

Sexual dimorphism in insulin sensitivity and susceptibility to develop diabetes in rats

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Abstract

The goal of this study was to evaluate gender-related differences of some metabolic determinants of insulin sensitivity and of susceptibility to the effects of diabetes. Changes in body weight, blood glucose, and serum insulin concentrations were compared between female and male Wistar rats in prepubertal, pubertal, and adult stages of life. A diabetic model was induced by streptozotocin (STZ) under nicotinamide protection in both sexes and metabolic patterns were evaluated during the next 4 weeks. Finally, the pancreases were processed for morphometric analysis. In the three age groups, at similar blood glucose levels, higher fasting

serum insulin levels were found in female as compared with age matched male rats. After STZ treatment, female rats show lower insulin and higher glucose levels, and a worse survival rate as compared with male rats. The more severe disease phenotype observed in female animals is associated with a more dramatic perturbation of pancreatic islet morphology. Significant differences exist in insulin sensitivity between sexes, females being less sensitive to insulin than males at all age groups and more susceptible to the rapid development of a more severe form of diabetes than males.

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Introduction

Diabetes mellitus is a common metabolic disease. Type 2 diabetes is rapidly becoming pandemic, and although the origin of this disease is not entirely clear, it is accepted that insulin resistance is important in its pathogenesis and that defects in insulin secretion by pancreatic β -cells lead to hyperglycemia and the onset of diabetes (King *et al.* 1998, Wild *et al.* 2004). It is interesting that the proportion of diabetes is higher in women than in men (King *et al.* 1998). Recent studies in young populations show that 5-year-old girls and female adolescents show higher insulin resistance than boys (Hoffman *et al.* 2000, Murphy *et al.* 2004). It has been proposed that sex-related genes could be important in explaining this difference (Murphy *et al.* 2004).

Different rodent models also show differences in insulin sensitivity and secretion between genders. For example, glucose-induced insulin secretion by isolated pancreatic islets from female Wistar rats at 4, 8, and 21 days of age is twofold higher than the respective secretion in males (Lopes Da Costa *et al.* 2004). Also, the spontaneous incidence of type 1 diabetes in the non-obese diabetic mice is 85% in females and 25% in males (Hawkins *et al.* 1993, Winer *et al.* 2002).

In this study, we analyzed differences between sexes in blood glucose concentration and serum insulin levels at different ages in Wistar rats. We also measured differences between both sexes in some metabolic and morphologic

parameters in a diabetic rat model induced with streptozotocin (STZ) and nicotinamide (Masiello *et al.* 1998).

Materials and Methods

Materials

Reagents were obtained from the following sources: STZ, nicotinamide, mouse anti-rat glucagon, Triton X-100, propidium iodide, sodium citrate, and salts from Sigma-Aldrich; rat insulin ELISA (ALPCO Diagnostics, Windham, NH, USA); Paraplast (Sherwood Medical Co., St Louis, MO, USA); insulin-antiserum (ICN, Irvine, CA, USA), fluorescein isothiocyanate (FITC)-conjugated goat anti-guinea pig IgG, CY5-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

Animals

Groups of 12 prepubertal (21 days), 12 pubertal (45 days), and 12 adult (2 months) female and male Wistar rats were obtained from the local animal facility, housed separated by sex, maintained in a 14 h light (0600–2000):10 h darkness cycle, and allowed free access to standard laboratory rat diet and distilled water. Animals were handled according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH No. 85-23, revised 1985).

All methods used in this study were approved by the Internal Council and the Animal Care Committee of The Institute of Cellular Physiology, Universidad Nacional Autonoma de Mexico (UNAM).

Insulin sensitivity

Insulin sensitivity was assessed using fasting insulin and glucose, as described by McAuley *et al.* (2001). These parameters have been validated as an appropriated single laboratory measure to describe insulin sensitivity in individuals (Laakso 1993, McAuley *et al.* 2001).

Insulin tolerance test

Rats were injected intraperitoneally with insulin (1 U/kg body weight) and glucose was measured in the tail vein 0, 15, 30, and 60 min after injection, using an automatic glucometer (Precision, QIDTM, MediSense, Inc., Abbott Laboratories Company). This test was always performed at 1100–1400 h (Goren *et al.* 2004).

