OPEN

ORIGINAL STUDY

Treatment with a dual amylin and calcitonin receptor agonist improves metabolic health in an old, obese, and ovariectomized rat model

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Abstract

Objectives: Menopause is often characterized by detrimental metabolic changes, such as obesity, insulin resistance, and impaired glucose tolerance, often requiring treatment. KeyBioscience Peptides (KBPs) are Dual Amylin and Calcitonin Receptor Agonists which have shown promising metabolic effects in rats. The objective of this study was to investigate the in vivo effect of KBP on the metabolic health in a model driven by unhealthy diet, age, and menopause.

Methods: Female Sprague Dawley rats were fed a high-fat diet (HFD) for 3 months before the initiation of the study. At 6 months of age the rats were randomized into groups ($n = 12$) and subjected to ovariectomy surgery and treatment with KBP: (1) Lean-Sham, (2) HFD-Sham, (3) Lean-OVX, (4) HFD-OVX, (5) HFD-OVX-KBP (10 μg/kg/d), (6) HFD-OVX-KBP (20 μg/kg/d), (7) HFD-OVX-EE2 (30 μg/d 17a-ethynylestradiol). Body weight, food intake, oral glucose tolerance tests (OGTTs), subcutaneous fat, visceral fat, liver weight, and uterus weight were assessed during the 6-month study. Statistical analyses were conducted by one-way ANOVA with Tukey post-hoc test for multiple comparisons.

Results: Combination of OVX and HFD led to significant induction of obesity (31% weight increase, $P < 0.001$) and insulin resistance (13% increase in tAUC$_{glucose}$ during OGTT $P < 0.01$) compared with the relevant control groups ($P < 0.05$), and this could be completely rescued by EE2 therapy confirming the model system ($P < 0.05$).

Treatment of OVX-HFD rats with KBP for 26 weeks led to a significant reduction in body weight (13%, $P < 0.001$) in the high dose and 9% ($P < 0.01$) in the low dose, with corresponding improvements in fat depot sizes, all compared with HFD-OVX controls. As expected, food intake was suppressed, albeit mainly in the first 2 weeks of treatment, resulting in a reduction of overall caloric intake by 6.5% ($P < 0.01$) and 12.5% ($P < 0.001$) in the low and high doses respectively. Furthermore, treatment with KBP reduced the weight of visceral and subcutaneous fat tissues. Finally, KBP treatment significantly improved glucose tolerance, assessed using OGTTs at weeks 8, 16, and 24.

Conclusions: The data presented here clearly indicate a positive and sustained effect of KBP treatment on body weight loss, fat depot size, and improved glucose tolerance, illustrating the potential of KBPs as treatments for metabolic complications of overweight and menopause.

Key Words: Animal models – Dual amylin-calcitonin receptor agonist – HFD – OVX – Treatment – Weight loss.

M enopause, in middle-aged women, is known to increase the risk of developing central obesity.[1-3] It is well known that obesity is associated with altered menstrual cycles, reduced fertility, and altered hormone patterns in both premenopausal women and rodents.[4,5] Experimental studies have shown that experimentally induced menopause, ie, ovariectomy (OVX) is associated with hyperphagia, decreased energy expenditure,

increased adiposity, and body weight. Furthermore, estrogen deficiency and ovariectomy lead to the development of obesity with metabolic disturbances, such as peripheral insulin resistance, increased body weight and fat mass, fat accumulation in the liver as well as in the heart and skeletal muscle.[6-10]

In addition to menopause status, several risk factors have also been associated with metabolic disturbances such as the consumption of fat-enriched diets (high-fat diet [HFD]).[11,12] Chronic feeding with HFD has previously been demonstrated to increase fat accumulation, leptin levels, and consequently causes the development of obese-insulin resistance.[13,14] Importantly, estrogen deficiency increases the sensitivity to the deleterious effects of HFD possibly due to inflammation and dyslipidemia,[15,16] and there are indications of a strong involvement in the regulation of energy homeostasis between menopause and obesity. Therefore, postmenopausal women who constitute a high percentage of the obese population might benefit from antiobesity agents.[17]

Dual amylin and calcitonin receptor agonists (DACRAs) are a group of peptides that activate both the amylin receptor and calcitonin receptor potently and for a prolonged period of time compared with the natural ligands.[18,19] KBP (KeyBioscience Peptide), the DACRA tested in the present study, have been shown to induce weight loss as well as improve insulin sensitivity superior to those of the amylin therapy.[20] Furthermore, DACRAs have been shown to elicit positive effects on both fasting hyperglycemia and HbA1c levels in rat models of type 2 diabetes.[21-23] Overall, due to their beneficial effects on body weight, blood glucose, and insulin sensitivity, DACRAs are considered to be novel candidates for the treatment of obesity and type 2 diabetes.

