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Insulin sensitivity in male sheep born to ewes treated with testosterone during pregnancy

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Original Article

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Abstract

In animal models, exposure to excess testosterone during gestation induces polycystic ovary syndrome (PCOS)-like reproductive and metabolic traits in female offspring, suggesting that the hyperandrogenemic intrauterine environment may have a role in the etiology of PCOS. Additionally, few studies have also addressed metabolic and reproductive outcomes in male offspring. In the present study, the intravenous glucose tolerance test (IGTT) was used to assess the insulin–glucose homeostasis at various ages during sexual development in male sheep born to testosterone-treated ewes. To further analyze the programming effect of testosterone on insulin–glucose homeostasis, indexes of insulin sensitivity were assessed in orchidectomized post-pubertal males born to testosterone-treated ewes (Torq-males) and orchidectomized post-puberal controls (Corq-males) before and 48 h after a testosterone injection. There was no difference in insulin sensitivity indexes between males born to testosterone-treated ewes (T-males) and control males born to control ewes (C-males) at 5, 10, 20 and 30 weeks of age, representing the infantile, early and late pre-pubertal, and early post-pubertal stage of sexual development, respectively. In orchidectomized males, basal levels of insulin and glucose were not different between both groups before and after the testosterone injection; however, Torq-males released more insulin before and after T challenge during the first 20 min of the test. Despite this, plasma glucose concentrations were not different in both groups during IVGTT, resulting in an insulin sensitivity index composite similar between groups. We concluded that the effect of prenatal exposure to excess testosterone may reprogram the pancreatic β-cells insulin release in ovine males, with effects more evident in castrated males versus intact males.

Introduction

Programmed development of an organism is a series of synchronized events guided by genetic instructions acquired during conception. During early critical periods of fetal life, the growing fetus has the ability to respond to environmental stimulus through adaptations at cellular and molecular levels. Early adaptations to external or internal environmental stimulus may permanently change anatomy and physiology, with effects expressed even if the initiating stimulus or insult is no longer present.[1,2] Experimental studies in animals and epidemiological data in humans have shown the adverse consequences in postnatal life due to individual tissues programmed *in utero*.[3] The hypothesis of the 'fetal origin of adult diseases' proposes that alterations in nutrition or in the endocrine status of the mother produce adaptations in the development of the growing fetus that permanently change its anatomy and physiology, and predispose individuals to cardiovascular, metabolic, and endocrine diseases later on.[4] In addition to nutrition, hormones also play a central role in programming, as they regulate fetal growth and the development of individual tissues.[3] One hormone that has received greater attention in fetal reprogramming, particularly females, is testosterone (T), which can modify development patterns of the gonadal axis[5] and program energetic homeostasis, either modifying insulin secretion or tissue insulin sensitivity (IS).[6]

Administration of testosterone to pregnant monkeys or sheep resulted in changes in the adult female offspring similar to those observed in women with polycystic ovary syndrome (PCOS), for example, hyperinsulinemia, insulin resistance, hypersecretion of Luteinizing Hormone (LH), and androgen excess,[7–9] which suggests that females prenatally exposed to excess testosterone could be used as a model for understanding the pathophysiology of this disease.

However, less attention has been paid to the reproductive or metabolic consequences on the male offspring of these mothers who received testosterone during pregnancy. In our laboratory, after administration of testosterone to pregnant sheep, as an animal model of PCOS, it was possible to observe substantial alterations in the reproductive axis of their male offspring.[10–12] However, the impact on some aspects of metabolism, such as glucose homeostasis of these

descendants, is less known. In human, males born to mothers affected by PCOS, the metabolic disarrangements are present from childhood to adulthood. For instance, young adults show dyslipidemia and insulin resistance.[13] In the present study, in order to evaluate the programming effect of testosterone exposure during gestation on postnatal glucose homeostasis, we analyzed IS in a set of intact males born to testosterone-treated mothers (T-males). IS was thus evaluated by means of the intravenous glucose tolerance test (IVGTT) at four different stages of postnatal development, namely, infancy, early and late pre-puberty and post-puberty. This metabolic evaluation has been previously used in similar studies.[6] Additionally, to analyze the potential contribution of T as a second hit during puberty in prenatally exposed and non-exposed males, we assessed IS in orchidectomized males before and after a single T injection.

Materials and methods

General management, breeding, prenatal T exposure and group allocation

A group of adult Suffolk Down females was estrous-synchronized in late March and early April, during the natural breeding season at the Chillán Campus of the Universidad de Concepción, Chile (36° 36′ south latitude, 71° 30′ west longitude, 144 m above the sea level), and mated with fertile males as described previously.[14] After pregnancy was confirmed by ultrasound, pregnant sheep was allocated randomly to one of two treatments. One group of pregnant sheep received twice weekly intramuscular injections of 30 mg of testosterone propionate (T; Steraloids, Newport, RI, USA) dissolved in cottonseed oil as vehicle, between days 30 and 90 of pregnancy followed by injections of 40 mg T from day 90 to 120 of pregnancy. The other group served as control and received injections of cottonseed oil vehicle during the same time frame and in the same schedule than the group of mothers injected with T. This regimen of T administration was chosen to reflect the higher levels of androgens seen in women with PCOS during their pregnancies and has been used in previous studies,[14,15] in addition, the period in which T was administered covers all stages of pancreatic development and the period in which insulin-sensitive tissues develop their ability to respond to insulin. Blood samples were collected 24 h after each testosterone injection to follow the plasma T profile during this regimen of T administration.

