Tungstate administration improves the sexual and reproductive function in female rats with streptozotocin-induced diabetes

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BACKGROUND: Diabetes induces great alterations in female reproductive function. We analyzed the effects of tungstate, an anti-diabetic agent, on the reproductive function of healthy and diabetic female rats. METHODS: Healthy and streptozotocin-induced diabetic rats were treated with sodium tungstate (2 mg/ml in their drinking water) for 12 weeks. Markers of reproductive function and diabetes were measured in serum, and in uterus and ovaries by Western blot or RT–PCR. Reproductive function was also assessed by mating. RESULTS: Diabetic rats showed great impairment of libido, which was accompanied by a total loss of fertility (P<0.05) and a decrease in the serum levels of FSH (P<0.05) and LH (P<0.05) compared with healthy rats. Tungstate treatment of diabetic rats partially recovered libido while fertility rate increased to 66.6%. This improvement was accompanied by a recovery of serum FSH (to a level higher than healthy rats) and LH. Moreover, tungstate treatment normalized ovarian expression of GLUT 3 hexose transporter, and estrogen, progesterone and FSH receptors, whereas only GLUT 3 and FSH receptors were normalized in the uterus. CONCLUSIONS: Our results indicate that the alterations in female reproduction in diabetes were partially reversed after tungstate treatment by a mechanism(s) involving the normalization of serum FSH and LH levels, and ovarian and uterine expression of FSH receptors and GLUT3.

Keywords: diabetes; female rats; reproductive function; tungstate; streptozotocin

Introduction
Female reproductive function is greatly altered in insulin-dependent diabetes. Diabetes causes alterations in the timing of the estrous cycle, associated with modifications in ovary function, which induces a decrease or even absence of ovulated oocytes in female rats (Bestetti et al., 1985). Furthermore, insulin-dependent diabetes affects fertility by increasing embryonic resorptions, congenital abnormalities and perinatal mortality in female rats (Diamond et al., 1989) and mice (De Hertogh et al., 1992).

Alterations in female reproduction in diabetes have been implicated in the metabolic disturbances caused by the lack of insulin and by hyperglycemia (Diamond et al., 1989; De Hertogh et al., 1992). It has been shown diabetes causes a decrease in serum levels of FSH and LH in different animal models (Bestetti et al., 1985; Ballester et al., 2004), which is accompanied by a loss of sensitivity of ovary cells to these two hormones (Katayama et al., 1984). Impaired action of FSH and LH on the ovary has as a primary consequence, a concomitant alteration in the capacity of this organ to synthesize ovarian reproductive hormones; mainly estrogens from follicular cells and progesterone from luteal cells (see Everett, 1988, for a general review in mammals). Follicular growth, oocyte maturation and estrus behavior are regulated by FSH/estrogen action, whereas ovulation and maintenance of gestation, at least in early stages, are controlled mainly by the LH/progesterone system, although FSH also plays a relevant role (see Everett, 1988, as a general review for mammals). This implies that the alteration of the hypothalamic/hypophyseal-gonadal hormonal axis in diabetes is reflected in changes in all these ovary functions, in addition to the pathological changes caused by the metabolic disturbance induced by diabetes itself.

Insulin treatment of insulin-dependent diabetes induces a marked recovery of female reproduction (Bestetti et al., 1987; Diamond et al., 1989; De Hertogh et al., 1992). This recovery occurs through the normalization of glycemia, but also probably via a direct action of insulin on the female
reproductive system: insulin stimulates the synthesis of FSH in cultured cells, which concomitantly stimulates the in vitro synthesis of estrogens and progesterone (Davoren and Hsueh, 1984). This poses the question of whether anti-diabetic therapies that are not based on insulin also produce a recovery in female reproductive function.

Sodium tungstate shows a remarkable normoglycemic effect in several long-term treatment models of diabetes, and low toxicity in diabetic and healthy animals (Barberà et al., 1994, 1997; Muñoz et al., 2001). The streptozotocin (STZ) model of diabetes has been used extensively to study the immunological pathways that lead to insulinitis and β-cell death. STZ is a nitrosurea derivative isolated from Streptomyces achromogenes and has broad-spectrum antibiotic and anti-neoplastic activity. STZ-induced diabetes is an interesting model because in susceptible rodents it triggers insulinopenic diabetes in which immune destruction is involved, as in human Type 1 diabetes (Rees and Alcolado, 2005). Here, we analyzed the effects of tungstate on the overall female reproductive function in healthy and diabetic (STZ model) rats. We examined serum levels of several hormones and expression of functional female reproductive markers.