With this in mind, this study aimed at determining the effects of DACRAs in a model combining the metabolic consequences of age, HFD, and estrogen deficiency, ie, a model of older menopausal obese women.

METHODS

Animals

All animal procedures were performed in accordance with guidelines from the Animal Welfare Division of the Danish Ministry of Justice under the institutional license issued to Nordic Bioscience (2015-15-0201-00469). Animals were housed pairwise in enriched standard type IV cages under controlled temperature ($21°C$ $-23°C$, 55%–65% relative humidity) and a normal 12-hour light-dark cycle with ad libitum access to food and water.

Peptide and estrogen therapy

Synthetic KBP (American Peptide Company, Sunnyvale, CA) was dissolved in saline (NaCl 0.9%) for subcutaneous injection. The doses chosen for this study were 10 µg/kg and 20 µg/kg, once daily, based on previous KBP studies in obesity.[24] Animals treated with 17α-ethynylestradiol (EE2) had a SC pellet inserted in the neck, releasing 28 µg/kg/d for 90 days (17α-ethynylestradiol, Product no.: NE-241, 2.5 mg/pellet, 90 d release, Supplier: Innovative Research of America, Sarasota, FL). Animals were anesthetized with isoflurane and surgically equipped with a pellet subcutaneously in the neck region. Drug administration was initiated 5 days after OVX surgery and continued for 6 months.

In vivo study

The study was performed in a total of 84 female Sprague Dawley rats (Harlan Laboratories, Indianapolis, IN) obtained at 3 months of age. Upon arrival and throughout the study period, animals were given normal chow (Altromin 1328 Fortified, Altromin Spezialfutter, GmbH & Co. KG, Germany (500 mg/kg of phytoestrogens)) or HFD containing 62 kcal% fat of which 25 g of soybean oil, 18 kcal% protein, and 20 kcal% carbohydrates (#D12492, Research Diet, New Brunswick, NJ). Animals were housed for 3 months prior to study start, to allow acclimatization and induce obesity in HFD groups. Following that the rats were allocated into treatment groups based on body weight (n = 12 rats/treatment group) and were ovariectomized or sham-operated. Treatment groups were HFD-OVX, HFD-Sham, Lean-OVX, Lean-Sham, HFD-OVX-KBP 10 µg/kg, HFD-OVX-KBP 20 µg/kg, and HFD-OVX-EE2. Body weight and food intake were monitored throughout the whole study. At the end of the study, the rats were euthanized and the weight of the subcutaneous (inguinal) and visceral (epididymal and perirenal) adipose tissues as well as the liver and uterus were weighed.

Bilateral OVX

Prior to surgery, the rats were anesthetized with Hypnorm and Midazolam (2 mL/kg). The ventral aspect of the animal was shaved and the skin was disinfected. A single midline-long incision (2-3 cm) was made through the abdomen where the ovaries were located and excised. The incision was sutured using aseptic techniques. For the Sham animals the same procedure for OVX surgery was followed with the exception that the ovaries were left intact. All animals received 5 mg/kg Rimadyl immediately after surgery.

Oral glucose tolerance test

Oral glucose tolerance tests (OGTT) was performed in overnight fasted rats. A glucose bolus (2 g/kg) (Sigma-Aldrich, Copenhagen, Denmark) was administrated p.o. gavage (4 mL/kg) at time 0. Blood samples (EDTA tubes) were collected from the tail vein before glucose administration (0 min) and then the following 15, 30, 60, and 120 minutes post glucose challenge. Blood glucose was monitored 0, 15, 30, 60, 120, and 180 min post-glucose challenge. Blood glucose was monitored by Accu-Check Avia monitoring system (Roche Diagnostics, Rotkreuz, Switzerland).

Statistical analysis

The statistical analyses were conducted in two separate steps. Step 1 compared the effects of HFD, OVX, the

combination of HFD and OVX with the corresponding controls lean-sham and EE2. Step 2 compared the treatment effects of KBP with the corresponding HFD-OVX-vehicle group. All data were analyzed, and the plots were generated using GraphPad Prism 7.01 (Graph Pad Software, San Diego, CA). For time course studies, the group differences were assessed by calculating the area under the curve for the entire time frame, and then using one-way ANOVA followed by Tukey post-hoc test for multiple comparisons of parametric data. For endpoint data, one-way ANOVA followed by Tukey's post-hoc test for multiple comparison of parametric data was used. All data are presented as mean ± standard error of the mean. A P value <0.05 was considered statistically significant.