Mothers were maintained on pasture and supplemented with hay and pelleted food to meet energy requirements of pregnant sheep. Lambs were born in mid-September by normal delivery, after full-length gestation (mean of 147 d), and were weaned at 8 weeks of age. They were maintained under natural photoperiod and given free access to water, pasture, and supplemented twice a day with hay and commercial pelleted food for ruminants as detailed elsewhere.[14] Body weight of males was recorded at each experimental study day.

The group allocation of the male offspring proceeded as follows: T-males ($n = 5$) and C-males ($n = 7$) were intact males born either to mothers treated with T during pregnancy or to control mothers, respectively. This set of males was used to study the programming effect of prenatal exposure to testosterone excess on IS. Furthermore, a subset of males, Torq ($n = 8$) and Corq ($n = 7$), were castrated at 24 weeks of age and underwent an IVGTT at 30 weeks of age, both before and after an intramuscular administration of 40 mg of T propionate. This subset of males was used to study the effect of T administration, under a condition of lower IS due to androgen deprivation (activational effects), on the programming effects induced by prenatal exposition to a T excess (organizational effects).

Intravenous glucose tolerance test

The IVGTT was conducted in intact males at 5, 10, 20, and 30 weeks of age and in orchidectomized males only at 30 weeks of age, before and 48 h after T administration. Five weeks of age corresponds to childhood, 10th and 20th week correspond to the early and late pre-pubertal stage, respectively, and 30th week corresponds to the early post-pubertal stage.

One or two d before the IVGTT, an indwelling jugular catheter was placed under local anesthesia in both jugular veins. The IVGTT started at 08.00 after 12 h of fasting. The IVGTT procedures have been published elsewhere.[6] Briefly, 300 mg glucose/kg $BW^{0.75}$ was infused over 2 min through one catheter. Blood samples (2 ml) were drawn through the other catheter at −15, −10, 0 (glucose infusion), 3, 5, 7, 10, 13, 15, 17, and 20 min and placed in two separated set of tubes conserved on ice. One set contained heparin (10 IU/µl) was used to collect blood for insulin measurements, whereas the other set contained heparin and sodium fluoride for glucose measurements. Twenty minutes after the beginning of glucose administration (time 0), one insulin bolus (1 IU/kg BW, Humulin; Elli Lilly, Indianapolis) was administered and blood samples were collected at 23, 25, 27, 30, 33, 35, 37, 40, 50, 60, 80, 100, 120, 140, 160, and 180 min after time 0 and allocated in the two sets of tubes. Blood samples were centrifuged at 1000×*g* for 15 min at 4°C to separate plasma, which was collected and preserved at −20°C for subsequent measurements of insulin and glucose.

The blood sample at time 0 was also used for measuring testosterone concentrations.

Orchiectomy procedure

Orchiectomy was conducted at 24 weeks of age in 8 males born to testosterone-treated mothers (Torq-males) and in 7 control males born to control mothers (Corq-males). On the day of surgery, and after 18 h of fasting, males were pre-medicated with Acepromazine (0.02 mg/kg, Acedan; Holliday-Scott, Chile, Santiago) and atropine (0.02 mg/kg atropine sulfate; Sanderson, Chile, Santiago) 30 min prior to surgery. Ketamin (KETAMIL; Troy Laboratories, Chile, Santiago) at a dose of 5 mg/kg was intravenously administered, and 2−3 ml of Mepivacaine (2% Vetacaine; Troy Laboratories) was instilled in the mouth to facilitate intubation with an endotracheal tube (7 mm internal diameter), equipped with a balloon which was inflated after intubation was completed. Anesthesia was induced with Isofluorane (Forane, Baxter, Chile) mixed with 3.5% oxygen for the rest of the surgery. During the period of anesthesia, flow of fresh oxygen was 100 ml/kg per minute. The surgical area was neatly shaved and desinfected with povidone iodine, and an incision was made through the scrotal major axis, the blood vessels were clamped and both testicles removed. Once surgery was completed, oxygen was infused to a volume of 4 l/min until the swallowing reflex was restored. The orchidectomized males received antibiotic treatment for 7 d (Pentril, Laboratorio Chile) and an anti-inflammatory (Ketoprofen 10%, Vetanco, Chile) for 5 d after surgery.

As stated above, the subset of orchidectomized males was subjected to IVGTT at 30 weeks of age, both before and 48 h after the administration of a single dose of T.

