Original Article

Effect of PPAR-γ Agonist Rosiglitazone on Bone Mineral Density and Serum Adipokines in C57BL/6 Male Mice

(rosiglitazone / bone metabolism / adipokines)

[1]P. D. BROULÍK, [2]L. ŠEFC, [1]M. HALUZÍK

[1]Third Department of Medicine – Department of Endocrinology and Metabolism, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Prague, Czech Republic.
[2]Institute of Pathological Physiology and Centre for Experimental Haematology, First Faculty of Medicine, Charles University in Prague, Prague, Czech Republic

Abstract. Thiazolinediones (TZD) are widely used to treat type 2 diabetes, but their mechanism of action still remains only partially understood. Although the *in vitro* effects of TZD on osteoblastogenesis are well recognized, the *in vivo* consequences of these compounds on bone turnover are less understood and rather controversial. We demonstrate that a 9-week oral treatment with rosiglitazone in C57BL/6 male mice resulted in significant bone loss that was not dose dependent. The bones of the rosiglitazone-treated mice were characterized by reduction of bone density, and ash, calcium and phosphorus content. Rosiglitazone-treated mice had significantly thinner cortical widths. In contrast to serum TrACP expressed by action of osteoclasts, serum B-ALP activity, which serves as a marker of osteoblastic activity, was significantly lower in the rosiglitazone-fed animals. We did not find any differences in circulating levels of adipokines that could eventually explain rosiglitazone action. As the decrease in osteoblastic activity was demonstrated after rosiglitazone treatment, we anticipated changes in the haematopoietic stem cell pool. These cells seed in endosteal niches which comprise osteoblasts in order to maintain their stem cell function. In our study we did not see any significant influence of rosiglitazone administration on stem cells or any impairment in the lineage restrictions of rosiglitazone-treated stem cells. Our data demonstrate that rosiglitazone administration causes a loss of bone mass in cortical bone, possibly through a decrease in bone formation expressed by decreased B-ALP in male C57BL/6 mice. The levels of adipokines do not play any role.

Introduction

Although thiazolinediones (TZD) are widely used to treat type 2 diabetes, their complex mechanism of action still remains only partially understood. TZD act as agonists for the transcription factor PPAR-γ (peroxisome proliferator-activated receptor-γ) and, in addition to their insulin-sensitizing effects, they can promote adipogenesis and control gene expression in adipose tissue. TZD improve insulin sensitivity in muscle, liver and adipose tissue, inhibit hepatic gluconeogenesis and reduce levels of plasma glucose**.** Osteoblasts share a common mesenchymal precursor in the bone marrow with adopocytes.TZD activate PPAR-γ and diminish osteoblast differentiation while increasing bone marrow adipogenesis. PPAR-γ agonists in the treatment of type 2 diabetes mellitus have been associated with increased incidence of osteoporosis in postmenopausal women**.** Observational study suggested that TZD administration results in progressive bone loss in older diabetic postmenopausal women but not in older diabetic men (Kahn et al., 2008). However, Yaturu et al. in their study show a significant increase in bone loss both at total hip and femoral neck area in type 2 diabetic men on rosiglitazone treatment (Yaturu et al., 2007).

Studies in animals treated with TZD yielded conflicting results concerning bone marrow fat cell diferentiation and bone status: either significant loss of bone mineral density (Rzonca et al., 2004; Lazarenko et al., 2007)

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Corresponding author: Petr Dan Broulík, Third Department of Medicine – Department of Endocrinology and Metabolism, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, U nemocnice l, 128 08 Prague, Czech Republic. e-mail: pbrou@lf1.cuni.cz

Abbreviations: B-ALP – bone alkaline phosphatase, BAT – brown adipose tissue, BMD – bone mineral density, GGT – γ-glutamyl transferase, LTRC – long-term repopulating cells, MCP-1– monocyte chemotactic protein 1, PBS – phosphate-buffered saline, PPAR-γ – peroxisome proliferator-activated receptor γ, TPAI-1 – tissue plasminogen activator inhibitor, TrACP – tartrate-resistant acid phosphatase, TZD – thiazolinedione(s), WAT – white adipose tissue.

and increased fat content in bones (Alli et al., 2005; Ackert-Bicknell et al., 2009) or no adverse effect in intact rats (Sottile et al., 2004). Troglitazone treatment increases bone marrow adipose tissue volume but does not affect trabecular bone volume in mice (Tornvig et al., 2001). For example, adult female C57BL/6J mice showed increases in marrow adiposity without associated changes in trabecular bone after TZD treatment (Ackert-Bicknell et al., 2009).