RESULTS

Characterization of the high fat diet—ovariectomy model

To characterize the effects of combined surgical menopause and HFD on body weight and food intake, the rats, in the obese groups, were initially fed a high fat diet for 3 months, which, as expected resulted in a weight gain compared with the lean group (Fig. 1A). Following induction of obesity, the rats underwent OVX or sham surgery, while continuing their respective diets for an additional 6 months, which resulted in significant increases in body weight of 18% ($P < 0.001$) in the lean-OVX and 11% ($P < 0.001$) in the HFD-OVX compared with their respective sham groups, and a 31% ($P < 0.001$) increase in the HFD-OVX compared with lean-sham (Fig. 1C). Caloric intake was initially elevated in the OVX (7%, $P < 0.05$) and the HFD-OVX (8%, $P < 0.01$) groups, whereas the HFD did not reach statistical significance, when compared with lean-sham OVX (Fig. 1B, D). Additionally, OVX and/or HFD led to significantly higher adipose tissue weights compared with all the other groups, with the combination (HFD-OVX group) showing the largest gain in adipose tissue weight, (Fig. 1E, F). Importantly, uterine weight confirmed ovariectomy (Fig. 1G), whereas liver weights were unchanged by the interventions. Treatment with estradiol significantly reversed the changes induced by both HFD and OVX ($P < 0.001$ for all parameters) (Fig. 1).

High-fat diet and ovariectomy promote impaired glucose handling

To assess changes in glucose handling as a function of HFD, OVX, or the combination, three OGTTs were performed after 8, 16, and 24 weeks of OVX surgery. In the HFD-OVX whole-body glucose tolerance was impaired at all three tests, with no obvious worsening over time, whereas neither the Lean-OVX nor the HFD-Sham alone produced significant impairments in glucose tolerance, although a trend was seen in the HFD-Sham group at all time points (Fig. 2A–F). Estrogen treatment resulted in an improvement in glucose tolerance with the HFD-OVX group at all timepoints, as seen in Figure 2D to F. Overall, these illustrate the usefulness of the OVX-HFD combination as a model of menopausal obesity, as expected.[25]

KBP treatment induces weight loss and reduces overall adiposity

To assess the effect of KBP on metabolism, HFD-OVX rats were treated using single daily injections for 6 months. Treatment with KBP led to 10% ($P < 0.01$) and 13% ($P < 0.001$) compared with HFD-OVX (Fig. 3A, C). As expected[22,24] KBP treatment led to significant reduction in food intake 6.5% ($P < 0.01$) and 12.5% ($P < 0.001$) in the low and high-dose groups, as well as a significant difference ($P < 0.01$) between low and high doses (Fig. 3B, D). In conjunction with the significant reduction in body weight, the weights of both adipose tissues were significantly reduced in both KBP-treated groups (inguinal depot 30%, $P < 0.05$ low dose, 48%, $P < 0.001$ high dose; visceral depot 34%, $P < 0.05$ low dose, 41%, $P < 0.01$ high dose) (Fig. 3E, F). There was no significant difference between the two dosing groups. None of the treatments significantly altered uterus weight and liver weight at study end (Fig. 3G, H).

KBP treatment reduced blood glucose levels during an OGTT

To assess the impact of KBP on glucose tolerance in this model, OGTTs were performed three times during the 6 months treatment. Both KBP treatments, 10 and 20 μg/kg, resulted in significant improvements in glucose tolerance 8, 16, and 24 weeks after both interventions (Fig. 4A–F). Regardless of concentration, KBP therapy significantly lowered the blood glucose levels and proved to have an acute as well as a chronic effect in the improvement of the glycemic control, data that are consistent with previous findings.[18,20,23]

DISCUSSION

The objective of this study was to develop a model of postmenopausal obesity and applying it to study the metabolic effects of DACRAs. Using a combination of HFD induction and ovariectomy we obtained a markedly obese model with impaired glucose tolerance. Utilizing the model, we here present data showing that DACRA therapy is efficacious for weight reduction and glucose control over an extended time period of 6 months.