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Hormones and glucose determinations

Glucose was quantified in all samples from the IVGTT using a commercial kit (Farmalatina, Chile), based on glucose oxidase and peroxidase reaction with measurement of light absorbance at 505 nm in a spectrophotometer. Plasma insulin concentrations were determined by Immunoradiometric assay (IRMA) (Biosource, Louvain-la-Neuve, Belgium) in samples before the infusion of glucose and in those from the first 20 min of the IVGTT. Minimal detectable level of insulin concentrations was 1 μU/ml. Intra- and interassays coefficient of variation were 2.96% and 3.88%, respectively. Testosterone concentrations were determined by Radioimmunoassay (RIA) (Biosource), with a minimal detectable dose of 50 pg/ml and intra- and interassays coefficients of variation of 2% and 5%, respectively (12).

Calculations of IS indexes

IS was assessed by the insulin/glucose ratio (I/G ratio), the insulin sensitivity index composite (ISI-C), the glucose utilization constant (kG), and the rate of glucose disappearance in response to exogenous insulin injection (Rate of Glucose Disappearance (RGD)). The I/G ratio corresponds to the mean plasma concentrations of insulin or glucose from time −15 to 0 of the IVGTT. The ISI-C was calculated using the formula proposed by Matsuda and De Fronzo[16], where ISI-C = 10000/square root of ((fasting glucose × fasting insulin) × (mean glucose × mean insulin during the first 20 min of IVGTT)). The kG (%/min) was calculated using the slope of the log-linear regression of plasma glucose concentration from 10 to 20 min post-glucose administration. The effect of exogenous insulin on RGD was determined with the formula: Δglucose/glucose 20 from Grulet et al.,[17] where glucose 20 is the glucose concentration before exogenous insulin administration and Δglucose is the variation between the glucose at 20 min and the plasma glucose obtained at 40 min (20 min after insulin administration), which is derived from the regression plot.

Statistical analysis

In the first study, each index was analyzed by analysis of variance (ANOVA) for repeated measurements with treatment as the main factor and age as the repeated factor. *Post hoc* comparisons were made with the Newman Keuls's test. Pearson's correlation coefficients were calculated to analyze the correlation between body weight and each IS index.

In the second study, IS indexes were compared between Corq-males and Torq-males by two-way ANOVA. *Post hoc* comparisons were made with the Newman Keuls's test. For all analyses, a $P < 0.05$ was considered as statistically different. Data are shown as mean ± SEM.

Results

Maternal and male offspring plasma testosterone concentrations

Plasma concentrations of testosterone from week 5 to 7 of pregnancy in T-treated mothers ($n = 11$) were elevated throughout T treatment, with a mean of 4.86 ± 0.17 ng/ml, whereas plasma T concentrations in control mothers ($n = 9$) were undetectable.

Plasma T concentrations in C-males and T-males at 5, 10, 20, and 30 weeks of age and after orchidectomy are shown in Table 1. No difference in plasma T concentration was observed between groups at 5 and 10 weeks of age. Nevertheless, at 20 and 30 weeks of age, plasma T was significantly higher in T-males respect to C-males.

Six weeks after orchidectomy, T concentrations dropped to undetectable levels in both Torq and Corq groups. Forty-eight hours after the injection of T, plasma concentrations of this androgen increased to a similar extent in both groups of orchidectomized males (Table 1).

Table 1. Mean ± SEM of plasma concentration of testosterone in male sheep (ng/ml)

Weeks of age	C-males	T-males
5	0.14 ± 0.31	0.13 ± 0.18
10	0.24 ± 0.31	0.35 ± 0.60
20	0.45 ± 0.27[a]	3.02 ± 1.70[b]
30	0.53 ± 0.10[a]	6.48 ± 2.98[b]
30 orq-males before T	0.01 ± 0.00	0.01 ± 0.00
30 orq-males 48 h after T	1.54 ± 0.25	1.37 ± 0.22

[a,b] Different letters mean statistically difference between groups ($P < 0.05$).

Table 2. Mean ± SEM of body weight of males at birth, and at 5, 10, 20, and 30 weeks of age in C-males ($n = 7$) and T-males ($n = 5$)

Age (weeks)	C-males	T-males
0	4.84 ± 0.26	3.86 ± 0.37
5	11.91 ± 0.63	10.46 ± 0.71
10	19.00 ± 1.18	19.83 ± 1.23
20	31.57 ± 1.88[a]	36.72 ± 1.60[b]
30	47.83 ± 1.26	46.18 ± 2.10

[a,b] Different letters mean statistically significative difference between groups ($P < 0.05$).

Body weight in males

Body weights of intact males recorded at birth and at the time of the IVGTT are shown in Table 2. At 20 weeks of age, body weight was significantly different. T-males were 16.3% heavier than C-males ($P < 0.05$). At other ages, body weight was similar between groups.

In orchidectomized males, body weight was similar between groups both before and 48 h after T treatment; however, their weight was significantly lower than the body weight of intact males at the same age (Table 2).