Materials and Methods

Suppliers
All chemical reagents were of analytical grade and were obtained from Sigma (St. Louis, MO, USA), Merck (Darmstadt, Germany), Bio-Rad Laboratories (Hercules, CA, USA) and EMS (Fort Washington, PA, USA).

Animals and treatment protocols
Adult female Wistar rats (200 g) were kept under a constant 12-h light-dark cycle and were allowed to eat and drink ad libitum. When stated, diabetes was induced by a single i.p. injection of STZ (70 mg/kg of body weight) in 0.9% NaCl with 100 mM sodium citrate buffer (pH 4.5). Diabetes was confirmed by measuring glucosuria and glycermia (glucose and glycermia strips Boehringer Mannheim; Mannheim, Germany). Diabetic rats were used 5–7 days after STZ injection, once serum glucose was higher than 500 g/dl. At the beginning of the experiment, animals were divided into four groups, each with 24 animals. The first two groups were untreated healthy (UH) and untreated diabetic (UD) rats, which received a solution of 0.9% NaCl as drinking water. The remaining two groups were treated healthy (TH) and treated diabetic (TD) rats, given a drinking solution of 2 mg/ml sodium tungstate in 0.9% NaCl. The treatment was carried out for 12 weeks. During this period, glycemia, food and water intake, and body weight were measured regularly. At the end of the experiment, all the rats in the UH and TH groups were still alive, whereas only 16 animals in the TD group and 12 in the UD group survived (weight loss meant these animals were euthanased before the end of the experiment). Sexual and reproductive function was assessed in all rats before decapitation. Blood was collected immediately to measure serum parameters. All the animals were then anesthetized with diethyl ether and killed by decapitation between 9:00 and 11:00 h. Ovaries horns were prepared in two ways. First, part of the tissues were immediately fixed in 3% formaldehyde in a buffer solution containing 54 mM NaH2PO4 and 28 mM Na2HPO4 (pH 7.4) at 4°C (buffered formaldehyde). This material can be stored for up to 5 weeks. These tissues were used for optical microscope histology. The rest of the tissues were immediately frozen in liquid N2 and stored at −90°C until Western blot and semi-quantitative RT–PCR analyses were performed.

Measure of reproductive performance
To evaluate reproductive performance, after 10 weeks of treatment, individual females were placed in a cage with one healthy adult male (body weight: 250 g). The animals were kept together overnight, and then separated the following morning. Immediately after each separation, a vaginal examination and scrape were carried out to determine if sexual intercourse had occurred. When intercourse was positive (presence of a vaginal tap and/or spermatozoa), the night/day routine was discontinued and the female was housed individually during the estimated period of gestation. At parturition, litter size was determined and the mother was anesthetized with diethyl ether and killed by decapitation. The neonates were killed by CO2 inhalation. When females showed no sexual contact with the male during 9 consecutive days, they were not used for further mating and were labelled ‘unable’, whereas those that showed sexual activity were labelled ‘able’.

The reproductive performance of the rats was measured as the proportion of ‘able’ females to the total number tested (the percentage of positive vaginal scrapes, i.e. those with presence of spermatozoa, with respect to the total number of vaginal scrapes performed in one experimental group). This parameter was named as ‘mating index’. The number of vaginal scrapes varied depending on the time required by animals to show positive intercourse. Furthermore, we also calculated the percentage of parturitions with respect to the number of positive scrapes. This parameter was named ‘fertility’. Finally, the mean litter size was also calculated and was defined as ‘prolificacy’. These definitions do not necessarily coincide with the common definitions for mating index, fertility and prolificacy published elsewhere.