The combination of HFD and menopause by OVX showed the expected accumulation of fat and deterioration of glucose tolerance, consistent with literature showing that exposing animals to a series of obesity risk factors, such as high-fat diet feeding, prior to ovariectomy contributes to greater weight and fat-mass gain.[26] Moreover, except the fact that chronic HFD consumption induces an increase in body weight and fat accumulation in ovarian-intact rats compared with rats fed a lean diet[27,28] we also observed that ovariectomy dramatically enhanced the body weight and fat gain.[28,29] During menopause in women and OVX rodent models, there is a reduction in circulating E2 levels which is directly associated with fat accumulation[30,31] and as expected administration of E2 led to a normalization of the phenotype.[32] It should be mentioned that our lack of uterine dry weights is a limitation in terms of
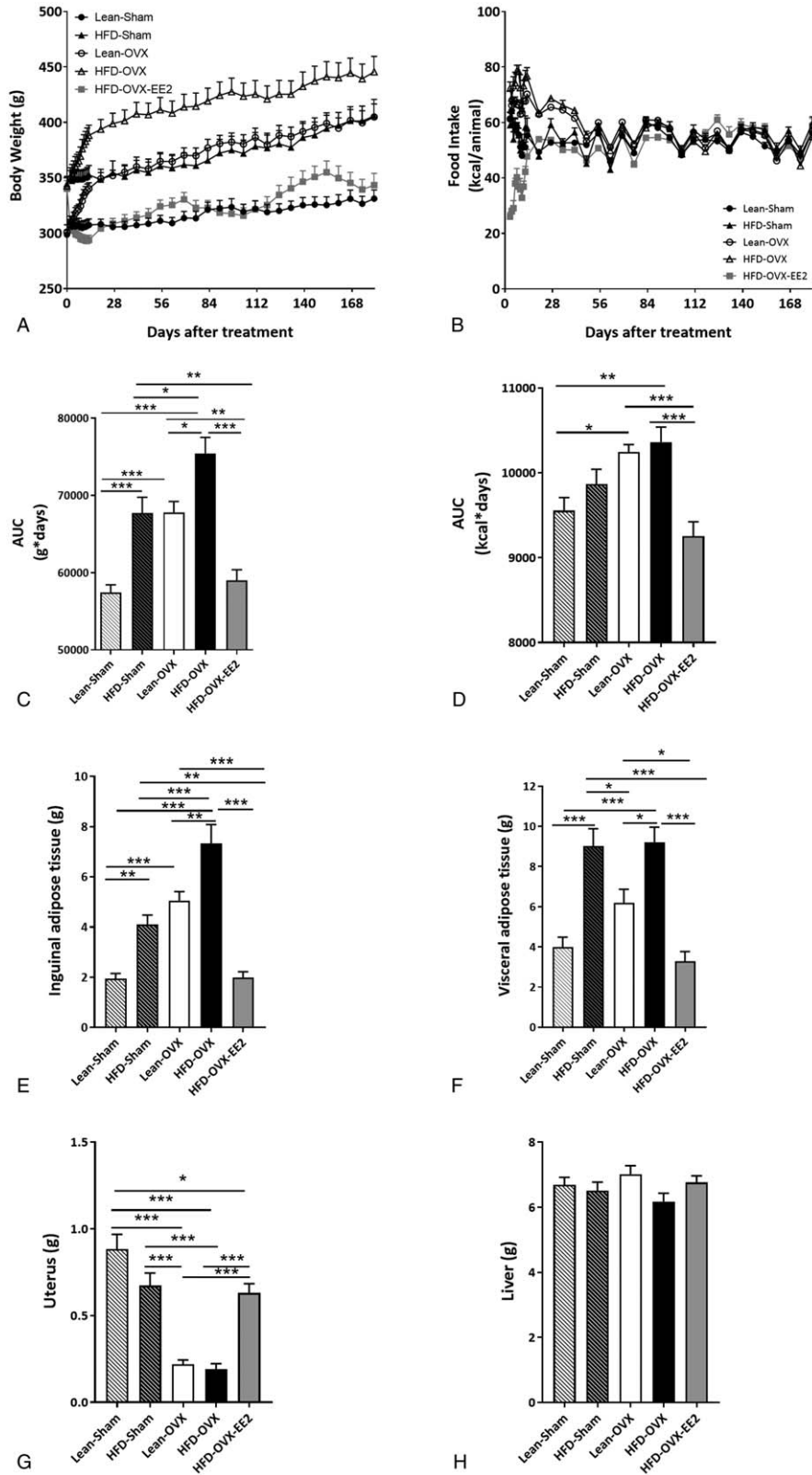
FIG. 1. The combination of HFD and OVX increases food intake, body weight, and fat adipose tissues. Body weight (**A**) and food intake (**B**) during the 6-month study. The incremental area under the curve as shown for body