First study: IS indexes at different stages of sexual development

IS indexes calculated from IVGTT at different stages of somatic development in intact males are shown in Table 3. Fasting glucose concentration was similar between and within groups at all stages evaluated, and within groups through the study, fasting insulin concentration showed no differences between C-males and T-males, but within the C-males, there was a 32.1% significantly decrease in basal plasma concentration of insulin at 30 weeks of age compared to 5 weeks of age (Table 3). Consequently, fasting insulin/glucose ratio was not different between groups at 5, 10, 20, and 30 weeks.

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Table 3. Mean ± SEM of insulin sensitivity indexes calculated from IVGTT made at different stages of somatic development (5, 10, 20, and 30 weeks of age) in control and prenatally exposed to a testosterone excess male sheep

Parameter	5 weeks		10 weeks		20 weeks		30 weeks	
	C-males	T-males	C-males	T-males	C-males	T-males	C-males	T-males
Fasting insulin (μUI/ml)	9.10 ± 0.79^{b}	7.65 ± 0.75	7.70 ± 0.73^{ab}	6.85 ± 0.44	8.17 ± 1.20^{ab}	6.26 ± 0.61	6.18 ± 0.44^{a}	6.39 ± 0.85
Fasting glucose (g/l)	0.76 ± 0.05	0.82 ± 0.05	0.73 ± 0.04	0.84 ± 0.04	0.77 ± 0.02	0.75 ± 0.04	0.77 ± 0.02	0.71 ± 0.03
Insulin/glucose ratio (μUI × ml^{-1}/g × l^{-1})	16.74 ± 2.82	12.46 ± 1.33	14.23 ± 2.11	11.50 ± 0.77	14.33 ± 2.30	9.90 ± 1.34	12.81 ± 1.05	13.25 ± 1.35
ISI-composite	9.31 ± 1.23	12.34 ± 2.07	8.20 ± 0.74	11.17 ± 1.21	10.46 ± 1.32	12.98 ± 1.89	12.46 ± 1.29	12.76 ± 1.53
Mean insulin (μUI/ml)	22.03 ± 3.14^{aby}	12.53 ± 2.32^{x}	29.22 ± 3.69^{by}	14.81 ± 2.20^{x}	18.82 ± 2.42^{a}	13.36 ± 1.76	19.03 ± 1.84^{ab}	15.31 ± 1.72
AUC-I insulin (μUI × ml^{-1} × 20 min)	328.74 ± 74.30^{ab}	135.10 ± 22.02	558.73 ± 84.40^{by}	200.68 ± 49.27^{x}	276.58 ± 35.57^{a}	184.55 ± 37.35	327.86 ± 45.98^{ab}	213.96 ± 37.78
Mean glucose (g/l)	0.76 ± 0.04^{abx}	0.82 ± 0.04^{y}	0.73 ± 0.02^{ax}	0.84 ± 0.03^{y}	0.77 ± 0.02^{b}	0.75 ± 0.03	0.72 ± 0.03^{a}	0.71 ± 0.03
Glucose desaparition rate (mg/min)	0.34 ± 0.10	0.34 ± 0.09	0.46 ± 0.05	0.52 ± 0.05	0.33 ± 0.04	0.30 ± 0.18	0.40 ± 0.07	0.49 ± 0.10
Glucose utilization constant (%/min)	1.55 ± 0.58	0.50 ± 0.31	1.68 ± 0.17	1.36 ± 0.19	1.82 ± 0.09	1.77 ± 0.67	1.35 ± 0.29	1.81 ± 0.80

Only superscripts are indicated in those stages in which there is a statistically significant difference within or between groups.
^{a,b} Within a row, means without a common superscript different inside a group, C-males or T-males in different stages of somatic development ($P < 0.05$).
^{x,y} Within a row, means without a common superscript different between groups, C-males versus T-males at the same weeks of age ($P < 0.05$).

During the IVGTT, in both groups and at all ages studied, blood glucose concentration peaked 3–5 min after glucose infusion and subsequently declined. The insulin bolus, which was administered 20 min after the infusion of glucose, caused blood glucose concentrations to gradually decrease until 70 or 80 min. Then, glucose began to reach basal concentrations observed during fasting (Fig. 1a–d, rights panels). Mean plasma glucose during IVGTT only was significantly higher in T- versus C-males at 10 weeks, while there were no differences in this parameter between groups at the other ages (Fig. 1b, right panel). In C-males, there was a significant increase in mean glucose concentration, at 20 weeks respect to 10 weeks of age, during IVGTT; additionally, there were no differences in these groups of intact males between other stages of somatic development. Mean plasma insulin during the first 20 min of the IVGTT was significantly lower in T-males versus C-males at 5 and 10 weeks of age (Fig. 1a and b, left panels). At 20 and 30 weeks, mean insulin secretion in T-males was no different than the C-males, while this latter group showed a decrease respect to 10 weeks (Table 3, Fig. 1c and d, left panels).

At 20 weeks of age, T-males present a significantly lower AUC-I of insulin compared to C-males at the same age (Table 3). Within the C-male group, there is significantly higher insulin Incremental Area Under the Curve (AUC-I) at 10 weeks of age compared to 5, 20, and 30 weeks of age (Table 3). There were no differences in other IS indexes: ISI-C, glucose disappearance rate (GDR), and kG, calculated from IVGTT, between C- and T-males and within groups at any other stage of the study.