Analytical procedures in serum samples
Glycemia was measured by the hexokinase method (Glucocquant, Boehringer Mannheim); alanine aminotransferase (ALT) was measured spectrophotometrically (ALT monotest, Boehringer Mannheim); alanine aminotransferase (ALT) was determined by enzyme-linked immunosorbent assay (ELISA) [Intra-assay coefficient of variation (CV) = 3.5% and inter-assay CV = 6.3%; assay range: 156–10 000 pg/ml], using a commercial kit (Cristal Chem; Chicago, IL, USA). Serum progesterone levels were determined by ELISA kit (Progesterone: intra-assay precision CV = 4.6% and inter-assay CV = 5.1%; sensitivity: 0.13 ng/ml) (Diagnostic Systems Laboratories, Inc; Webster, TX, USA). Serum levels of FSH and LH were also measured using a commercial ELISA kit (FSH: within-assay precision; percentage determined by measuring optical density of multiple replicate controls = 7.4% and by measuring concentration = 11.4%, sensitivity: 8.66 ng/ml; LH: within-assay precision by optical density = 10.9% and by concentration = 7.6%, sensitivity: 0.1 ng/ml) (Habersham; Buckinghamshire, UK).

Histological techniques for optical microscope observation
Formaldehyde-fixed ovaries were embedded in paraffin and sliced (thickness: 3–4 μm) onto slides pre-coated with silane. Xylol was used to remove paraffin from the slices, and histological analysis was performed following a hematoxilin-eosin stain. For long storage, slides were mounted with a commercial mounting medium (Adh CLINIC®; Clinic Services, Barcelona, Spain). Optical microscope observation was done through a Nikon® Eclipse E800® (Kanagawa, Japan).
Western blot analyses were performed using frozen samples that were homogenized (1:8, w/v) in a 25 mM HEPES buffer (pH 7.4) containing 4 mM EDTA, 250 mM sucrose, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin A and 0.2 mM phenylmethylsulfonyl fluoride.

Western blot analysis was based on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transfer to nitrocellulose (Laemmli, 1970; Burnett, 1981). This analysis was done with a Mini-PROTEAN 3 System (Bio-Rad laboratories). The protein profile obtained after SDS–PAGE was also examined to test for differences in loading among groups. Antibodies were used at a dilution of 1/200 (v/v). Immunoreactive proteins were tested with peroxidase-conjugated anti-rabbit, anti-mouse or anti-goat secondary antibodies (Amersham) and the reaction was developed with an enhanced chemiluminescence detection system (Amersham).

Anti-rabbit estrogen receptor, progesterone receptor, insulin receptor and FSH receptor were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-rabbit GLUT 3 hexose transporter was from Chemicon International (Temecula, CA, USA).

Oligonucleotide Primers
Three pairs of 20-mer oligonucleotide primers were synthesized. These oligonucleotides were from the complementary DNA (cDNA) sequence of the rat FSH receptor (Sprengel et al., 1990), the rat LH receptor (Zhang et al., 1995) and rat glucose 6-phosphate dehydrogenase, the latter used as an internal control with constitutive expression (Ouhtit et al., 1993). Primers for the intracellular domain of prolactin receptor were: the 5'-primer (5'-TCTTGTGAA TGGATGCGCATTAGCGCTG-3') corresponded to nucleotides 1158–1187 and the 3'-primer (5'-TTCAAGGAACCCAGGC TTCGTCTGTTGTT-3') to nucleotides 1765–1794 (Ouhtit et al., 1993). The primers for the FSH receptor were as follows: the 5'-primer (Forward) (5'-ATCTGATGCTACATCTGCT-3') corresponded to nucleotides 45–65 of the FSH receptor cDNA, and the 3'-primer (Reverse) (5'-AATGCA TCGTGGTGGTAGG-3') to nucleotides 1035055. These amplify a fragment of ~1000 kb, localized between the extracellular and transmembrane domains (Sprengel et al., 1990). The primers used for the LH receptor were: the 5'-primer (5'-CGAGTC CCGACTCTGAGA-3') corresponded to nucleotides 10–29 of the LH receptor cDNA, and the 3'-primer (5'-CACGCCA TGAGGGAAC-3') to nucleotides 943–962. These amplified a fragment of ~950 kb localized in the extracellular domain of the protein (Zhang et al., 1995). Finally, the 5'-primer for glucose 6-phosphate dehydrogenase (5'-GACCTG CAGTCCATACAAC-3') and the 3'-primer (5'-CAGGACCCTCA GTACCAAGGG-3') amplified a fragment of ~150 kb (Ouhtit et al., 1993).