weight (**C**) and food intake (**D**). Weight of inguinal (**E**) and visceral (**F**) adipose tissues as well as uterus (**G**) and liver (**H**) tissues at termination. *n* = 12/group. Statistical analysis between groups was evaluated by one-way ANOVA with Tukey multiple comparisons test $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Error bars indicate the SEM. EE2, 17α-ethynylestradiol; HFD, high-fat diet; LEAN, normal-chow diet; OVX, ovariectomy; SHAM, nonoperated.
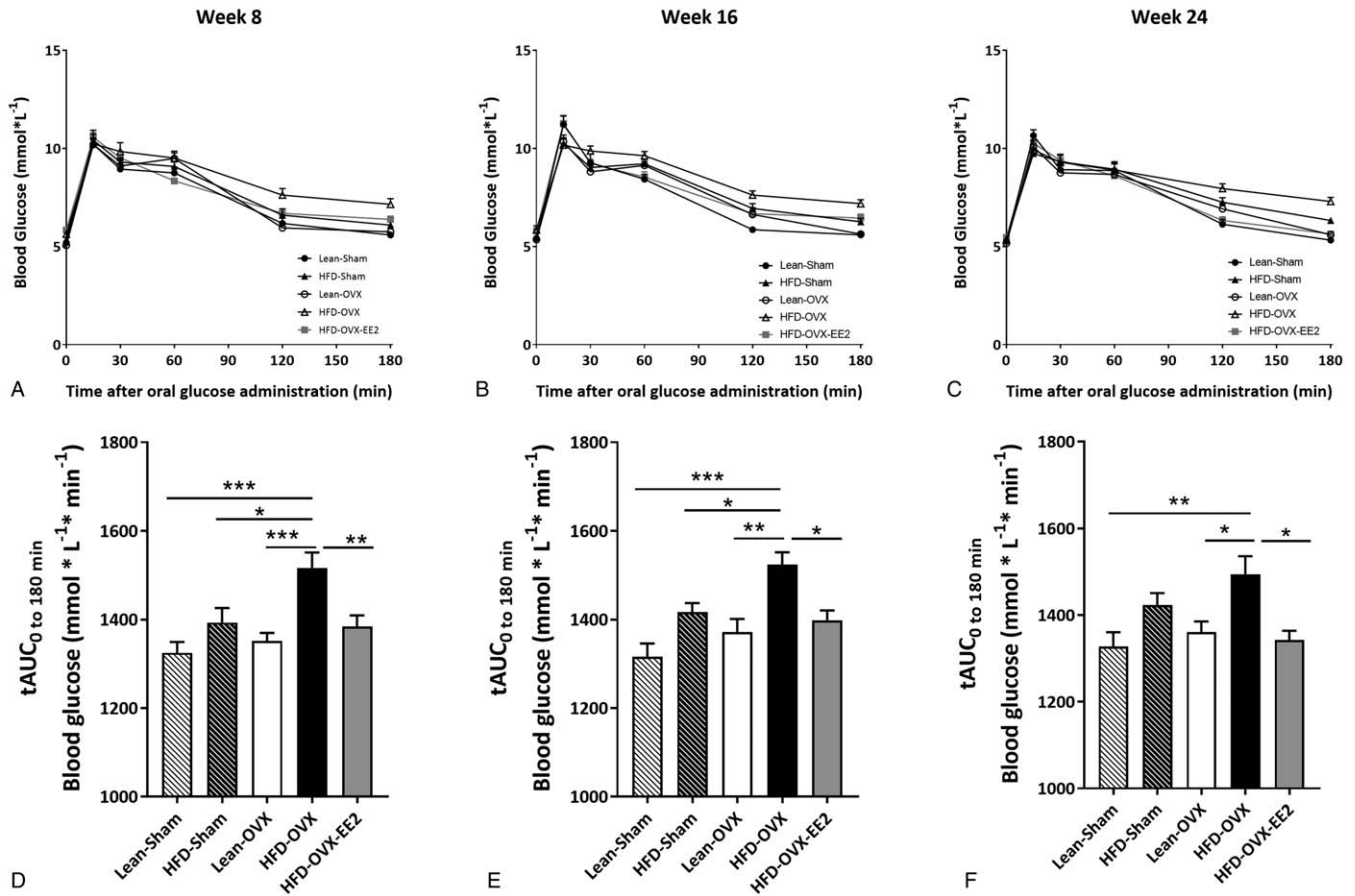
FIG. 2. Decreased glucose tolerance following the combination of HFD and OVX. Oral glucose tolerance tests (OGTTs) were performed after 8 (**A**), 16 (**B**), and 24 (**C**) weeks of diet and OVX surgery start. Total AUC for the relative blood glucose levels during the OGTT are shown in (**D**–**F**) for the weeks 8, 16, and 24 respectively. $n = 12$/group. Statistical analysis between groups was evaluated by one-way ANOVA with Tukey's multiple comparisons test $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Error bars indicate the SEM. EE2, 17α-ethynylestradiol; HFD, high-fat diet; LEAN, normal-chow diet; OVX, ovariectomy; SHAM, nonoperated.