In addition, Pearson's correlation test showed no correlation between body weight and the ISI-C, the GDR or the kG within a group or between treatments (data not shown).

Second study: IS in orquidectomized males before and after a T challenge

There was no difference in the fasting plasma insulin concentrations either before or after the injection of T between Torq and Corq groups, whereas fasting glucose concentrations were lower in Torq versus Corq both before and after T injection ($P < 0.05$). However, mean plasma insulin was no different between groups or within groups before or after testosterone administration, but mean plasma glucose was significantly higher in Torq-males after testosterone challenge; no difference was evident in the group of Corq-males. Despite the difference in glucose concentrations, the insulin/glucose ratio was not different between both groups, either before and 48 h after T administration (Table 4).

The mean secretion of insulin in Torq-males during the first 20 min of the IVGTT before and after the T administration was similar. The same was observed in Corq-males. Nevertheless, Torq-males exhibited a mean higher concentration of insulin than those of Corq-males in both experimental periods, but this difference was not significant (Fig. 2a and b). Therefore, the incremental area under the curve of insulin secretion was higher ($P < 0.01$) in Torq-males compared to Corq-males. Besides, Torq-males, after the T administration, tended to release more insulin than before the T ($P = 0.053$) (Table 4).

The ISI-C, as a reflection of the insulin and glucose values during the first 20 min of the IVGTT, was not different between Torq-males and Corq-males before and after the T administration (Table 4).

There were no significant differences within a group or between groups for kG or GDR during both experimental periods (Table 4).

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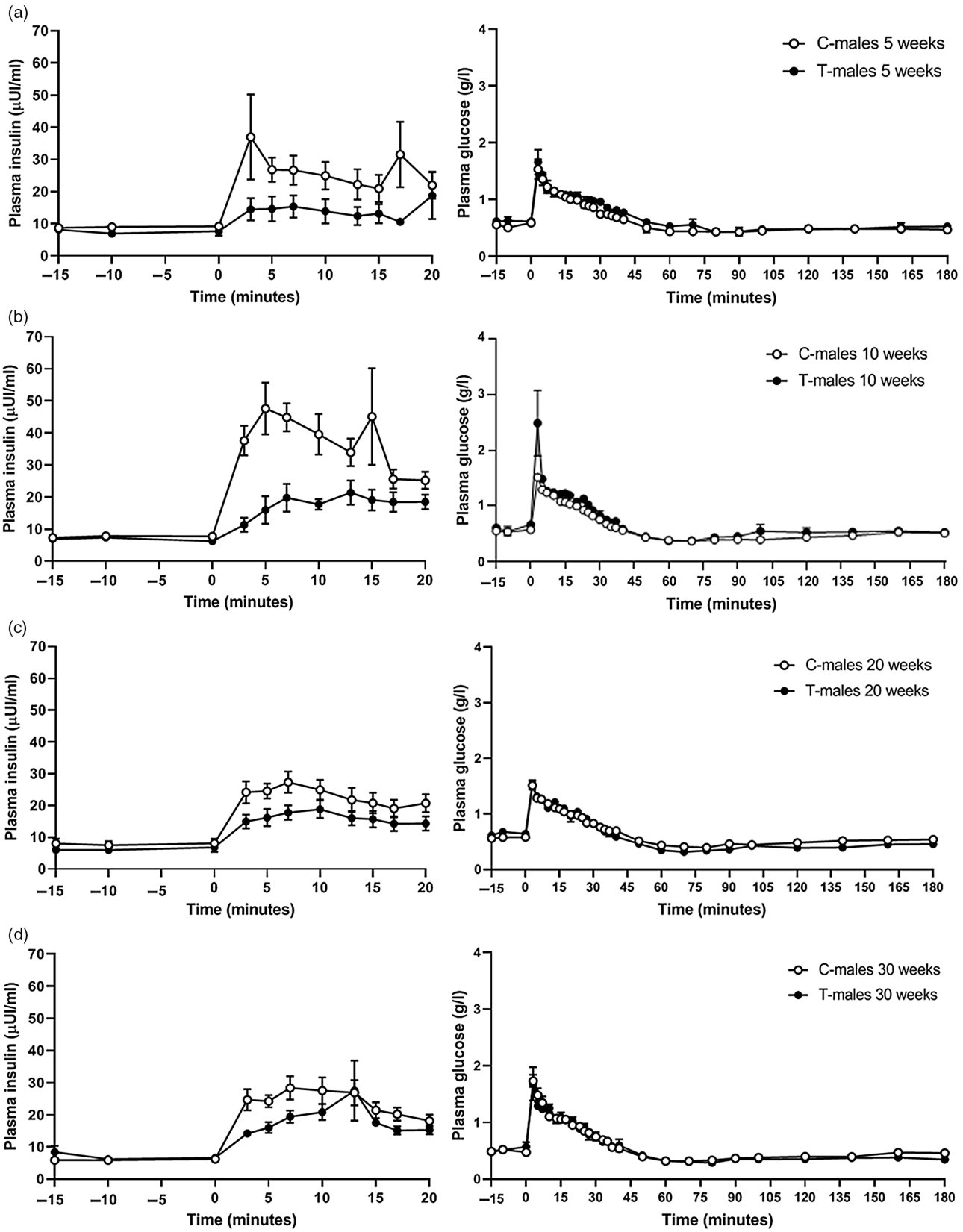