Although the *in vitro* effects of TZD on osteoblastogenesis are well recognized, the *in vivo* consequences of these compounds on bone turnover and bone mineral density are less understood and rather controversial, especially in the case of males. The objective of this study was to determine whether chronic thiazolidinedione treatment is associated with changes in true bone mineral density, activity of osteoblasts and osteoclasts, and whether these changes are related to modulation of endocrine function of adipose tissue. To this end, we examined the *in vivo* effect of rosiglitazone administration on the skeletons of adult (3-month old) male non-diabetic C57BL/6 mice.

Material and Methods

Three-month old male C57BL/6 mice weighing approximately 25 grams were used for the experiment. They were maintained on standard laboratory diet containing 23 % protein, 4.5 % fat, 1.2 % calcium and 0.6 % phosphorus with water *ad libitum*, and were kept in an indirectly illuminated room with controlled temperature at 22 ± 2 °C. After one-week acclimatization to the new environment, one group of 12 mice was fed by rosiglitazone (Avandia, GlaxoSmithKline, Brentford, United Kingdom) 10 mg/kg/day. The second group of 12 mice was fed by rosiglitazone 20 mg/kg/day and the third group of 12 mice served as a control group. The drug was administered in the animal diet. Each animal received 5.0 g of food per day and treatment continued for nine weeks. The dose of daily administration of rosiglitazone (10 and 20 mg/kg /day) was chosen based on previous studies that demonstrated its effectiveness in lowering blood glucose levels in diabetic KK-Aγ mice similarly to the pharmacological doses used in humans (Rzonca et al., 2004; Alli et al., 2005). By weighing the food dish before its placement into the cage and after its removal it was determined that each mouse consumed more than 90 % of the chow throughout the course of the experiment.

After nine weeks of treatment, the animals were killed under ether anaesthesia and the femurs, spleen, kidneys, liver, and seminal vesicles from individual donors were removed. The wet weights of seminal vesicles, kidneys and livers were determined. Fat depots (epididymal white adipose tissue, WAT, and interscapular brown adipose tissue, BAT) were dissected and their wet weights were determined on an analytic balance to the nearest 0.01 g. Animals were fasted 18 h before the end of the experiment. Blood was collected by cardiac puncture at the time of killing and serum interleukin 6, insulin, leptin, MCP-1, resistin and TPAI-1 were measured using Lincoplex kit (LINCO, St. Charles, MO). The activity of plasma tartrate-resistant acid phosphatase (TrACP) (EC 3.1.3.2) was determined according to Tietz (1995). The high amount of TrACP is expressed by bone resorbing osteoclasts and activated macrophages. The activity of serum bone alkaline phosphatase (B-ALP) was determined by electrophoretic system using Hydragel 7 Iso-Pal kit (Sebia, Norcross, GA) (Van Hoof et al., 1988). Gamma glutamyl transferase (GGT) calcium and phosphorus were determined by Modular Roche automatic device (Roche, Indianapolis, IN).

Each femur was removed and cleaned of muscle and tendon. The left femurs were collected, stripped of soft tissues, and stored in glass vials at –20 °C. To determine the bone density, left femurs were placed in unstoppered glass vials filled with deionized water. Vials were placed into a desiccator that was connected to a vacuum for 60 min so that the trapped air diffused out of the bone. All bones were weighed before being immersed in deionized water previously equilibrated to room temperature. Bone density was calculated by Archimedes principle as described previously (Deyhim et al., 2006). Briefly, according to the Archimedes principle, a buoyant force on submerged object in a fluid equals the density of the fluid multiplied by the volume of the fluid that was displaced. The latter is the ratio of buoyant force to fluid density. The density of the object is then obtained as the mass divided by the volume that is displaced. However, it is important to note that the buoyant force does not depend on the weight or shape of the submerged object but on the weight of the displaced fluid.

