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Research article

The intake of an extract from seeds of *Tamarindus indica L.* modulates the endocrine function of adult male mice under a high fat diet

Frank Cloutier [a], Pauline Roumaud [a], Sabrina Ayoub-Charette [b], Subrata Chowdhury [c], Luc J. Martin [a,*]

[a] Biology Department, Université de Moncton, Moncton, New-Brunswick, E1A 3E9, Canada
[b] Department of Nutritional Science, University of Toronto and St. Michael's Hospital, Toronto, Ontario, M5S 1A8, Canada
[c] RPS Biologiques Inc., PEI Food Tech Center, 101 Belvedere Av, Charlottetown, Prince Edward Island, C1A 6B3, Canada

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ABSTRACT

TBP is a natural product from *Tamarindus indica L.* seeds used as a natural remedy in India. This product is an antioxidant and may have beneficial effects on endocrine and metabolic functions. However, the regulatory mechanisms involved remain to be elucidated. In males, testosterone is synthesized by Leydig cells from the testis. With aging and obesity, testis function declines, leading to decreased testosterone synthesis. The aim of the current research is to determine how TBP improves testosterone production in male mice under a high fat diet leading to hypoandrogenic condition. Using C2C12 myoblast cells, we have found that TBP increased mitochondrial mass and oxygen respiration, as well as the production of the IGF-1 hormone. In addition, treatment of TM3 Leydig cells with TBP resulted in increased testosterone production. In mice under a high fat diet, TBP lowered blood glucose level and corticosterone production and improved total testosterone production after five weeks of treatment. In addition, testicular expressions of genes encoding the mitochondrial transporter of cholesterol (*Star*) and steroidogenic enzymes (*Cyp11a1*, *Hsd3b1*, *Cyp17a1* and *Hsd17b3*) were increased by TBP. Hence, TBP may prevent the detrimental effects of long-term consumption of a high fat diet and may have health benefits on the endocrine function.

1. Introduction

Obesity leads to increased risks for a number of critical health problems such as type 2 diabetes, cardiovascular and respiratory diseases, endocrine disorders, immune deficiency, common cancers (such as prostate, endometrial and breast cancers) and reduced fertility (Pi-Sunyer, 2002). Abdominal obesity in males is associated with reduced androgen production by Leydig cells from the testis. Such condition may be exacerbated when low plasma levels of testosterone contribute to the increase in abdominal fat through inhibition of lipolysis (Cohen, 2001; De Maddalena et al., 2012; Mårin and Arver, 1998). These fat cells secrete several hormones (including leptin) that inhibit testosterone synthesis from Leydig cells (Lin et al., 2009; Roumaud and Martin, 2015), creating a vicious cycle that exacerbates men's obesity. Indeed, testosterone in men with a body mass index (BMI) $>$ 30 is about 30% lower than in men with a BMI$<$25 (Huhtaniemi, 2014). This decrease is associated with lower energy and muscle strength, reduced fertility, depressed mood, reduced cognitive function, increased risks of cardiovascular diseases such as atherosclerosis and a shorter life expectancy. Thus, the use of TBP, an extract from tamarind (*Tamarindus indica L.*) seeds, may help overcome these symptoms associated with low testosterone production and break potent worsening cycle between obesity and testosterone production. However, the regulatory mechanisms of TBP on androgen production remain to be elucidated. Other commercial herbal supplements, such as Testofen®, have been shown to improve testosterone production in healthy aging males (Rao et al., 2016). Testofen® is a standardized formulation containing *Trigonella foenum-graecum* seed extract combined with magnesium, zinc, pyridoxine and methylcobalamin. It is known to have a positive effect on libido and to contribute in maintaining normal healthy testosterone levels (Steels et al., 2011).

Androgens are important not only for initiation and adequate spermatogenesis, but also for the development of secondary sex characteristics in males (Wang et al., 2009). In addition, androgens are also important to avoid andropause related symptoms associated with aging. Starting in their 30s, serum and testicular testosterone levels decline gradually at an average rate of 1% per year (Matsumoto, 2002). In the testis, testosterone synthesis from cholesterol involves the cholesterol

* Corresponding author.
E-mail address: Luc.Martin@umoncton.ca (L.J. Martin).

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transporter (Star) and different steroidogenic enzymes (Cyp11a1, Cyp17a1, Hsd3b1 and Hsd17b3) within Leydig cells (Figure 1).