understanding whether the DACRA potentially could affect the endometrium.

In this obese-menopausal model, we proceeded to test a very long treatment regimen, namely 6 months. As expected, we initially observed a suppression of food intake, which then returned to normal, or very close to normal levels within 2 to 3 weeks of treatment.[20,22] More importantly, we observed a marked reduction in body weight, which importantly was sustained throughout the full treatment period, clearly indicating no loss of durability of the treatment, even when treating much longer than previously.[20,22,23] A previous study has also investigated the pharmacological effect of amylin therapy to understand its antiobesity potential in a state of estradiol deficiency.[33] Furthermore oral salmon calcitonin has demonstrated improvement of impaired fasting glycemia and obesity in HFD-OVX rats before.[34] However, it should be noted that the effect of diet and the duration of the model and treatment was not taken into account. Given the DACRAs ability to activate the amylin and calcitonin receptors for an extended period of time, it is expected to see markedly superior effects on classical amylin-induced responses in vivo.[20]

We found that upon ovariectomy in rats, the fat deposition prevailed in the visceral (perirenal) adipose tissue, more than the subcutaneous (inguinal) adipose tissue. This is in agreement with many reports showing that lack of estrogens in rodents is higher in the visceral than subcutaneous fat[16,35,36] and reflects what's being described in human studies.[37] Increase of the visceral fat tissue is highly associated with an increased risk of developing metabolic diseases.[38,39] The KBP changes in adiposity were confirmed as the KBP treated animals had significant lower subcutaneous and visceral adipose tissues compared with the OVX-HFD animals. This indicates that the body weight loss observed is due to loss of fat mass and not lean body mass. Moreover, other studies have also linked the amylin receptor activation with a fat mass decrease rather than lean mass.[40]