Fig. 1. Insulin (left panel) and glucose (right panel) dynamics during IVGTT in sheep males at 5 (a), 10 (b), 20 (c), and 30 (d) weeks of age control and prenatally exposed to a testosterone excess.

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Table 4. Mean ± SEM of insulin sensitivity indexes during IVGTT before and 48 h after a single dose of testosterone in orquidectomized control ($n = 7$) and prenatally expose to a testosterone excess ($n = 8$) male sheep

	Corq-males pre-T	Torq-males pre-T	Corq-males post-T	Torq-males post-T
Fasting glucose (g/l)	0.70 ± 0.04^{y}	0.56 ± 0.02^{x}	0.75 ± 0.07^{y}	0.59 ± 0.03^{x}
Fasting insulin (μUI/ml)	15.56 ± 1.57	11.90 ± 1.79	17.01 ± 3.10	13.20 ± 2.14
Insulin				
AUC incremental (μUI/ml × 20 min)	293.10 ± 86.62^{x}	483.81 ± 85.20^{y}	299.91 ± 34.82^{x}	624.82 ± 149.16^{y}
Mean insulin (μUI/ml)	31.25 ± 5.80	37.40 ± 5.65	33.23 ± 4.61	45.88 ± 7.82
Glucose				
AUC incremental (g/l × 20 min)	10.64 ± 1.04	11.36 ± 0.63^{a}	11.68 ± 1.14	13.37 ± 1.06^{b}
Mean glucose (g/l)	0.82 ± 0.05	0.71 ± 0.03^{a}	0.91 ± 0.05	0.80 ± 0.05^{b}
Insulin/glucose ratio (μUI×ml^{-1}/g × l^{-1})	22.51 ± 2.69	21.07 ± 3.00	22.52 ± 2.56	21.56 ± 2.96
ISI-C	5.09 ± 0.55	7.01 ± 1.10	4.54 ± 0.71	5.97 ± 1.29
kG (%/min)	1.11 ± 0.19	1.23 ± 0.13	1.10 ± 0.10	1.18 ± 0.07
GDR (mg/min)	1.16 ± 0.22	1.19 ± 0.15	1.20 ± 0.40	1.30 ± 0.19

Only superscripts are indicated in those stages in which there is a statistically significant difference between or within the groups.
[a,b] Within a row means without a common superscript difference inside a group ($P < 0.05$).
[x,y] Within a row means without a common superscript difference between groups ($P < 0.05$).