RNA was extracted from ovaries and uterine horns stored at −80°C using the TriPure Isolation Reagent, one-step method (Roche Inc., Basel, Switzerland), following the manufacturer’s instructions. Three microgram of the total RNA was mixed with the PCR reaction buffer [10 mM Tris–HCl (pH 8.3), 1.5 mM MgCl2, 50 mM KCl, 200 µM deoxy-NTPs and 1 mM of each primer] and added to 40 U RNAase inhibitor (RNasin; Promega, Madison, WI, USA) in a final volume of 50 µl. The PCR mixture was then supplemented with 12.5 U of avian myeloblastosis virus reverse transcriptase (Promega), and samples were placed in a thermal cycle GeneAmp PCR System 9600 (Perkin Elmer, Inc.; Waltham, MA, USA). Reverse transcriptase reaction was performed after incubation for 10 min at 50°C and denaturation by incubation for 3 min at 97°C. PCR cycles started with incubation for 1.5 min at 96°C, followed by incubations for 1 min and 3.5 min at 57°C and 72°C, respectively. These steps were performed 40 times and were followed by a final extension for 10 min at 72°C.

Semi-quantitative analyses of RT–PCR results were carried out using 2 µl aliquots of the RT–PCR reaction mixture together with 5 µl of a 5:1 (v/v) formamide loading buffer containing 25 mM EDTA and 50 mg/ml dextran blue (pH 8). A further 0.5 µl of molecular weight markers (GeneScan 2500 ROX Size Standard ABI PRISM; Applied Biosystems, Foster City, USA) was added to the mixture. The resultant sample was denatured by heating at 95°C for 2 min. Samples were loaded into a polyacrylamide gel system of Applied Biosystems 373 DNA Sequencer STRETCH. To quantify differences in band intensities we used the software GeneScan Analysis 3.1 (Applied Biosystems).

Statistics
All values are means ± SEM. Statistical comparisons of the means were performed by analysis of variance, followed by the Student–Neumann–Keuls test. This test makes multiple pairwise comparisons, where the cut-off point to which comparisons are made changes between them. Thus, this methodology shows greater sensitivity than other similar multiple pairwise comparison tests, such as the Student’s t-test. A P-value of 0.05 was considered to be statistically significant.

Results
Effects of tungstate on body weight
Before STZ injection, mean body weight was 247.2 ± 2.4 g; food intake 55.6 ± 3.0 and water intake 79.3 ± 3.4. On the 5–7th day of the experiment (i.e. after STZ treatment), body weight in UH and TH was 255.3 ± 2.8 g and in UD and TD 242.3 ± 2.0; food intake and water intake was similar in all groups at 63.4 ± 2.2 and 78.6 ± 7.1, respectively. STZ-induced diabetes had a dramatic effect on body weight. UD animals showed a mean positive weight gain of only 20.0 g after 12 weeks, whereas TD rats showed a weight loss of 38.8 g (Table 1). Tungstate treatment also caused a decrease in the body weight gain of healthy rats (Table 1). In contrast, UD rats showed a significant (P < 0.05) increase in the daily food and water consumption compared with healthy animals. Tungstate treatment did not modify daily food and water consumption in healthy rats, but caused a significant (P < 0.05) decrease in both in TD rats, which reached values only slightly higher than those observed in the UH and TH groups (Table 1).

Effects of tungstate treatment on serum parameters
UD rats showed very high serum levels of glucose and ALT activity and low levels of serum insulin, progesterone, FSH and LH (Table 1). Tungstate treatment did not modify these parameters in healthy rats. In contrast to healthy rats, tungstate completely counteracted the effects of diabetes on LH levels, and FSH was higher in the TD group than in UH or TH rats (Table 1). However, tungstate treatment failed to restore progesterone levels (Table 1).

Effects of tungstate on reproductive performance
Diabetes also induced a clear impairment of libido, with a very low percentage of ‘able’ females (Table 2). This effect was
Of 45 kDa, whereas tungstate treatment almost completely
clearly increased the intensity of these bands, especially that
the intensity of the bands in healthy rats (Fig. 2A). Diabetes
and 90 kDa. Tungstate treatment induced a slight decrease in
Western blot against GLUT 3 showed two bands of ~45 kDa
and insulin receptors in ovary and uterus extracts
Changes in the expression of GLUT 3 hexose transporters
(Western blot against the progesterone receptor indicated the
presence of a specific band of ~70 kDa in ovary and uterus.
In contrast, the uterine expression of the progesterone receptor in healthy
rats was not affected by tungstate treatment. Diabetes
did not affect any reproductive parameter in healthy rats.