Glucose tolerance test

Rats were fasted overnight (16 h) and injected intraperitoneally with glucose (2 mg/kg); 0, 15, 30, 60, and 120 min after injection, glucose was measured in tail vein blood with a glucometer (Goren *et al.* 2004).

Assessment of abdominal fat

Prepubertal female and male Wistar rats were killed by sodium pentobarbital (60 mg/kg) overdose. In male rats, epididymal fat pads were removed and weighed, whereas in female rats, parametrial and retroperitoneal fat pads were removed and weighed together.

Diabetic model

Three independent groups of ten young adult male and female rats received a single i.p. dose (87.5 mg/kg) of nicotinamide dissolved in saline solution. After 15 min, β -cell destruction was induced by a single i.p. dose (90 mg/kg) of STZ, dissolved in citrate buffer (pH 4.5) immediately before use. In parallel, a control group of animals received vehicles of both substances. A pilot group of five male and five female adult rats received a single nicotinamide i.p. dose (175 mg/kg), dissolved in saline solution. After 15 min, β -cell destruction was induced as described above. Also parallel controls received vehicles.

Blood extraction and glucose and insulin measurements

For the next 4 weeks, we measured once a week, in the morning, body weight, blood glucose, and serum insulin concentrations. Rats were previously fasted for 4 h (from 0700 to 1100 h) and anesthetized with ether before blood

extraction by intraorbital retrobulbar plexus puncture. Sera were obtained and stored at -20°C until assayed.

Blood glucose concentrations were determined as described above. Only nicotinamide- and STZ-treated rats with blood glucose concentrations above 16 mmol/l were included in the study.

To determine the insulin concentration in the sera samples, we used an ultra sensitive rat insulin ELISA as instructed by the fabricant. The absorbance of the enzymatic reaction was measured at 450 nm in an ELISA reader (Bio Rad). All determinations were performed in duplicate. Assay sensitivity by this method was 10 pg/ml and the total interassay coefficient of variation was 3.2%.

Morphometric analysis

After 4 weeks, animals were anesthetized with sodium pentobarbital (40 mg/kg) and the pancreases were removed for morphometric and immunohistochemical analysis. Finally, animals were killed by cervical dislocation.

The pancreases from six control and six diabetic rats of each sex were removed, fixed overnight in 4% paraformaldehyde in PBS, dehydrated, and embedded in Paraplast. Four serial sections 5 μm thick were selected, each 100 μm from each tail pancreas, and mounted on slides. Paraffin sections were deparaffinized, rehydrated, permeabilised, and subsequently incubated overnight with guinea pig anti-porcine insulin antibody (1:4000) as recommended by the technical bulletins suppliers. Then, sections were incubated with a second FITC-conjugated goat anti-guinea pig IgG antibody for insulin detection (1:100), then incubated for 4 h with mouse anti-rat glucagon (1:6000) and a second CY5-conjugated goat anti-mouse IgG (1:100). Sections were counterstained with propidium iodide at 10 $\mu\text{g}/\text{ml}$ to facilitate nuclear identification.

Sections were observed by confocal microscopy using a Bio Rad MRC-1024 system, equipped with a Kr/Ar laser attached to an inverted Nikon Diaphot TMD 300 microscope, with an oil-immersion 40 \times objective (Nikon Corporation, Tokyo, Japan). Iris aperture, gain, and laser power remained fixed in each session, FITC was excited with a 494 nm wavelength, and emitted light was band-passed with a 520 nm filter, while CY5 was excited with a 650 nm wavelength, and emitted light was band-passed with a 670 nm filter. Confocal images were viewed and processed using Confocal Assistant 4.02 (Todd Clark University of Minnesota, MN, USA).

The following controls were performed to achieve a reliable double immunostaining. (1) Negative control of antibodies: experimental protocols were carried out without the addition of primary or secondary antibody. (2) Antisera specificity controls were performed by overnight preadsorbing anti-insulin and anti-glucagon antibodies with their respective antigens, followed by the same protocol. No fluorescent signal could be detected in either control (not shown).

The percentages of β - and α -cell areas in the pancreas were calculated by dividing insulin-positive or glucagon-positive areas in one section by the total area of this section and multiplying this ratio by 100. As described above, only islets containing 15 or more endocrine cells were measured (Xu *et al.* 1999).

