There were relative increases in fibrinogen factors V, VII, and XI when the insulin-treated group was compared with the controls. The changes are consistent with an increased risk of thrombosis and may explain, in part, the higher incidence of thrombosis in infants of diabetic mothers. ***(Pediatr Res* 38: 169–172, 1995)**

**Abbreviations**

**$D_5W$,** dextrose 5% in water solution
**WBC,** white blood cell

Infants of diabetic mothers have an increased incidence of both arterial and venous thromboses. Oppenheimer and Esterly (1) reported a 15.8% incidence of venous thrombosis in 45 newborn infants of diabetic mothers. Renal vein thrombosis is the most common thrombotic complication in infants of diabetic mothers (2, 3). More recently, Van Allen *et al.* (4) first reported an infant of a diabetic mother with an *in utero* brachial artery thrombosis and neonatal gangrene who developed postnatal aortic and mesenteric artery thrombosis. After review of the literature, they found that 7 of 32 infants with neonatal gangrene of a limb presenting at birth were infants of diabetic mothers. David *et al.* (5) reported a case of oromandibular limb hypogenesis thought to be associated with an intrauterine vascular accident in an infant of a diabetic mother.

The hemostatic alterations predisposing to thrombosis and the specific factors responsible for these alterations are unclear. Hemostatic alterations found in infants of diabetic mothers who experience a thrombotic event could simply be secondary to the thrombotic event. There could also be primary alterations of the normal development of homeostasis in these infants exposed to both hyperglycemia and hyperinsulinemia during intrauterine life leading to a hypercoagulable state. The chronically catheterized fetal lamb provides a model whereby the intrauterine environment can be altered and the effects measured. Using twin lamb fetuses, it is possible to modify the environment of one fetus while allowing the other fetus to serve as control. This model was used successfully in studies of the effects of glucocorticoids and thyroxin on the development of hemostasis in the fetus (6, 7). We have, therefore, undertaken similar studies to measure the effects of hyperinsulinemia on the development of hemostasis in fetal lambs during the last trimester of pregnancy.

## METHODS

Pregnant mixed-breed Dorset-Suffolk ewes were obtained from existing sources and were housed throughout the study at the University of Iowa Animal Quarters. The gestational ages of the fetuses were known on the basis of induced ovulation technique (8). Before surgery the ewes were fasted for 48 h. Anesthesia of the ewe and surgery on the fetus were performed as described previously (9).

Ten pregnant ewes each with twin pregnancies were used in this study. The gestational ages of the twins averaged 120 d (109–130 d). Twins were used to control for natural intrauterine environmental differences. Both fetuses in each pregnant ewe were catheterized. Arterial and venous catheters were placed in the femoral arteries and veins of each of the twins. After a 5-d rest period, one of each set of twins was infused i.v. with insulin in $D_5W$ at a rate of 2.5 U/h for 48 h. The twin sibling was infused with an equal volume (10 mL/24 h) of

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$D_5W$. Constant infusion pumps (Cormed, Inc., Middleport, NY) were used for the infusions. Both micropumps were housed in pockets of special jackets designed by our laboratory which allow the ewes complete freedom of movement during the infusions.

Before and at the completion of the infusions at 48 h, fetal blood samples were obtained through the chronically placed catheters for measurements of coagulation factor activities in both test and control fetuses. A 7-mL aliquot of blood was obtained from each fetus with each phlebotomy. All samples were collected in plastic, and samples for measurement of coagulation factor activities were diluted 9 parts of blood to 1 part of 0.01 M sodium citrate, pH 5.0. Blood samples were centrifuged at 1800 × *g* for 30 min; the supernatant platelet-poor plasma was removed, stored at −70°C in aliquots, and thawed just before measurement of clotting factor activities.

Standard techniques were used for measuring the prothrombin time (10) and the partial thromboplastin time (11). Specific factor activities II, V, VII, VIII, IX, X, XI, and XII were assayed with the one-stage assays measuring the ability of the test plasma to correct plasma known to be deficient in the factors tested (12). Fibrinogen was measured by thrombin clotting time according to the method of Clauss (13). Human deficient plasmas were used, and an adult sheep standard pool consisting of plasma from 10 normal nonpregnant ewes was used for calibration. Antithrombin III was measured by the method of Henriksen and Owen (14). Plasminogen was measured using the amidolytic method described by Mussoni *et al.* (15). Protein C activity was measured as described with Laurell rocket immunoelectrophoresis using a monospecific polyclonal rabbit anti-ovine protein C antibody (our unpublished experiment). Fibrin monomer was measured by the method of Kisker (16). All activities measured are quoted as percentage of the reference standard pool of sheep plasma. Insulin levels were measured by RIA kit (Diagnostic Products Corp., Los Angeles, CA). The hemoglobin, hematocrit, and WBC count were measured with a Coulter model S (Coulter Electronics, Inc., Hialeah, FL) on a 0.5-mL aliquot of blood that was anticoagulated with EDTA. Platelet counts were measured by phase microscopy on this EDTA sample.

The differences before and after insulin or saline infusion in each group and the differences between groups before and after insulin or saline infusions were statistically analyzed using paired *t* tests.