Microscopic morphology of ovaries after tungstate treatment
Histological analyses of ovaries did not show evident differences between groups. Thus, active ovaries showing evolutive
follicles, corpora lutea and follicular glands were detected in all
groups (Fig. 1A). Ovaries were generally covered by a significant
amount of adipose tissue (data not shown). However, 7 out
12 UD rats showed some degenerative signals of ovarian structures, such as small cells, with picnotic nuclei spread in the
ovary, scavenger cells in follicles, an increase in intracellular vacuoles in follicles, corpora lutea and epithelial oviductal
cells and clear signs of neovascularization in this organ (Fig. 1B).

Changes in the expression of GLUT 3 hexose transporters
and insulin receptors in ovary and uterus extracts
Western blot against GLUT 3 showed two bands of ~45 kDa
and 90 kDa. Tungstate treatment induced a slight decrease in
the intensity of the bands in healthy rats (Fig. 2A). Diabetes
clearly increased the intensity of these bands, especially that
of 45 kDa, whereas tungstate treatment almost completely
counteracted this increase (Fig. 2A). Similar results were
observed in uterine extracts (Fig. 2B).

Western blot against the insulin receptor showed the presence of a specific band of ~130 kDa. There were no discernible differences in the intensity of these bands in ovaries or in uterus among the four experimental groups (data not shown).

Expression of ovarian and uterine estrogen, progesterone
and prolactin receptors
Western blots against estrogen receptors showed the presence of a specific band of 50 kDa in ovarian and uterine extracts (Fig. 3A and B). Diabetes increased the intensity of the band in the ovary, whereas it decreased intensity in the uterus. Tungstate treatment counteracted the effect only in the ovary (Fig. 3A and B). There were no differences in the expression of the estrogen receptor in the two groups of healthy rats (Fig. 3A and B).

Western blots against the progesterone receptor indicated the presence of a specific band of ~70 kDa in ovary and uterus. Tungstate treatment had a considerable effect on the expression of this receptor, decreasing the intensity of the band in ovaries from TH animals. Diabetes induced a great increase in band intensity (Fig. 3C). The effect of diabetes on ovaries was partially counteracted by tungstate treatment (Fig. 3C). In contrast, the uterine expression of the progesterone receptor in healthy rats was not affected by tungstate treatment. Diabetes induced a clear decrease in progesterone receptor signal, which was not counteracted by tungstate treatment (Fig. 3D).

Finally, prolactin is a key modulator of follicular growth and differentiation, and therefore, we measured the expression of its receptor in ovarian extracts. The semi-quantitative RT–PCR analyses did not show a significant change in the amount of ovarian prolactin receptor mRNA among the four groups (data not shown).

Table 1: Effects of tungstate treatment for 12 weeks on physical and serum parameters in female rats

<table>
<thead>
<tr>
<th></th>
<th>UH (n = 24)</th>
<th>TH (n = 24)</th>
<th>UD (n = 12)</th>
<th>TD (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain (g/period of treatment)</td>
<td>77.2 ± 3.0a</td>
<td>45.7 ± 3.5b</td>
<td>20.0 ± 7.1c</td>
<td>−38.8 ± 4.9d</td>
</tr>
<tr>
<td>Food intake at day 85 of treatment (g/Kg body weight)</td>
<td>56.1 ± 2.4a</td>
<td>53.2 ± 2.0a</td>
<td>174.2 ± 6.7n</td>
<td>67.3 ± 4.0h</td>
</tr>
<tr>
<td>Water intake at day 85 of treatment (ml/Kg body weight)</td>
<td>78.1 ± 3.1a</td>
<td>77.6 ± 2.8a</td>
<td>802.5 ± 5.3b</td>
<td>96.4 ± 4.0f</td>
</tr>
<tr>
<td>Serum glucose (g/dL)</td>
<td>104.2 ± 2.5a</td>
<td>107.5 ± 3.3a</td>
<td>586.2 ± 6.8b</td>
<td>255.7 ± 10.2c</td>
</tr>
<tr>
<td>Serum insulin (ng/mL)</td>
<td>1.1 ± 0.3a</td>
<td>1.1 ± 0.1a</td>
<td>N.D.a</td>
<td>0.4 ± 0.1b</td>
</tr>
<tr>
<td>Serum ALT (U/L)</td>
<td>47 ± 3a</td>
<td>43 ± 2a</td>
<td>99 ± 5b</td>
<td>42 ± 6c</td>
</tr>
<tr>
<td>Serum FSH (ng/mL)</td>
<td>65.2 ± 9.8a</td>
<td>68.5 ± 5.3a</td>
<td>45.9 ± 3.3b</td>
<td>116.5 ± 6.3c</td>
</tr>
<tr>
<td>Serum LH (ng/mL)</td>
<td>2.1 ± 0.2a</td>
<td>2.1 ± 0.4a</td>
<td>1.2 ± 0.1a</td>
<td>1.8 ± 0.1b</td>
</tr>
<tr>
<td>Serum progesterone (ng/mL)</td>
<td>16.1 ± 0.8a</td>
<td>20.2 ± 4.9a</td>
<td>8.3 ± 1.5a</td>
<td>6.9 ± 0.9b</td>
</tr>
</tbody>
</table>