Morphometric measurements were performed using a manual optical picture image analyzer; Laser Pix program Bio Rad version 4.0.0.13 on a projected image of the histological sections of the pancreas. Nearly 140 islets per rat were analyzed.

Statistical analysis

All data are presented as the mean \pm S.E.M.; *n* denotes the number of the evaluated subjects. Statistical significance was assessed by one-way ANOVA followed by Fisher's multiple range tests and *P*-values less than 0.01 were considered statistically significant (Stat view 4.57.0.0, Abacus Concepts Inc., Berkeley, CA, USA).

Results

Metabolic development

Body weight, blood glucose, and serum insulin were measured and compared between female and male, pre-pubertal (21 days), pubertal (45 days), and adult (2 months) rats (Table 1).

As described above, pubertal and adult males were nearly 20% heavier than females (Engelbregt *et al.* 2000). In contrast, we found no difference in body weight in prepubertal rats between sexes. However, interestingly, the percentage of abdominal fat at this developmental stage was significantly higher in females ($4.3 \pm 0.07\%$) than in males ($2.09 \pm 0.3\%$).

Fasting blood glucose values were similar between sexes at all stages. However, glucose concentration in prepubertal animals was 20% higher (Table 1) and insulin concentration was lower, when compared with pubertal and adult rats.

At the three developmental stages studied, fasting insulin concentration in females was 45% higher compared with males (Table 1).

Table 1 Metabolic status of development in female and male rats

	Prepubertal		Pubertal		Adult	
	Male	Female	Male	Female	Male	Female
Body weight (g)	82 \pm 4†	87 \pm 5†	253 \pm 7	209 \pm 4*	303 \pm 3	278 \pm 5*
Blood glucose (G) (mmol/l)	9 \pm 0.03†	9 \pm 0.02†	7 \pm 0.03	7 \pm 0.02	7 \pm 0.02	7 \pm 0.03
Serum insulin (I) (pmol/l)	14 \pm 3†	47 \pm 16*†	110 \pm 9	160 \pm 12*	104 \pm 10	151 \pm 9*
I/G ratio (pmol/mmol)	1.6 \pm 1†	5.2 \pm 1*†	14.7 \pm 1	22.2 \pm 1*	14.5 \pm 1	20.2 \pm 1*

Data represent mean \pm S.E.M. for at least 12 rats in each condition. **P* < 0.01 with respect to male age matched; †with respect to pubertal and adult.

Glucose tolerance test

This test was performed in animals that had fasted for 16 h (Fig. 1A and C) and reflects insulin-stimulated glucose clearance and inhibition of glucose release by the liver (Goren *et al.* 2004). We did not observe differences between the sexes in glucose values. As described above, compared to pubertal rats, prepubertal rats showed higher glucose concentrations.

Insulin sensitivity test

We evaluated insulin sensitivity with an insulin tolerance test (Fig. 1B and D). A pharmacological dose of insulin was injected to non-fasted rats. At 15 min, a similar decrease in plasmatic glucose was observed in both sexes. However, at 30 and 60 min after injection, male glucose concentrations were 20% lower, compared to females. These observations suggest that prepubertal female rats are less sensitive to insulin than males (Fig. 1B). A similar response was observed in pubertal female rats (Fig. 1D).

Diabetic model

To further explore sex differences in insulin sensitivity, we developed an experimental diabetic model using STZ (95 mg/kg) and nicotinamide (87.5 mg/kg; see Methods), and compared the metabolic patterns between sexes during 4 weeks of diabetes development.

Previously, the same amount of STZ combined with a higher concentration of nicotinamide (175 mg/kg) was used in a pilot group. In this experiment, no changes were observed in body weight in experimental animals, compared to controls (not shown). Only one female and male developed hyperglycemia before week 5 and all the animals survived more than 8 weeks. In order to have less protection to STZ toxic effect, we decided to use a lower nicotinamide dose (87.5 mg/kg).

In diabetes loss of body weight and hyperglycemia are observed

While body weight in control males increased throughout the experiment, it did not significantly change in control females. In contrast, diabetic males did not gained weight and a 13% mean reduction was observed in diabetic females (Fig. 2A).