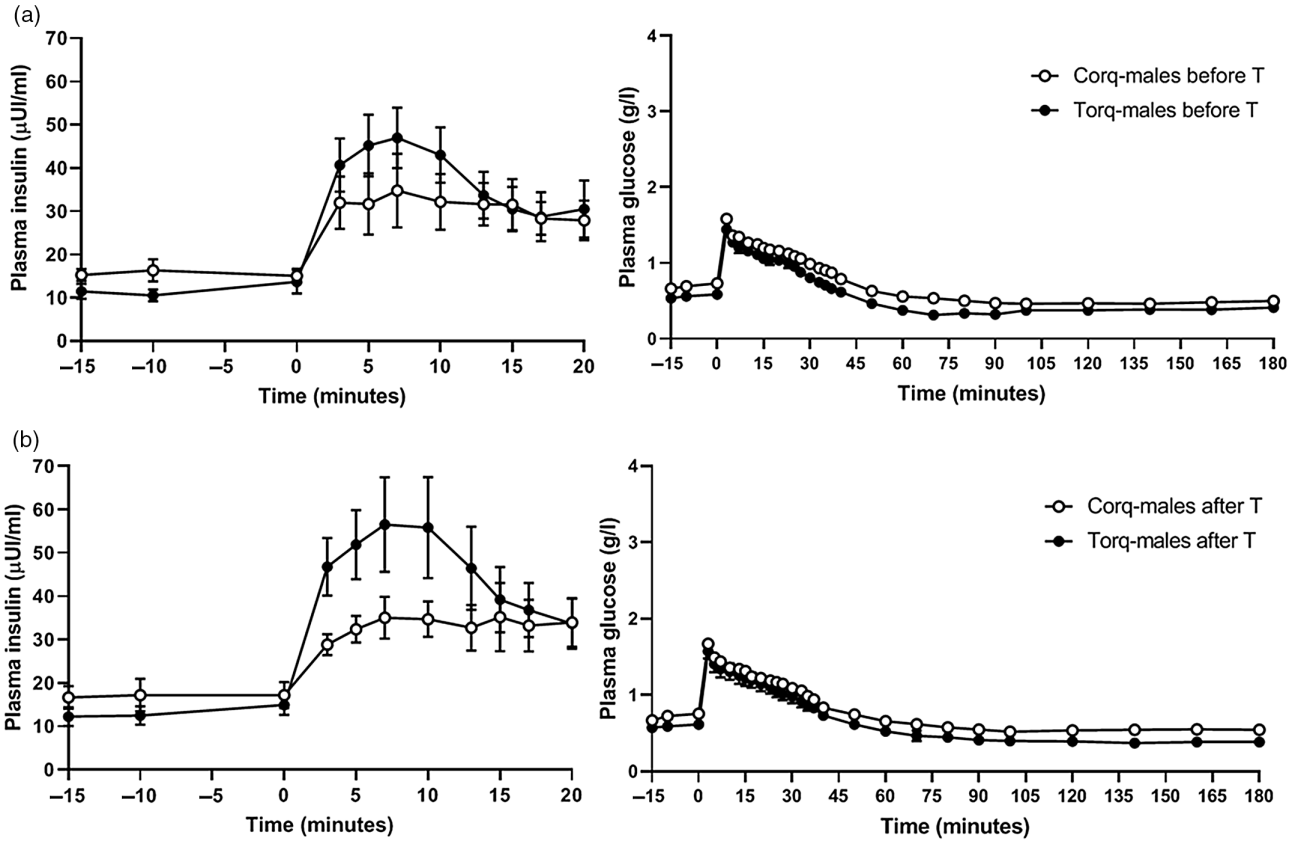

Fig. 2. Plasma insulin (left panel) and glucose (right panel) dynamics during IVGTT in orquidectomized male sheep control and prenatally exposed to an excess of testosterone at 30 weeks of age before (a) and 48 hours after (b) testosterone administration.

Discussion

In this study, the administration of T to pregnant ewes had no significant effect on IS indexes in their male offspring throughout sexual development. T-males had the same values in IS indexes from 5 to 30 weeks of age compared to control males born to non-exposed mothers. The apparent lack of programming effect of testosterone given to pregnant ewes on IS of their male offspring is in contrast to the effects observed in females under similar conditions and

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independent of the dose of T given to the mothers.[6,18] Perhaps male sheep is born insulin resistant due to the effect of testosterone secreted by fetal gonads in programming insulin release by β-cells, and consequently, exogenous testosterone given to the pregnant ewes did not worsen this condition. Certainly, there is an increase in plasma testosterone concentrations in the male fetus when the mother was given testosterone,[19] and this testosterone may diffuse from the mother to the fetus and join to the endogenous testicular hormones without further biological effect.

Pancreatic beta cells mature during early embryonic development.[20] Responsiveness to glucose is detected during the first part of gestation in humans and at 4–5 d of gestation in rats. However, final capacity may be acquired postnatally, due to increased cell proliferation in pancreatic islets.[21–25] Effects of increased prenatal T in pancreas development in males are not well understood. In a study, using an ovine model of PCOS, pregnant ewes injected twice weekly with 100 mg of T via maternal injection, from day 62 to 102 of gestation, there was an increased number of β-cells in female offspring and fetal pancreas had upregulated expression of androgen receptor. Therefore, androgen overexposure modified fetal pancreatic development and β-cell numbers in female offspring.[26,27] It is tempting to predict a similar change in fetal male pancreas due to effects of fetal endogenous testosterone or due to exogenous testosterone given to the mother. In that regard, when males were orchiectomized to prevent effects of endogenous testosterone on IS, a testosterone challenge elicited a greater insulin release in response to glucose infusion in Torq-males before and 48 h after T administration. In that case, it appeared that fetal testosterone improved capacity of β-cells to release insulin, since Corq-males also had increased insulin secretion, but it was of lower magnitude than in Torq-males (organizational effects). However, since insulin secretion was similar in Torq-males before and after the T challenge, it was not possible to attribute the higher insulin secretion to the exogenous T challenge (activational effect).

The absence of changes in IS during sexual development in the present study in control males differed from results obtained by Gatford et al.[28] in male sheep. Those authors proposed that secretion of insulin and IS decreases during sexual development in males and females, being more pronounced between 38 and 130 d of age. The discrepancy between our study and that of Gatford et al.[28] may have been due to differences in breed and in experimental methodology, since they evaluated the IS by the hyperinsulinemic euglicemic clamp, in addition to using the IVGTT. Nevertheless, IVGTT is highly correlated with the clamp (16, 29–31 and unpublished work from our laboratory). Notwithstanding, the present results were in accordance with a previous study from our laboratory where pregnant ewes received 60 mg T twice weekly from days 30 to 90 of pregnancy. Males born to these mothers subjected to a glucose challenge in the form of an IVGTT had no differences in ISI-C throughout sexual development,[32] or with those of control males. Therefore, neither dose nor extent of the treatment altered IS during postnatal development in male sheep at 5, 10, 20, and 30 weeks of age. However, same treatment with 30 mg of T as in the present study to pregnant sheep has a dramatic effect on the male gonadal axis,[12,14,33,34] making a difference between fetal programming of metabolism and reproductive function in male sheep.