The aim of the current research is to determine how TBP improves testosterone production in male mice under a high fat diet leading to hypoandrogenic condition. This research includes *in vitro* data to determine the biological properties of TBP on hormone production and *in vivo* research using the high-fat diet mice model. In male mice on a prolonged high-fat diet for 10 weeks, serum testosterone may be reduced by more than 90% (Palmer et al., 2012). Thus, six groups of 6 CD1 male mice of 10 weeks of age will be fed the following diets for 10 weeks: (1) normal diet, (2) normal diet supplemented daily with TBP (50 mg/kg of mice bodyweight), (3) normal diet supplemented daily with Testofen (50 mg/kg of mice bodyweight), (4) high-fat diet, (5) high-fat diet supplemented daily with TBP and (6) high-fat diet supplemented daily with Testofen.

2. Material and methods

2.1. Chemicals and TBP preparation

TBP, an extract from tamarind (*Tamarindus indica L.*) seeds, was obtained from RPS Biologiques Inc. (Charlottetown, PEI, Canada) and was approved by the FDA (NDIN#2018001098) and Health Canada (NPN 80091363). Briefly, tamarind seeds were sieved and roasted. After cooling, the roasted seeds were placed in a rotary mixer to remove the coating and retain the seeds' embryo and endosperm. The seeds were then crushed and were strained with a 200 μm mesh filter to obtain a tamarind seed powder consisting of polysaccharides with residual proteins, lipids and minerals. It contained no more than 10% moisture and had a viscosity of less than 450 mPa-second. The compounds present in TBP were then activated by performing a subcritical extraction procedure on the powder to activate and optimize the bioavailability of the functional components and increase their solubilities. The powdered material was treated at a high temperature (121 °C) and pressure (15 psi) for 20 min, followed by drying in a drum dryer to yield the TBP powder. Testofen® was obtained using the NUGENIX supplement (28% Testofen®) (NUGENIX, Salt Lake City, UT, USA). Caffeine was purchased from Sigma-Aldrich (Saint-Louis, MO, USA).

2.2. Cell culture

Mouse TM3 Leydig (Mather, 1980), mouse C2C12 myoblast (Yaffe and Saxel, 1977) and human H295R adrenal (Gazdar et al., 1990) cell lines were acquired from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM/F12 containing 5% horse serum with 2.5% fetal bovine serum, DMEM containing 10% fetal bovine serum and ATCC formulated DMEM supplemented with 2.5% ITS Premix (BD Biosciences, Mississauga, Canada), respectively. Cells were incubated at 37 °C and 5% CO_2.

2.3. Mitochondrial mass assay

C2C12 cells were seeded in 96 well culture plates at a density of 20,000 cells/ml and incubated 24 h before the mitochondrial mass assay. Following treatments of C2C12 cells with water (control), TBP at 500 μg/ml or caffeine at 10 mM in serum-free medium for 24 h, mitochondrial mass was assayed using the nonyl acridine orange (NAO) probe (100 ng/ml) diluted in culture medium. The probe was incubated with cells for 30 min at 37 °C, 5% CO_2, followed by measurement of changes in fluorescence (excitation 380 nm, emission 640 nm) using a fluorescence microplate reader.

2.4. Mitochondrial oxygen respiration assay

C2C12 cells were seeded in 96 well plates at a density of 20,000 cells/ml, followed by an incubation of 24h. Following an incubation in serum-free medium containing water (control), TBP at 500 μg/ml or caffeine at 10 mM for 24 h, extracellular oxygen consumption in differentiated C2C12 cells was measured by assessing phosphorescence of porphyrine based water soluble oxygen sensitive probe using the MitoXpress® Xtra-HS kit (Agilent technologies Inc., Santa Clara, CA, USA) according to the manufacturer's protocol in the absence or presence of treatments. Probe fluorescence was quenched by molecular oxygen (O_2), resulting in lower probe signal. The rate of increase in fluorescence was related to the rate of cellular oxygen consumption.

2.5. Insulin like factor-1 (IGF-1) level determination assay

To evaluate the effects of treatments with water (control), TBP at 500 μg/ml or caffeine at 10 mM in serum-free medium for 24 h on IGF-I concentration in differentiated C2C12 cells, the cell culture medium was assessed using the mouse IGF-I ELISA kit (Sigma-Aldrich, Saint-Louis, MO, USA) according to the manufacturer's instructions. Colorimetric measurements were performed for absorbance at 450 nm using a microplate reader.