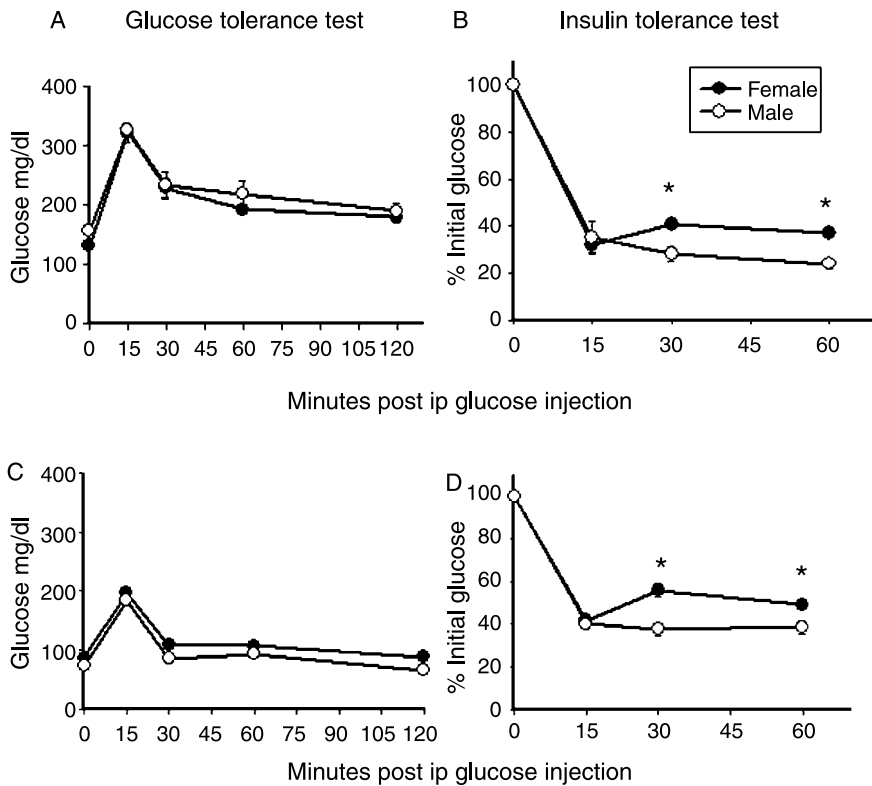


Figure 1 Glucose homeostasis in rats at 21 (A, B) and 45 (C, D) days of age. Open circles represent males and closed circles, females. After 16 h of fasting animals were subjected to glucose tolerance test (A, C) and insulin tolerance test (B, D), as described in Materials and Methods. Data represent mean \pm s.e.m. of at least six rats in each group. * $P < 0.01$ vs male.

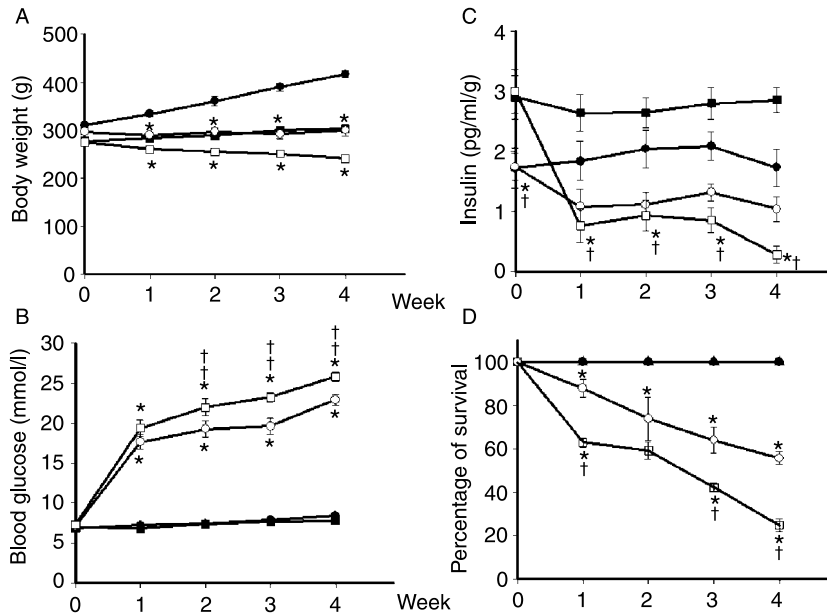


Figure 2 Body weight (A), blood glucose (B), serum insulin (C) and survival (D) was monitored weekly in control and diabetic rats. Data represent the mean \pm s.e.m. of at least ten rats in each experimental condition. Male control (MC; ●), male diabetic (MD; ○), female control (FC; ■), and female diabetic (FD; □). * $P < 0.01$ vs MC; † $P < 0.01$ vs FC, ‡ $P < 0.01$ vs MD.