More importantly, there is considerable evidence that suggest that expanded adipose tissue accumulation, particularly in the visceral depot, plays a causative role in the development of peripheral insulin resistance, which is closely linked with impaired glucose control.[41] Clearly the HFD-OVX rats in this study showed an increase in their blood glucose, during an OGTT, which was significantly different
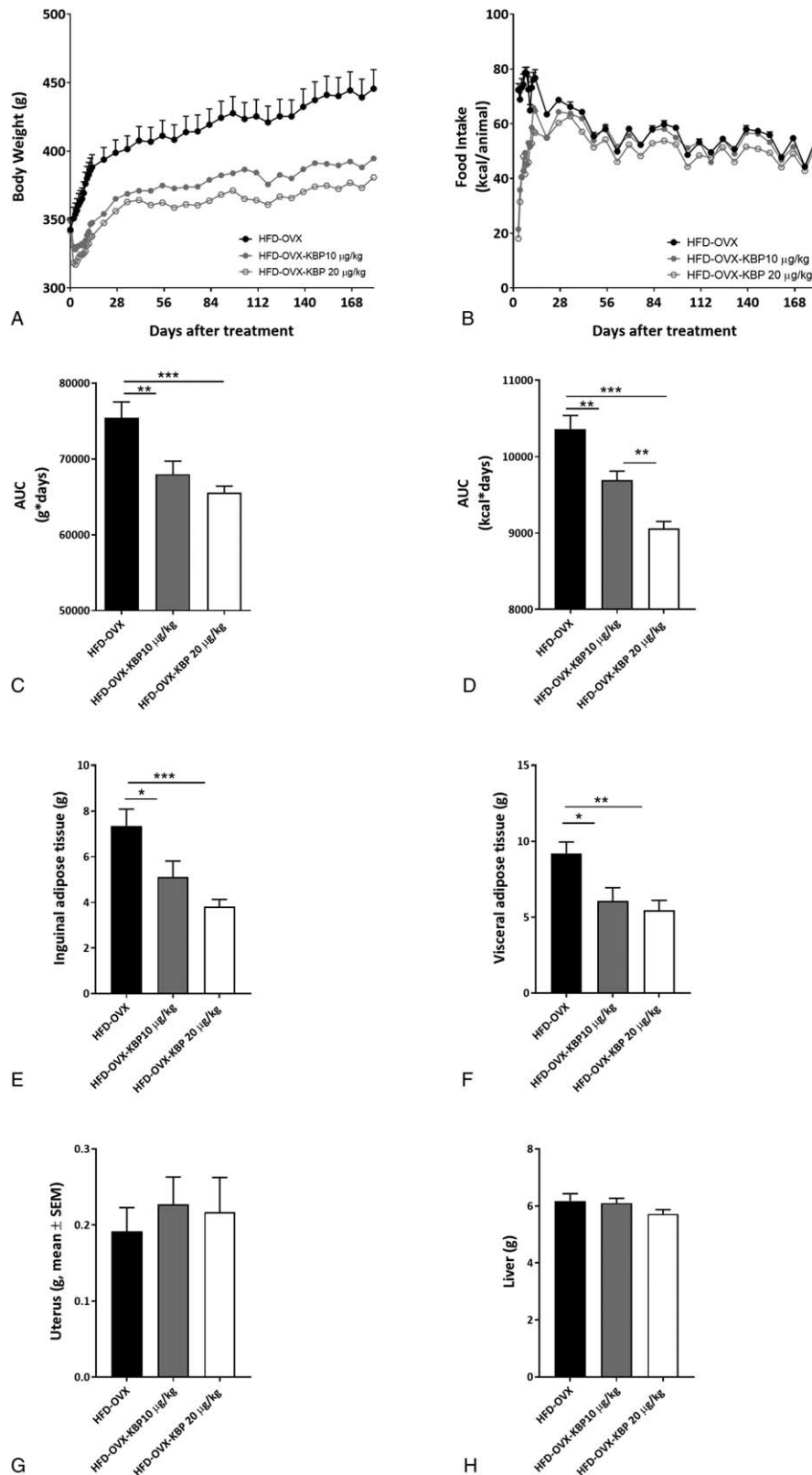
FIG. 3. Body weight loss and adipose tissue reduction by KBP treatment under HFD and OVX conditions. Body weight (**A**) and food intake (**B**) during the 6-month study. The incremental area under the curve as shown for body weight (**C**) and food intake (**D**). Weight of inguinal (**E**) and visceral (**F**) adipose tissues as well as uterus (**G**) and liver (**H**) tissues at termination. $n = 12$/group. Statistical analysis between groups was evaluated by one-way ANOVA with Tukey multiple comparisons test $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Error bars indicate the SEM. HFD, high-fat diet; OVX, ovariectomy; KBP, a dual amylin and calcitonin receptor agonist.
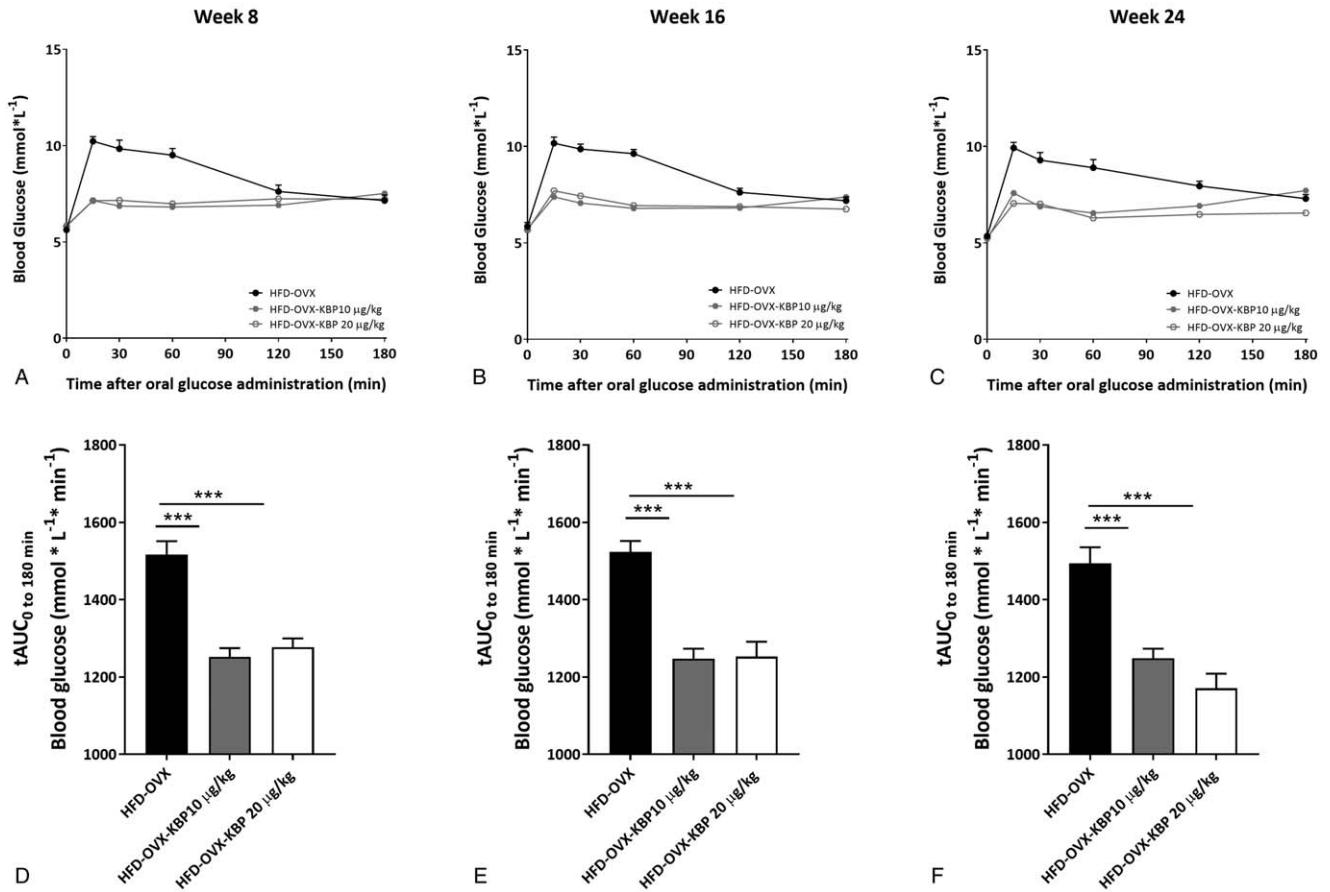
FIG. 4. Decrease of blood glucose levels with KBP treatment in HFD and OVX rats, independent of dosing. Oral glucose tolerance tests (OGTTs) were performed after 8 (**A**), 16 (**B**), and 24 (**C**) weeks of diet and OVX surgery start. Total AUC for the relative blood glucose levels during the OGTT are shown in (**D**–**F**) for the weeks 8, 16, and 24 respectively. $n = 12$/group. Statistical analysis between groups was evaluated by one-way ANOVA with Tukey multiple comparisons test $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Error bars indicate the SEM. HFD, high-fat diet; OVX, ovariectomy; KBP, a dual amylin and calcitonin receptor agonist.