## RESULTS

In Table 1 are presented the means and standard deviations of coagulation factor activities, hematocrit, WBC counts, platelet counts, insulin levels, and serum glucose concentrations before and after insulin infusion in control and treated twin pairs. The *p* values for the differences before and after treatment in each group are presented and the between group before and after treatment *p* values are also included.

Figure 1 shows that protein C significantly decreased after insulin infusion in the insulin treated group. In the insulin-treated animals, as shown in Figure 1, the level of protein C decreased from 64 to 44% ($p = 0.003$), whereas in the control animals the level decreased from 66 to 56% ($p = 0.141$). The between group differences did not, however, reach statistical significance ($p = 0.0895$). As expected, insulin also showed a statistically significant increase within the insulin-treated group

**Table 1.** *Treatment results*

| TEST | Insulin (n = 10) Before | Insulin (n = 10) After | B-A* $p =$ | Control (n = 10) Before | Control (n = 10) After | B-A† $p =$ | GROUP‡ $p =$ |
|---|---|---|---|---|---|---|---|
| Prothrombin time (s) | 16.3 ± 1.2 | 16.2 ± 1.3 | 0.812 | 16.9 ± 2.0 | 16.8 ± 1.5 | 0.960 | 0.8605 |
| Partial thromboplastin time (s) | 70.9 ± 21.9 | 61.3 ± 12.8 | 0.250 | 74.8 ± 21.9 | 70.0 ± 13.9 | 0.556 | 0.1105 |
| Thrombin time (s) | 17.4 ± 2.9 | 19.4 ± 4.7 | 0.282 | 17.2 ± 2.5 | 17.9 ± 2.8 | 0.554 | 0.4445 |
| Fibrinogen (mg/dl) | 108.9 ± 14.8 | 109.4 ± 13.7 | 0.938 | 118.7 ± 22.7 | 108.5 ± 17.9 | 0.276 | *0.0153* |
| Factor II (%) | 33.8 ± 6.24 | 33.14 ± 9.27 | 0.860 | 32.1 ± 6.7 | 30.1 ± 7.3 | 0.526 | 0.4952 |
| Factor V (%) | 50.6 ± 911.34 | 54.7 ± 11.22 | 0.435 | 50.74 ± 11.7 | 45.5 ± 10.2 | 0.299 | *0.0230* |
| Factor VII (%) | 44.2 ± 8.34 | 49.6 ± 9.3 | 0.196 | 48.5 ± 7.1 | 46.3 ± 6.9 | 0.497 | *0.0399* |
| Factor VIII (%) | 30.92 ± 9.4 | 32.6 ± 11 | 0.712 | 31.9 ± 11.9 | 33.8 ± 16.3 | 0.770 | 0.9591 |
| Factor IX (%) | 30.4 ± 8.2 | 30.1 ± 8.4 | 0.943 | 28.7 ± 5.6 | 27.1 ± 8.4 | 0.623 | 0.6223 |
| Factor X (%) | 30.2 ± 4.2 | 27.8 ± 7.1 | 0.372 | 31.3 ± 6.3 | 26.9 ± 4.9 | 0.097 | 0.3544 |
| Factor XI (%) | 53.9 ± 21.8 | 59.1 ± 22.5 | 0.608 | 58.8 ± 28.5 | 47.7 ± 16.1 | 0.298 | *0.0063* |
| Factor XII (%) | 42.5 ± 10.9 | 44.4 ± 11.2 | 0.708 | 43.1 ± 13.1 | 42.7 ± 18.3 | 0.947 | 0.4998 |
| von Willebrand factor (%) | 10.8 ± 7.5 | 10.2 ± 7.5 | 0.866 | 11.5 ± 8.3 | 10.9 ± 8.9 | 0.871 | 0.9169 |
| Protein C (%) | 63.9 ± 12.4 | 44.0 ± 13.1 | *0.003* | 65.9 ± 16.6 | 56.2 ± 10.9 | 0.141 | 0.0895 |
| Antithrombin III (%) | 96.8 ± 3.1 | 102.4 ± 13.3 | 0.259 | 95.9 ± 3.7 | 100.4 ± 11.5 | 0.303 | 0.3925 |
| Plasminogen (%) | 50.3 ± 7.7 | 51.06 ± 8.9 | 0.838 | 48.3 ± 6.7 | 52.6 ± 8.2 | 0.201 | 0.1673 |
| Fibrin monomer | 1.6 ± 2.1 | 2.0 ± 2.1 | 0.673 | 1.6 ± 2.1 | 2.0 ± 2.1 | 0.673 | 1.0000 |
| Hematocrit | 32.5 ± 4.4 | 33.2 ± 4.4 | 0.734 | 30.8 ± 4.9 | 32.5 ± 4.5 | 0.430 | 0.5947 |
| WBC | 3240 ± 1088 | 3660 ± 1359 | 0.456 | 2990 ± 664 | 3340 ± 729 | 0.276 | 0.8310 |
| Platelet | 389.0 ± 103.7 | 403.8 ± 141.9 | 0.465 | 488.5 ± 224.2 | 497.4 ± 211.6 | 0.276 | 0.9496 |
| Insulin | 7.8 ± 5.1 | 220.6 ± 111.6 | *0.000* | 8.3 ± 6.2 | 8.9 ± 6.8 | 0.863 | *0.0002* |
| Glucose | 13.0 ± 7.5 | 12.1 ± 14.0 | 0.863 | 19.7 ± 24.0 | 16.0 ± 14.6 | 0.679 | 0.7185 |

Results are presented as mean values ± 1 SD.