The bones were then dried to constant weight and incinerated for 24 h at 600 °C to white ash, which was weighed. The ash weight was expressed per millilitre of volume of unashed femur. Bone ashes were then dissolved in 3 mol/l hydrochloric acid before determination of the calcium and phosphorus content.

For the bone morphology we used the method presented by Beall et al. (1984) and Vanderschueren et al. (1992). Before the femurs were incinerated, standardized roentgenographs of femur were made using Philips mammo diagnost 3000 Mammo Unit (Philips Deutschland GmbH, Hamburg, Germany) at controlled exposures of 26 kV at 5.5 mA. Morphometric measurements were performed directly on the X rays after magnification by fine calliper. On the roentgenographs at 40 % of the total length starting from the distal end the external, inner bone diameter and cortical width were measured after magnification with fine calliper. Analyses were blinded. Livers and seminal vesicles were cleaned and weighed and the weight was expressed in relative values (mg/100 g body weight).

Plasma glucose levels were determined for each animal at the beginning and end of the experiment. Plasma glucose levels were determined based on the enzymatic reaction of β-D-glucose with oxygen Beckman glucose analyzer 2 (Beckman Coulter, Inc., Brea, CA).

The method of bone marrow repopulation after transplantation into congenic recipients (Chang et al., 2005) was used to monitor the effects of rosiglitazone on haematopoietic stem cells. Bone marrow was flushed out from right femur into cold PBS (0.5% BSA), and nucleated cell counts were performed. Cells from each group were pooled and injected into five recipient Ly5.1 female mice sublethally irradiated with 4 Gy TBI (^{60}Co) in a dose representing 1/3 of the femur per mouse. Donor stem cells had to compete with surviving recipient stem cells to form blood cells. The chimerism (percentage of nucleated cells originated from donor cells – Ly5.1) was measured in peripheral blood and in bone marrow six months after transplantation. Briefly, blood was lysed by ammonium chloride solution. White blood cells and/or bone marrow cells were stained with PE-labelled anti-mouse CD45.1 antibodies (donor-origin Ly5.1 cells), FITC-labelled anti-mouse CD45.1 antibodies (recipient-origin Ly5.2 cells), and biotinylated antibody against particular blood cell lineages. Secondary staining was done by Streptavidin-PE-CY5. The following lineages were detected based on the surface lineage antigens: T lymphocytic cells – CD3, B lymphocytic cells – b220, granulocytic and macrophage lineage – CD11b (Mac-1) and Ly-6G/Ly-6C (Gr-1) antigens. All antibodies were purchased from BioLegend (San Diego, CA). Chimerism was measured by flow cytometry FACSCalibur (Becton Dickinson, Rockville, MD). The chimerism six month after transplantation both in bone marrow and blood reflects the action of long-term repopulating cells (LTRC) – haematopoietic stem cells.

Statistical analysis

Results are expressed as means ± SEM. Student's *t*-test or analysis of variance followed by multiple range test (Duncan, 1955) was used for comparison of the groups as appropriate. $P < 0.05$ denoted statistical significance.

Results

For the entire duration of the treatment, mice from both control and rosiglitazone-treated groups gained weight. During nine weeks of rosiglitazone administration no differences were observed between groups with respect to their eating behaviour. Rosiglitazone administration did not significantly affect animal body weight compared to the control group. Fasting plasma glucose level measured at the end of the experiment did not differ between the groups (rosiglitazone 20 mg/kg/day 6.8 ± 0.3 mmol/l, rosiglitazone 10 mg/kg/day 6.4 ± 0.3 mmol/l, control 6.7 ± 0.2 mmol/l).

Rosiglitazone administration had no effect on the wet weights of spleen, liver, or seminal vesicles. Analysis of epididymal WAT and interscapular BAT showed different changes in the weight of these tissues in rosiglitazone-fed vs. control animals (Table l). The weight of epididymal WAT was not increased, whereas the weight of interscapular BAT was increased by 38 % in the rosiglitazone group (10 mg/kg/day) and by 67 % in the rosiglitazone group (20 mg/kg/day). These changes were dose dependent.