2.6. In vitro cortisol determination assay

H295R cells were seeded in 24 well cell culture plates at a density of 75,000 cells/ml and incubated for 24h before treatments. Following an incubation with water (control), TBP at 500 μg/ml or caffeine at 10 mM

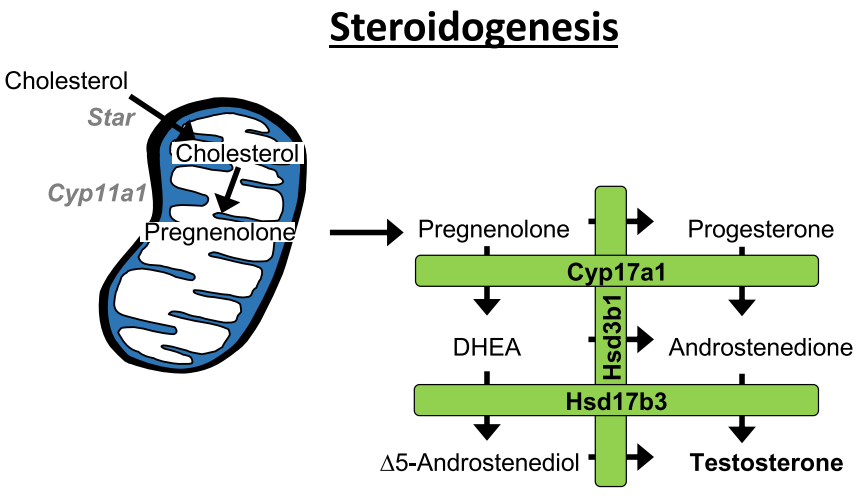

Figure 1. Steroidogenesis pathway involved in testosterone synthesis from cholesterol in testicular Leydig cells.

in serum-free medium for 24 h, measurements of cortisol concentrations from the cell culture medium were performed using the DetectX® Cortisol assay kit (Arbor Assays, Ann Arbor, MI, USA) according to the manufacturer's instructions.

2.7. In vitro testosterone assay

TM3 Leydig cells were seeded in 96 well plates at a density of 5,000 cells per well. After 24 h of incubation, cells were treated for 24h with TBP at 500 μg/ml or water (control) in serum-free medium. The cell culture medium was collected and stored at -20 °C until measurements. A testosterone ELISA kit (Enzo Life Sciences, Cat. # ADI-900-065) was used for quantification of testosterone according to the protocol from the manufacturer.

2.8. Animals

Male CD-1 mice (10 weeks) were purchased from Charles River International, Inc. (St-Constant, Canada) and kept at the animal facility of the Université de Moncton during the entire duration of the experimental protocol. These animals were maintained in a temperature- and humidity-regulated room (22 ± 2 °C, 55 ± 5%, respectively) under a 12-h light/dark cycle. A total of 36 mice were randomly divided into the following six groups of six animals according to the feeding diet: normal diet (ND), normal diet supplemented with TBP (50 mg/kg of bodyweight (bw)/day) (ND + TBP), normal diet supplemented with Testofen (NUGENIX® at 50 mg/kg bw/day) (ND + Testofen), high-fat diet (HFD), high-fat diet supplemented with TBP (50 mg/kg bw/day) (HFD + TBP) and high-fat diet supplemented with Testofen (NUGENIX® at 50 mg/kg bw/day) (HFD + Testofen). The normal diet consisted of 10% kcal% fat, 70 % kcal% carbohydrate and 20% kcal% protein (D12450B, Research Diets, Inc., New Brunswick, NJ, USA), whereas the high-fat diet consisted of 45% kcal% fat, 35 % kcal% carbohydrate and 20% kcal% protein (D12451, Research Diets, Inc., New Brunswick, NJ, USA). TBP and Testofen were resuspended in the daily drinking water of mice. Once the daily dosage was attained, normal drinking water was provided. Food intake and body weight were measured once a day, and blood samples (80 μl) were taken on weeks 1, 5 and 10. After receiving the above diets for 10 weeks, the mice were anesthetized with isoflurane, and blood samples were taken by cardiac puncture. Mice were then euthanized by cervical dislocation, and testes were removed, weighed and one was preserved in RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) (1:10 volume ratio) and frozen at -20 °C, whereas the other was fixed in 10% formalin (1:15 volume ratio) for histology. All experiments were conducted according to the Canadian Council for Animal Care and have been approved by the Animal Care and Ethics Committee of Université de Moncton (protocol 16–19).