* *p* values, *t* test for differences before and after insulin infusion.

† *p* values, *t* test for differences before and after control saline infusion.

‡ *p* values, paired *t* test for differences between groups before and after insulin.

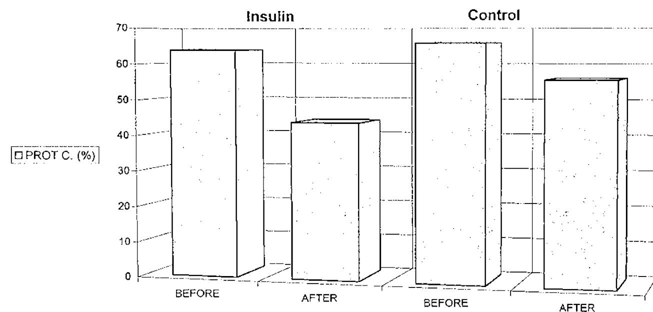


**Figure 1.** Protein C in fetal lambs before and 48 h after insulin and control treatment.

before and after treatment from a mean of 7.84 U to a mean of 220.6 U ($p = 0.0010$).

Fibrinogen and factors V, VII, and XI significantly increased in the insulin-treated group when compared with the control group. There were, however, no significant changes in these factors within the insulin or control groups.

## DISCUSSION

Blood coagulation factor activities, particularly the vitamin K-dependent factors including protein C, are low in newborn infants relative to adult standards (17–20). These deficiencies render the neonate at risk for developing either hemorrhage or thrombosis with minimal hemostatic stress.

Previous studies using a fetal lamb model have shown that the patterns of development of blood coagulation factor activities in the model are similar to those identified in the human infant (9). Major increases in many of the blood coagulation factor activities occur toward the end of the last trimester of pregnancy (9). Hormones may influence the normal patterns of development of coagulation factor activities and thus increase the likelihood of hemorrhage or thrombosis in the newborn. We previously reported a study of the effects of a 48-h infusion of glucocorticoids in the fetal lamb model. In this study, a 48-h exposure to increased glucocorticoids accelerated the development of coagulation factors (II, V, VII, IX, and X). More recently, a study of the effects of thyroxin demonstrated decreases in factor V and VII and increases in factor XII in thyroxin-treated fetal lambs (7).

A number of physiologic disorders in infants of diabetic mothers have been suggested as possible contributing factors to the development of thrombosis in these infants. These disorders include dehydration, polycythemia, increased platelet prostaglandin synthesis, and decreased fibrinolysis (1, 21, 22). A decrease in the anticoagulant protein C has also been suggested as potential factor leading to thrombosis. In a recent prospective survey of 193 newborn infants, protein C levels $<0.1$ U/mL were found in preterm infants with respiratory distress, infants of diabetic mothers, and infants of twin gestations (23). A protein C level $<0.1$ U/mL correlated with the subsequent onset of thrombosis even when the effects of gestational age and birth weight were excluded.

In the current studies fetal lambs were exposed to hyperinsulinemia using the protocol that had previously been successful in demonstrating changes in blood coagulation factors in fetal lambs exposed to glucocorticoids. In the current study we demonstrated a decrease in the level of protein C (mean = 63.9% before insulin, mean = 44.0% after insulin, and minimum = 25%, $p = 0.003$). The mean level (44%) has been associated with thrombosis in adults (24). The relative increases in fibrinogen and factors V, VII, and XI in the insulin-treated fetuses compared with their twin sibling controls are unexplained. In each case these factor levels increased in the insulin-treated fetuses, whereas the levels decreased in the control fetuses. However, none of the within group changes was significant. The volume of sample removed and the rate of infusion of saline or insulin was equal in both groups, and neither was sufficient to account for the decreased levels; there was no decrease in the hematocrit to suggest dilutional changes. The relative increases in fibrinogen and factors V, VII, and XI coupled with the low protein C levels all tend to shift the balance of hemostasis toward thrombosis. There were no counterbalancing decreases in the levels of other procoagulants or increases in the levels of other anticoagulants in the insulin-treated group which would shift the balance toward hemorrhage.

Unfortunately, not all factors predisposing to thrombosis could be measured in this experimental model. Because of the limitations on the volume of sample available, we were not able to measure the levels of free protein S, a cofactor of protein C. An increase in tissue factor pathway inhibitor might counterbalance the relatively increased levels of factors V and VII in the insulin-treated animals, but we were also unable to measure tissue factor pathway inhibitor in the fetal lambs. Despite these deficiencies, the findings demonstrated that exposure of the fetus *in utero* to hyperinsulinemia for 48 h results in alterations of the balance of hemostasis toward thrombosis.

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