All the experimental animals developed hyperglycemia. However, female glucose values were higher than male values. As shown in Fig. 2B, this increase was significantly progressive throughout the experiment.

Serum insulin concentration and animal survival decreased in diabetic rats

Figure 2C compares serum insulin concentration in control and diabetic animals. Clearly, diabetic animals secreted less insulin than their respective controls, the major decrease, of nearly 80%, being observed in females.

Interestingly, while 88% of diabetic males survived the first week of treatment, only 63% of the females remained alive. This tendency prevailed throughout the experiment (Fig. 2D).

Changes in pancreatic β -cells mass

After 4 weeks of treatment, we compared pancreatic islet morphology and insulin and glucagon distribution in

control and diabetic animals. Figure 3A and B shows that control islets from both sexes are rounded and composed of nearly 80% β -cells, located predominantly in the core of the islets. Most of the α -cells were surrounding β -cells and represented nearly 10% of the cells in the islets.

We observed many differences between pancreatic tissue obtained from female and male controls that can be summarized as follows: (a) mean islets area in females was 40% higher than in males (Fig. 3A). (b) Areas occupied by β - and α -cells in pancreas of females were nearly 22 and 50% higher respectively when compared with males (Fig. 3B and C). (c) Islet size and number in females was nearly 20% higher than males (Table 2).

Pancreatic islets in diabetic animals of both sexes were scarce, elongated and disorganized, as shown in Fig. 4C and D. In the islets that were not destroyed by the treatment, α -cells were located in the core, filling up the space previously occupied by β -cells (Fig. 4 and Table 2).