2.9. Blood and serum chemistry analyses

Blood glucose levels were measured at wk1, wk5 and wk10 using a commercial glucometer (Contour Next, Ascensia Diabetes Care, Mississauga, ON, CAN). Serum levels of LH, FSH, corticosterone, DHEA and testosterone were measured using ELISA kits: LH (E-EL-M0057, Elabscience, Bethesda, MD, USA), FSH (E-EL-M0511, Elabscience, Bethesda, MD, USA), corticosterone (501320-96, Cayman Chemical, Ann Arbor, MI, USA), DHEA (ADI-900-093, Enzo Life Sciences Inc., Farmingdale, NY, USA), testosterone (582701-96, Cayman Chemical, Ann Arbor, MI, USA).

2.10. Testicular histomorphometry analyses

After fixation for 72 h in 10% formalin at 4 °C, the testes were paraffin-embedded, sliced at 7 μm thickness, followed by hematoxylin-eosin staining. Sections were observed under light microscopy using the TI-U inverted microscope (Nikon Instruments Inc., Melville, NY, USA). Testicular histomorphometry analyses were evaluated by determining the surface area of seminiferous tubules compared to the interstitium. Twelve fields at 40X from four slices per testis were compiled for statistical analyses.

2.11. Quantitative real-time PCR

RNA has been isolated from mice testes previously stored at -20 °C in RNAlater. The concentration and purity of the extracted RNA were measured using a Nanodrop 1000, where the ratio of 260/280 was approximately 2.1. The mRNAs were reverse transcribed into cDNAs using qScript™ cDNA SuperMix (Quanta Biosciences, Beverly, MA, USA). Quantitative real-time polymerase chain reactions (qPCR) were performed using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA) on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The primers (Integrated DNA Technologies, Coralville, Iowa) used are depicted in Table 1. The primers' specificities were assessed by the analysis of the melt curves and the gel electrophoresis of DNA products. The levels of gene expressions were assessed using the ΔΔ threshold cycle (Ct) method after confirming that the efficiency of amplification was between 90% and 110% for all pairs of primers. The expressions of *Rpl19* and *Eif4h* were used as controls for total mRNA recoveries from mice testes. Samples have been quantified as technical duplicates.

2.12. Statistics

Experimental data was presented as means ± S.E.M. Statistical analysis was performed using two-way ANOVA followed by Fisher's LSD multiple comparisons test using GraphPad Prism version 8.2 (GraphPad Software Inc., San Diego, Ca, USA). $P < 0.05$ was considered significant.

3. Results

Before treating mice to determine the effect of TBP on the endocrine function, cell lines were used to evaluate the impact of this seed extract on mitochondrial activity and hormones productions. Following treatments of C2C12 cells with TBP, the mitochondrial mass and mitochondrial oxygen respiration were significantly increased when compared to non-treated cells (Table 2). Caffeine served as a positive control to confirm the increase in metabolism and mitochondria biogenesis as reported previously in skeletal muscle cells (Vaughan et al., 2012). Subsequently, the effects of TBP on the production of hormones involved in muscle growth and development were evaluated with cell lines treatments. Following stimulations of C2C12 cells, levels of IGF-1 hormone were significantly increased by 19% in response to TBP and by 66% with caffeine (Table 2). Although treatment of H295R adrenal cells with caffeine or TBP resulted in an increased trend in cortisol production, such effect was non-significant. Following the treatment of TM3 Leydig cells with TBP, testosterone production was increased by 17% (Table 2). Since TBP had stimulatory effects on mitochondrial activity and anabolic hormones productions, *in vivo* experiments on mice were undertaken.

After 10 weeks of treatment of CD-1 mice, all groups of mice underwent a significant increase in body weight, except those subjected to a ND + TBP (Figure 2A). We also investigated whether TBP supplementation could affect testicular function and steroid synthesis. To determine if TBP could have an effect on gonadal cells proliferation and testicular size, testis weight was measured and corrected to total weight of mice after 10 weeks of treatments (Figure 2B). Although mice treated with ND + TBP tended to have a higher % ratio of testicular versus total weight, none of the treatments indicated was different compared to normal diet alone. The Leydig cell population from the interstitium is highly correlated with the androgen synthesis capacity of the testis *in* vivo. Hence, we performed histomorphometric analyses on testis sections after 10 weeks of treatments to determine the surface area of the interstitium compared to seminiferous tubules. As indicated in Figure 2C and D, TBP supplementation had no effect on the proportion of the interstitium, where

Table 1. Oligonucleotide primer sequences used in this study during qPCR.