True bone density demonstrated a significant decrease in the bone mineral density (BMD) of animals fed for nine weeks by rosiglitazone compared with the control mice (1.460 ± 0.01 g/cm^3 vs 1.393 ± 0.02 g/cm^3 in rosiglitazone 10 mg/kg/day and 1.410 ± 0.03 g/cm^3 in rosiglitazone 20 mg/kg/day, $P < 0.01$). Ash and mineral content of the femur was significantly decreased in rosiglitazone-treated mice compared with intact ani-

Table 1. Variables of body weight, bone density, bone mineral content, glucose, ALP, ACP and levels of adipokines in individual groups of animals after 9 weeks of rosiglitazone or placebo administration (means ± SD)

	Controls N = 12	**Rosiglitazone 10 µg/g BW N = 12**	**Rosiglitazone 20 µg /g BW N = 12**
Final weight (g)	32.88 ± 2.29	33.07 ± 2.16	31.87 ± 1.21
Brown fat (mg/100 g BW)	212.0 ± 22.9	647 ± 82 •	925.0 ± 56.7 •
White fat (mg/100 g BW)	2128 ± 270	2370 ± 128	2064 ± 145
Liver (mg/100 g BW)	1492 ± 181	1443 ± 118	1411 ± 144
Sem.vesicl.(mg/100 g bw)	452.0 ± 50.3	438.0 ± 47.2	440 ± 52
Femur dry weight (mg)	55.22 ± 0.40	48.11 ± 0.30 •	48.78 ± 0.50 •
Density of femur (g/cm^3)	1.460 ± 0.030	1.393 ± 0.040 •	1.410 ± 0.030 •
Femur ash content g/cm^3	0.70 ± 0.05	0.64 ± 0.03 •	0.61 ± 0.02 •
Femur calcium (mg/cm^3)	310 ± 10	220 ± 10 •	219 ± 11 •
Femur phosphate mg/cm^3	120.0 ± 4.0	110.0 ± 3.0 •	109 ± 7 •
Tartrate ACP (µkat/l)	147 ± 12	145 ± 9	142 ± 13
B-ALP (µkat/l)	1.93 ± 0.16	0.68 ± 0.40 •	0.52 ± 0.38 •
GGT (µkat/l)	0.04 ± 0.12	0.050 ± 0.011	0.040 ± 0.014
Glucose (mmol/l)	6.8 ± 0.3	6.4 ± 0.3	6.7 ± 0.3
Interleukin 6 (pg/ml)	40 ± 1	33 ± 2	26 ± 10
Insulin (pg/ml)	146.9 ± 65.4	142 ± 87	110 ± 67
TPAI-1 (pg/ml)	1954 ± 1721	2869 ± 1411	2345 ± 1175
MCP-1 (pg/ml)	49.2 ± 14.0	89 ± 35	55 ± 16
Leptin (pg/ml)	4020 ± 3146	5526 ± 2948	3606 ± 989
Resistin (pg/ml)	1301 ± 611	1646 ± 293	1901 ± 807

• $P < 0.01$ vs. intact animals

Table 2. Variables of morphometric measurements on femur in individual groups of animals after 9 weeks of rosiglitazone or placebo administration (mean ± SE)

	Controls N = 12	Rosiglitazone 10 µg/g BW N = 12	Rosiglitazone 20 µg/g BW N = 12
Femur length (mm)	17.8 ± 0.1	16.9 ± 0.2	16.8 ± 0.4
Outer diameter (mm)	2.28 ± 0.06	2.25 ± 0.05	2.29 ± 0.05
Inner diameter (mm)	1.26 ± 0.03	1.35 ± 0.02 •	1.42 ± 0.02 •
Cortical width (mm)	1.02 ± 0.02	0.90 ± 0.03 •	0.87 ± 0.01 •

• $P < 0.01$ vs. intact animals

Table 3. Chimerism in white blood cells/cell lineages in mice transplanted with bone marrow cells from rosiglitazone-treated donors. No significant difference between control and treated groups was found.

	Chimerism	Controls	Rosiglitazone 10 µg/g BW	Rosiglitazone 20 µg/g BW
Blood	Overall	80.18 ± 1.36	57.81 ± 14.11	62.66 ± 15.62
	T cells	20.74 ± 0.86	27.22 ± 1.99	23.57 ± 2.11
	B cells	49.73 ± 3.09	48.20 ± 1.85	47.75 ± 2.72
	Granulocytes and macrophages	26.20 ± 0.64	16.33 ± 4.67	24.73 ± 2.74
Bone marrow	Overall	71.37 ± 6.52	45.96 ± 15.16	48.01 ± 11.45
	B lineage	16.78 ± 0.90	22.47 ± 4.09	16.13 ± 2.06
	GM lineage	67.36 ± 2.38	61.39 ± 5.55	70.77 ± 2.19

mals. However, the decrease in bone mineral density, ash and mineral content was not further potentiated by higher dose of rosiglitazone.