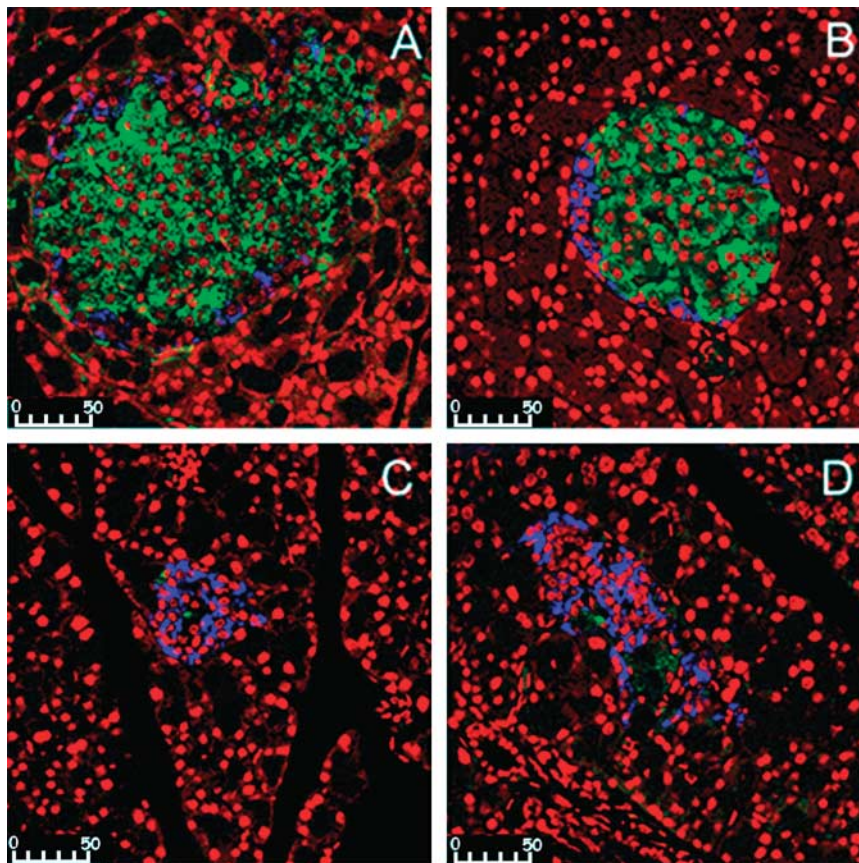


Figure 3 Representative histological sections of islets of female (A) and male (B) control rats and islets of female (C) and male (D) diabetic rats, incubated with anti-insulin (green) and anti-glucagon (blue) antibodies conjugated with a fluorescent dye, and counterstained with propidium iodide (red). Scale bar 50 μ m.

Table 2 Morphometric measurement of pancreatic islets in control and diabetic female and male rats

	Control male	Control female	Diabetic male	Diabetic female
Islet size (μm^2)	14 180 \pm 1320	17 156 \pm 1311*	9174 \pm 1224*	8073 \pm 1055†
Islets in pancreas (each 10 mm ²)	4.78 \pm 0.61	6.40 \pm 0.06*	3.10 \pm 0.77*	2.75 \pm 0.52†

Data represent mean \pm s.e.m. for at least six rats in each condition. * $P < 0.01$ with respect to male control; † $P < 0.01$ with respect to female control.

Discussion

We observed sexual dimorphism in insulin sensitivity, function and morphology of prepubertal, pubertal, and adult rat pancreatic islets, and susceptibility to develop diabetes. To our knowledge, this is the first report showing that serum insulin levels are higher in normal prepubertal, pubertal, and adult females than in age-matched males.

Further, we observed that females have more and bigger islets than males. However, both sexes showed similar fasting blood glucose concentrations. Moreover, plasmatic glucose response to an insulin challenge, which directly reflects insulin sensitivity of peripheral tissue, was lower in females than in males (Fig. 1).

Lower insulin sensitivity in females could be partially explained by the presence of higher fat proportions in females than in males (Schwartz & Porte 2005). In relation to their weight, we observed more abdominal fat in female prepubertal rats than in males. Other authors previously reported that this difference is present throughout life (Clegg *et al.* 2003). Abdominal and visceral adiposity has been linked to insulin resistance and metabolic syndrome. Adipocytes from this region produce more cytokines and show differences in metabolic activity compared with fat of other regions (Giorgino *et al.* 2005).

Prepubertal girls are more resistant to insulin than boys (Hoffman *et al.* 2000). Girls under 5 years show higher insulin levels, but similar blood glucose concentrations, than boys (Murphy *et al.* 2004). The 'EarlyBird Study' (Wilkin *et al.* 2004) shows that insulin resistance is inherited from mothers not from fathers and this agrees with the 'DECODE Study Group' (2003), which suggests that females are intrinsically more insulin resistant than males throughout life. Also in non-primate animal models, it had been observed that maternal obesity induced impaired glucose tolerance only in female offspring (Han *et al.* 2005).

Adipose tissue is an endocrine organ that secretes hormones such as leptin and adiponectin (Kershaw & Flier 2004). Leptin levels can be high in obesity due to insulin and leptin resistance by their different targets (Lazar 2005). Interestingly, leptin production and secretion is higher in female rats than in matched males (Pinilla *et al.* 1999, Engelbregt *et al.* 2000, Smith & Waddell 2003) and after STZ treatment, serum leptin levels decrease (Havel *et al.* 1998, 2000). This can be explained because there is a loss of weight and fat in diabetic animals.

On the other hand, estrogens and testosterone modulate pancreatic hormone secretion (Faure *et al.* 1988, Nadal *et al.* 1998, Sutter-Dub 2002, Morimoto *et al.* 2005). It has been reported that estrogens increase electrical activity and modulate insulin secretion (Nadal *et al.* 1998) and also decrease glucagon secretion by preventing low glucose-induced $[\text{Ca}]_i$