The apparent lack of effect of testosterone programming on IS contrasted with results in male rhesus monkey. Adult rhesus monkeys, prenatally exposed to testosterone at various stages of gestation, had a clear decrease in sensitivity to insulin and impaired function of pancreatic β-cells.[35] Males prenatally exposed to androgen did not exhibit hyperandrogenism and therefore was suggested that programming of tissues was due to intrauterine androgen excess.[35] Perhaps lambs with prenatal T exposure, upon reaching the adult stage with possible concomitant increased body fat, could develop some degree of insulin resistance similar to monkeys. As observed in monkeys, male rats exposed to testosterone excess during fetal development also had diminished IS, which was reversed by orchiectomy.[36]

Conversely, effects of testosterone dose or lesser susceptibility to testosterone between a non-ruminant and a ruminant can be considered as a factor explaining species differences. At 5 weeks of age, lambs are still suckling and could be considered as non-ruminant infants, while at later ages, they have a functional rumen and their metabolism is more dependent in free fatty acid for energy supply and by gluconeogenesis to obtain glucose.

The stimulatory effect of testosterone on insulin secretion has been demonstrated *in vitro* and *in vivo* in male rats,[37] with messenger RNA levels of insulin partially restored after 3 d of T administration,[37] a similar time lapse than that in our *in vivo* experiment. This stimulatory effect of testosterone is mediated by the androgen receptor in pancreas.[38] In human males, the relationship between testosterone and IS is well known.[39,40] Low androgens concentrations are related to insulin resistance and type 2 diabetes and other co-morbidities (for review see 41). In humans, obesity is linked to low secretion of T and low T concentrations are associated to insulin resistance and type 2 diabetes mellitus. Therefore, insulin resistance is associated to a low secretion of T by Leydig cells.[39] Consequently, there is a close relationship between low testosterone concentrations and low IS. However, this may be due to obesity. Testosterone treatment or substitution therapy improves the IS in hypogonadal men.[42] It is possible that because there was no difference in T concentration between Corq-males and Torq-males after its administration, that it was not possible to discern fetal programming of T on IS. In this aspect, following prenatal exposure to testosterone, adult male rats had higher testosterone and insulin concentrations than controls and an increase in the Homeostatic Model Assessment of Insulin Resistance-Insulin Resistance index.[43]

It may be argued that the acute effect (48 h) of testosterone in the present work was not long enough to discern the effects of T on β-cell response to a glucose challenge. In humans, T administration takes months to restore beneficial changes in physiological variables.[44] Since testosterone may stimulate β-cell through genomic and non-genomic mechanisms, a short duration stimulus may be enough to initiate, by either mechanism, effects on insulin secretion or on target cells of insulin action.

In the present study, there were no significant differences in birth weight between groups, during postnatal development, with the exception of 20 weeks of age, when T-males had a significantly higher body weight. Perhaps there was an effect of treatment, but without greater impact due to the transience and magnitude of the phenomenon. However, at 30 weeks, there were no longer differences in body weight. Results of this study differed from those obtained in Suffolk lambs whose mothers were given 100 mg testosterone propionate, twice a week, between 30 and 90 d of pregnancy. Male offspring had lower weight and size in comparison to control lambs.[45] In another study in rats, prenatal testosterone propionate treatment reduced size and weight at birth in both sexes.[46] Thus, it is possible to attribute differences in the body weight trajectory during postnatal development to the dose of testosterone given to the mothers. Most notable would be the effect of prenatal

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exposure to androgens in females on birth weight[47] and body development in early postnatal life. Ewe lambs treated with testosterone propionate prenatally had lower birth weight, smaller size, lower thoracic perimeter and exhibited compensatory growth during 2 to 4 months of life.[45] The last two effects were not present in males. In another study,[48] female offspring had smaller size, lighter body weight at birth, with compensatory growth at 16 weeks of age. In girls born to mothers with PCOS, they had a lower birth weight compared to those born to control mothers,[49] suggesting that the hyperandrogenemia present during pregnancy in PCOS may contribute to reduced body weight in female offspring.[15] On the contrary, boys born to PCOS mothers had greater body weight from infancy to adulthood.[50]

In summary, prenatal exposure of male sheep to testosterone had not significantly impact in IS during sexual development. In addition, orchiectomy of pubertal males allowed facilitated differentiating effects of a glucose challenge on insulin secretion between males with and without prenatal exposure to testosterone.

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Conflicts of Interest. None.

Ethical standards. All procedures, management, and experimental methodologies were previously revised and approved for the Ethical Committee in Animal Research of the Faculty of Veterinary Science of the Universidad de Concepción, Chile, and are in agreement with the International Guiding Principles for Biomedical Research Involving Animals and Bioethics Advisory Committee of the Chilean National Commission for Scientific and Technological Research (CONICYT, Chile).

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