Plasma tartrate-resistant acid phosphatase (TrACP-EC 3.1.3.2) levels, marker of osteoclastic resorption and γ-glutamyl transferase (GGT), in rosiglitazone-treated mice and control mice were not significantly different. Serum bone alkaline phosphatase (B-ALP) as an estimate of osteoblastic activity significantly decreased in rosiglitazone-fed mice.

To determine the effects of rosiglitazone on markers of adipose tissue metabolism we measured the circulating levels of selected adipokines. We did not observe any differences between controls and rosiglitazone-treated mice with respect to serum leptin and resistin levels. TPAI-1, interleukin 6 and MCP-1 as well as serum insulin levels were not affected by rosiglitazone treatment.

Femoral length and outer diameter were not significantly different between the groups. Rosiglitazone-treated mice had significantly thinner cortical widths at that time. This decrease in cortical width was not more pronounced after higher dose of rosiglitazone (Table 2).

Competitive repopulation assay using rosiglitazone-affected bone marrow showed a tendency towards lower engraftment of rosiglitazone-treated LTRC compared to controls. Nevertheless, this decrease did not reach statistical significance and it was not dose dependent. The distribution of blood cell lineages formed by these LTRC was not significantly affected by rosiglitazone treatment (Table 3).

Discussion

In this study we sought to delineate the *in vivo* consequences of rosiglitazone administration (10 and 20 mg/kg/day) on BMD and parameters of bone turnover in the murine skeleton. The weight of mice in both control and rosiglitazone-treated groups increased over time, but the changes in body weight were not significantly affected by rosiglitazone. In our study performed on C57BL/6 mice, neither blood glucose nor serum insulin levels were significantly affected by rosiglitazone treatment. We demonstrate that a nine-week oral treatment with rosiglitazone in C57BL/6 mice results in significant bone loss which is comparable in both rosiglitazone doses. The bones of our rosiglitazone-treated mice were characterized by reduction of bone density and ash, calcium and phosphorus content. At the same time, rosiglitazone-treated mice had significantly thinner cortical widths.

Decreases in bone volume in rosiglitazone-fed mice can arise either from an increase in bone resorption or a decrease in bone formation, processes that are regulated primarily by osteoclasts and osteoblasts, respectively. We therefore sought to determine which of these processes was altered by rosiglitazone treatment. Plasma tartrate-resistant acid phosphatase, which is expressed by active osteoclasts and therefore serves as a marker of osteoclastic activity, was not significantly different in rosiglitazone-treated mice and control mice. In contrast, serum B-ALP activity, which serves as a marker of osteoblastic activity, was significantly lower in the rosiglitazone-fed mice as compared with the control animals. Our results thus do not support published *in vitro* findings suggesting that TZD inhibit osteoclast-induced bone resorption (Okazaki, 2000).

Bone marrow adipocytes are now considered as active and potent secretory and endocrine cells. In addition to fatty acids they secrete adipokines, which are mainly regulators of adipogenic, osteogenic or haematopoietic development (Laharrague et al., 2000). In spite

of significant bone loss we did not find any changes in serum levels of adipokines and cytokines derived from adipose tissue. Serum leptin and resistin were unaltered by rosiglitazone treatment. It is noteworthy that our findings with respect to serum leptin levels do not differ from what has been reported in other studies. TZD transcriptionally inhibit leptin expression in adipocytes and serum leptin decrease (Boden et al., 2003; Watanabe et al., 2003) or remain unchanged (Sotoh et al., 2003) following TZD administration in patients with type 2 diabetes mellitus. In spite of the lack of changes in circulating adipokine levels we cannot exclude the possibility of local alterations in adipokine production within bone marrow adipocytes that could locally affect bone metabolism. It general, it is still not clear whether the effects of thiazolidinediones on bone cells are direct. It is possible that rosiglitazone alters the activity, production or binding of growth factors produced by the cells. Complete information concerning the mechanism of the skeletal effects of rosiglitazone is still lacking.