Body weight gain is expressed as the difference in body weight between the start and the end of the experiment. All data are expressed as mean ± SEM. Different superscript letters among each specific group indicate significant (P < 0.05) differences between groups. Significance of the differences was tested using the Students-Neumann-Keuls Test. ND, not detected; serum insulin concentration below sensitivity range of the kit used (156 pg/mL).

Table 2: Effects of diabetes on the reproductive performance of healthy and diabetic female rats

<table>
<thead>
<tr>
<th></th>
<th>“Able” females</th>
<th>Mating index %</th>
<th>Fertility %</th>
<th>Prolificacy (newborn/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UH (n = 24)</td>
<td>24</td>
<td>55.8% (24/43)</td>
<td>100.0% (24/24)</td>
<td>10.7a</td>
</tr>
<tr>
<td>TH (n = 24)</td>
<td>24</td>
<td>58.5% (24/41)</td>
<td>100.0% (24/24)</td>
<td>10.5a</td>
</tr>
<tr>
<td>UD (n = 12)</td>
<td>3</td>
<td>1.6% (3/187)</td>
<td>0% (0/3)</td>
<td>0b</td>
</tr>
<tr>
<td>TD (n = 16)</td>
<td>12</td>
<td>14.3% (16/112)</td>
<td>66.6% (8/12)</td>
<td>5.2c</td>
</tr>
</tbody>
</table>

‘Able’ females, mating index, fertility and prolificacy are defined in Materials and Methods. Different superscript letters among treatment groups for the same parameter indicate significant (P < 0.05) differences among groups. Significance of the differences was tested by a Chi-square test.
Figure 1: Rat ovaries after hematoxylin-eosin stain
Figures display images for a healthy (A) and a diabetic rat (B); and diabetic treated rat (C) showing the overall appearance of the ovary. Magnification: ×1000. Arrowheads indicate granulosa cells. Arrows indicate the location of luteal cells. Asterisk shows the presence of signs of degeneration in ovary structure.
Expression of FSH and LH receptors in ovary and uterus

Western blot showed a specific 70 kDa band against the FSH receptor in ovarian and uterine extracts. In UD rats, the amount of this receptor was diminished in both tissues. Tungstate treatment reversed this decrease (Fig. 3E and F).

In agreement with the results obtained by Western blot, the semi-quantitative RT–PCR analysis for FSH receptor mRNA showed a significant ($P < 0.05$) decrease in ovarian and uterine mRNA content in UD rats, which was reversed after tungstate treatment (Fig. 4A). This diabetes-induced decrease in FSH receptor mRNA was greater in uterine extracts than in ovary (Fig. 4A).

Semi-quantitative RT–PCR analysis of ovarian and uterine extracts showed that tungstate did not significantly affect LH receptor mRNA content in healthy rats (Fig. 4B). Diabetes induced a significant ($P < 0.05$) decrease in ovarian and uterine mRNA content for this receptor compared with UH and TH animals, and this decrease remained after tungstate treatment (Fig. 4B).