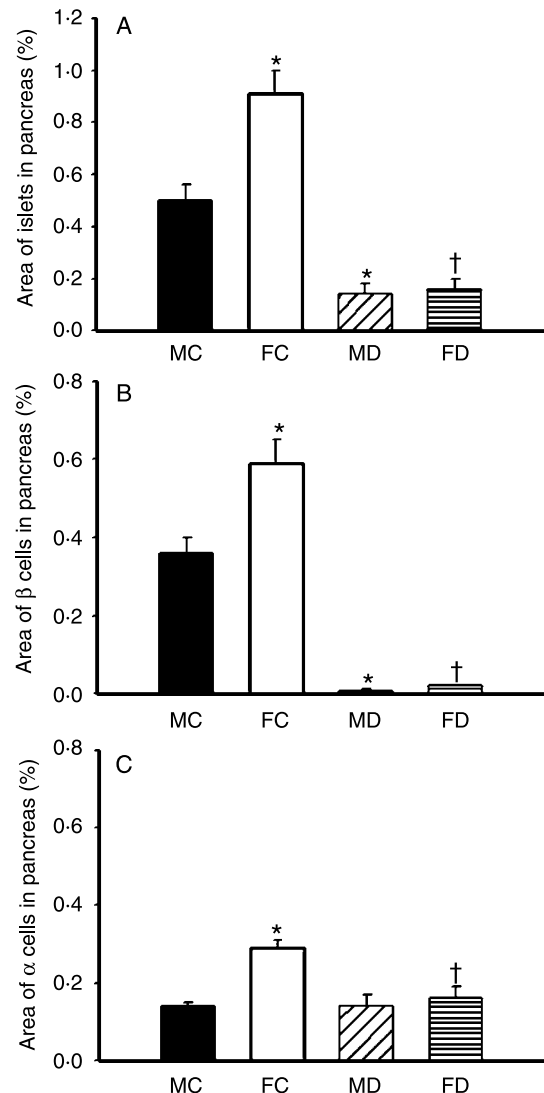


Figure 4 Endocrine area in pancreas of male control (MC), female control (FC), male diabetic (MD) and female diabetic (FD) rats. Data represent means \pm s.e.m. * $P < 0.01$ vs MC; † $P < 0.01$ vs female control.

oscillations in α -cells (Ropero *et al.* 2002). In addition, variations in serum insulin level and insulin mRNA in pancreas during the estrous cycle has been observed, suggesting that sexual steroid hormones modulate insulin secretion (Morimoto *et al.* 2001).

We observed that diabetes development in response to STZ treatment under nicotinamide protection is more severe in female rats than in males, because females developed the disease faster.

Diabetic males did not gain weight as did their matched-controls and a progressive disproportion in this parameter was observed during the experiment. Interestingly, when animals of both sexes began losing weight the risk of dying increased and females presented lower survival rates compared with males and with female controls (FCs).

Moreover, females showed more damaged β -cells and fewer, smaller pancreatic islets and accordingly, higher hyperglycemia and less serum insulin concentration than males.

In order to compare responses to males and females, we did the same experiments in castrated rats (unpublished results). One week after the treatment, castrated animals showed higher levels of hyperglycemia (26 ± 0.7 mmol/l) than diabetic males (20 ± 2 mmol/l) and females (21 ± 1 mmol/l). This observation is in accordance with previous results where we found that testosterone protects rat pancreatic β -cells against STZ-induced apoptosis (Morimoto *et al.* 2005).

Despite showing similar blood glucose concentration between sexes at all ages and in all the conditions studied, after 5 and 16 h of fasting and in fed animals, healthy females have more and bigger pancreatic islets, and higher serum insulin concentrations. The following factors could be implicated in the sexual dimorphism in insulin sensitivity: (1) insulin secretion is higher in females than in males at all ages studied. Since insulin stimulates glucose uptake and metabolism in adipose tissue, the greater fat-to-lean mass ratio seen in females of all ages, as previously observed, may be significant. (2) As shown in other studies, genetic background and (3) sex steroids might influence insulin biosynthesis or/and action, as well as β -cell survival. More studies are needed to entirely answer these questions.

After 4 weeks of diabetes, females show less and smaller pancreatic islets, and less serum insulin concentration than both FCs and diabetic males, and consequently, diabetic females have higher blood glucose values and lower survival rate than male diabetic rats, indicating a more severe response to the same treatment.

These results contribute to our understanding of the sexual differences in glucose homeostasis and may contribute to the future development of new and better therapeutic strategies for treating diabetes mellitus.

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