There was no evidence of liver disease or hypogonadism in any of rosiglitazone-fed mice in the present study; therefore, the confounding effects of these variables on bone mass were excluded. The weight of seminal vesicles is a very sensitive marker of testosterone activity.

The weight of interscapular BAT was increased by 38 % in the rosiglitazone group (10 mg/kg/day) and by 67 % in the rosiglitazone group (20 mg/kg/day). In a report of Aleo et al. (2003), darglitazone (a glitazone with 200-times higher potency compared to rosiglitazone) was a potent adipogenic agent in rats causing hyperplastic changes of dorsal thoracic BAT. Adipose tissue at different sites may have distinct responses to TZD. For instance, treatment with TZD increased subcutaneous adipose tissue while decreasing intra-abdominal fat accumulation (Mori et al., 1999). The PPAR-γ pathway represents an alternative potent and fully competent mechanism for BAT recruitment, which may be the cellular explanation for the enigmatic recruitment in prehibernation and prenatal states (Petrovic et al., 2008; Festuccia et al., 2009).

Rosiglitazone acts as a potent agonist to the nuclear hormone receptor peroxisome proliferator-activated receptor-γ (PPAR-γ). Activated PPAR-γ is required for the differentiation of bone marrow mesenchymal stem cells into adipocytes and is associated with the development of age-related marrow adiposity in mice. Osteoblasts or bone-forming cells share a common mesenchymal precursor in the bone marrow with adipocytes. Administration of rosiglitazone causes the imbalance in bone remodelling with diminished bone formation leading to the observed decrease in trabecular bone volume and BMD (Soroceanu et al., 2004).

Thiazolidinediones have been reported to induce adipocyte differentiation *in vitro* and there are limited data on their effects *in vivo*. Lazarenko et al. (2006) found that the pro-adipocytic and anti-osteoblastic activities of PPAR-γ can be separated *in vitro*; little evidence exists supporting this functional separation *in vivo*.

We have demonstrated that *in vivo* rosiglitazone administration to C57BL/6 mice resulted in significant bone loss**.** The divergent skeletal responses to rosiglitazone in the study of Ackert and colleagues suggest the existence of other important factors in rosiglitazone action on bone marrow (Ackert-Bicknell et al., 2009). Data from Tornvig et al. (2001) demonstrate that adipogenesis and osteogenesis can be regulated independently.

A comprehensive observational study revealed that TZD administration results in progressive bone loss in diabetic older postmenopausal women but not in older diabetic men (Schwartz et al., 2007). The only other data available on men is from the Health ABC observational study reporting a modest increase in bone loss that was not statistically significant (Schwartz et al., 2006). Fracture rates did not differ between treatment groups in a clinical study with rosiglitazone in male diabetic patients. However, in women taking rosiglitazone, an increased fracture rate was observed. Thomas et al. found that serum leptin correlated with BMD in women but not in men (Thomas et al., 2002).

In summary, we demonstrate that a nine-week oral treatment with rosiglitazone in C57BL/6 male mice resulted in significant bone loss that was not dose dependent. The bones of our rosiglitazone-treated mice were characterized by reduction of bone density and ash, calcium and phosphorus content. At the same time, rosiglitazone-treated mice had significantly thinner cortical widths. Treatment with rosiglitazone is associated with concomitant decreases in serum bone alkaline phosphatase, which serves as a marker of osteoblastic activity**.** We did not find any differences in circulating levels of adipokines that could eventually explain rosiglitazone action. As the decrease in osteoblastic activity was demonstrated after rosiglitazone treatment, we anticipated changes in the haematopoietic stem cell pool (represented by LTRC). These cells seed in endosteal niches which comprise osteoblasts in order to maintain their stem cell function (Arai et al., 2002). In our study we did not see any significant influence of rosiglitazone administration on stem cells or any impairment in the lineage restrictions of rosiglitazone-treated LTRC.

Our data demonstrate that rosiglitazone administration causes a loss of bone mass in cortical bone, possibly through a decrease in bone formation expressed by decreased B-ALP in male C57BL/6 mice. The levels of adipokines do not play any role. It still needs to be determined whether similar changes also apply to males with type 2 diabetes mellitus or whether they are limited only to male rodents.

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