from all the other groups. Estrogen treatment improved significantly the blood glucose of the OVX rats receiving high-fat diet which is in agreement with other reports[42] and was mainly due to the significant reduction of body weight. However DACRAs are known to delay gastric emptying, which can affect positively the postprandial blood glucose levels by delaying entry of glucose into the circulation.[24,43] The KBP, at both doses, managed to induce a reduction of the gastric emptying rate which corresponds well to the effects on blood glucose levels during OGTT. Previous studies have also shown an improvement in glucose tolerance induced by KBPs which is partly mediated through a lowering of gastric emptying rate, as previously described with amylin agonism[43,44] and DACRAs.[23,24] Furthermore KBPs have the ability to improve insulin action as previously observed[18,23] which is strongly correlated to body weight.[45]

Potential clinical value

The current study indicates that KBP induced a sustained weight loss over a 6-month study, leading to reduction in adipose tissue and improved glucose tolerance. This study demonstrates the promise of KBPs as treatments in the highly relevant population of older, menopausal, and obese women.

CONCLUSIONS

In conclusion, KBP induced a substantial weight loss, reduced food intake, and decreased adiposity, dose-dependently, in rats fed high-fat diet and being ovariectomized. In addition, both KBP groups managed to improve glucose tolerance, hence revealing that the KBP effect on oral glucose tolerance is independent of dosing concentration. Additionally, the present study proves the KBPs durability over a 6-month long-term study without a loss of its effect. KBP is a promising candidate and may overcome the challenges found in treating patients suffering from obesity, old age, and menopause.

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