Discussion

Our results show that tungstate treatment significantly improves the reproductive performance of female diabetic rats. This improvement appears to be due mainly to a normalization of serum FSH and LH levels. These two gonadotrophins are the most important regulatory hormones of ovarian and uterine function (Everett, 1988 as a general review for mammals). In turn, diabetes-related alteration of female reproductive function is caused by the lack of FSH- and LH-signaling. Our results suggest that the normalization of both serum FSH and LH levels in diabetic rats after tungstate treatment is related to the tungstate-induced increase in insulinenia in these animals. In this regard, we stress that previous studies have shown a strong, direct relationship between insulin and LH/FSH (Katayama et al., 1984; Bestetti et al., 1985; Ballester et al., 2004). Regarding female reproduction, insulin directly enhances FSH-stimulated steroidogenesis in cultured rat granulosa cells (Davoren and Hsueh, 1984). Moreover, LH and FSH secretion is independent of the glucose concentration in the medium for gonadotroph cells in culture (Adashi et al., 1981), suggesting that the sugar does not directly regulate the secretion of these two hormones. Another indication of a direct action of insulin on the regulation of hypophysary LH and FSH metabolism has been reported in...
transgenic mice that lack hypothalamic and hypophyysary insulin receptors (Brüning et al., 2000). These animals show an altered regulation of LH secretion by the hypophysis, indicating that insulin is involved in regulating LH secretion. On the basis of these data, we hypothesize that a lack of insulin is one of the most determinant factors in the failure of female reproductive function in insulin-dependent diabetes and, thus, that the tungstate-induced recovery of serum insulin levels would be one of the mechanisms involved in the improvement of female reproductive function in diabetic rats treated with tungstate.

FSH and LH are required for correct ovarian and uterine sex steroid metabolism. Thus, FSH controls estrogen synthesis in granulosa cells, whereas LH modulates progesterone synthesis in luteal cells (Baird, 1984). Notwithstanding, the tungstate-induced recovery of female reproductive function was not complete. In fact, our results show that the complete recovery of serum LH levels in TD rats was not accompanied by a concomitant recovery of progesterone levels, although serum LH and FSH levels were normalized. This would explain the observed incomplete recovery of the female reproductive fitness after tungstate treatment, since fertility and prolificacy are strongly regulated by progesterone (Niswender and Nett, 1988). Moreover, the decrease in ovarian and uterine expression of LH receptors in diabetes was not reversed by tungstate, indicating that ovarian and uterine LH and progesterone signaling were not normalized by either tungstate or serum LH. Since progesterone synthesis is strongly regulated by the combination of LH and progesterone action, in an endocrine-autocrine manner (Niswender and Nett, 1988), low serum progesterone would be a logical consequence of the combined effects of low LH receptors and low progesterone levels, despite normalized serum LH levels.

Our results show that tungstate treatment caused a dramatic decrease in body weight in both healthy and diabetic rats. The effect of this treatment on body weight has been reported previously in healthy and diabetic rats (Claret et al., 2005). This effect is important, since the decrease in body weight may contribute significantly to the lack of a complete recovery of diabetic female reproductive function after tungstate treatment. In this regard, reproductive function inversely correlates with body weight through a leptin-mediated mechanism (Swain et al., 2004). However, in male rats, tungstate-induced weight loss is not correlated with a concomitant decrease in serum leptin levels (Barberà, personal communication). These observations suggest that a leptin-mediated, weight loss-related mechanism by itself is not directly associated with the decrease in progesterone recovery observed after tungstate treatment. In fact, our results strongly suggest that incomplete recovery of female reproductive function in tungstate-treated diabetic rats can be explained by the lack of a full recovery from insulinemia combined with failure in recovering progesterone. We do not know, of course, if the same effect can be directly applicable to human patients. In this way, more studies focused on the putative involvement of leptin in reproductive alterations in human patients with diabetes and/or body weight alterations will be needed. Notwithstanding, the existence of a close relationship between insulinemia and reproductive hormones such as LH and progesterone in mammalian species, such as mice and rat (Brüning et al., 2000; Ballester et al., 2004; this work) clearly opens the possibility that a similar relationship may be present in human patients.

In conclusion, tungstate treatment improves the reproductive performance of diabetic female rats by several mechanism(s) related to recovery of the LH/FSH endocrine axis. One of these mechanisms would be related to the tungstate-induced increase in insulinemia in diabetic rats. The increase induced by tungstate would induce, in turn, a recovery of the serum gonadotrophin levels, which would explain the improvement in reproductive function observed. Although we cannot dismiss a direct effect of tungstate on reproductive function, we can only speculate about the exact nature of this action. Furthermore, although the recovery of reproductive function in TD rats was remarkable, it was not complete, probably due to a lack of a total recovery of both insulin and progesterone serum levels in these animals. These data should be taken into account when designing tungstate-based therapies for diabetes in women.

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References


Claret M, Corominola M, Saura J, Barcelo-Batlori S, Guinovart JJ, Gomis R. Tungstate decreases weight gain and adiposity in obese rats through...


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