Target gene	Forward primer	Reverse primer	NCBI Refseq.	Product size
StAR	5′-CAGCACTCAGCATGTTCCTCGCT-3′	5′-TCCCCGTTCTCCTGCTGGCTTT-3′	NM_011485	325 bp
Cyp11a1	5′-GATCCCGAGGCCCAGCGGTT-3′	5′-AGGGTCATGGAGGTCGTGTCCA-3′	NM_019779	323 bp
Hsd3b1	5′-GCCCCTGATCTTTTCAGCCACCAC-3′	5′-GGGTAACCCTTAGGAGGGCTGTTAA-3′	NM_008293	311 bp
Cyp17a1	5′-GCCCCAGATGGTGACTCAAAGCC-3′	5′-ACACATCTGGGTCCCGGCCT-3′	NM_007809	569 bp
Hsd17b3	5′-TGGAGTCAAGGAGGAAAGGCCTCA-3′	5′-GGAATCGTTGAGCGGTGCTGC-3′	NM_008291	366 bp
Sox4	5′-GGCTTCCTACCTTGCAACAA-3′	5′-TCGATTGCAGTTCACGAGAG-3′	NM_009238	171 bp
Rpl19	5′-AGTGTCCTCCGCTGCGGGAA-3′	5′-AGCCTCAGCCTGGTCAGCCA-3′	NM_009078 NM_001159483	447 bp
Eif4h	5′-GATACCTACGACGATCGGGC-3′	5′-TTGTCTCTGACTAGCCGCAC-3′	NM_033561 NM_001312867	215 bp 215 bp

Table 2. In vitro measurements following treatments of cell cultures with the indicated compounds.

	Control	Caffeine	TBP
Mitochondrial mass (% of control)[1]	100.0 ± 1.4a	138.4 ± 0.8b	115.5 ± 4.2c
Mitochondrial oxygen respiration[1]	37.77 ± 0.89a	65.97 ± 1.82b	48.60 ± 1.50c
IGF-1 concentration (pg/ml)[1]	102.4 ± 1.6a	166.2 ± 5.6b	119 ± 0.9c
Cortisol concentration (pg/ml)[2]	782.9 ± 29.4	828.9 ± 18.9	802.5 ± 28.7
Testosterone concentration (% of control)[3]	100.0 ± 1.3a	-	117.2 ± 1.3b

Note: Experiments were performed using C2C12[1], H295R[2] or TM3[3] cells (*see* the Material and Methods section for more information). Results with different letters are significantly different ($P < 0.05$).

Leydig cells are located, compared to seminiferous tubules. Interestingly, blood glucose levels remained relatively constant during the first 5 weeks of treatments and increased significantly for all treatments after 10 weeks (Figure 2E). Although the HFD treatment for 10 weeks resulted in increased blood glucose level compared to ND, cotreatment of TBP with HFD prevented such increase (Figure 2E).

At weeks 1, 5 and 10 of treatments, serum testosterone concentrations were determined by ELISA assays (Figure 3). Although most of the changes in testosterone levels were inconclusive due to important variability, the HFD + TBP treatment for 5 weeks resulted in an important increase in testosterone production compared to week 1. Interestingly, groups treated with HFD for 10 weeks tended to have lower testosterone production compared to the ND group and TBP cotreatment could not overcome such repression. However, TBP cotreatment with ND contributed to maintain testosterone production.

Next, we determined if testicular steroidogenesis could be altered at the gene expression level. Total mRNA extracts were prepared from the whole testes after 10 weeks of treatments. The expressions of the mitochondrial transporter of cholesterol (*Star*) and steroidogenic enzymes (*Cyp11a1*, *Hsd3b1*, *Cyp17a1* and *Hsd17b3*) were evaluated and normalized using *Rpl19* and *Eif4h* as reference genes. Interestingly, only TBP had a stimulatory effect on the expressions of all steroidogenic genes investigated (Figure 4A–E), whereas Testofen was only effective to increase *Cyp17a1* expression (Figure 4D). To confirm the specificity of this effect of TBP, *Sox4* (a non-steroidogenic gene) expression was also evaluated and was not altered by TBP or Testofen treatments (Figure 4F).

To determine if the modulatory effects of TBP and/or Testofen on steroidogenesis were specific to the testis or could also be observed for glucocorticoids production from the adrenal gland, we quantified serum corticosterone levels following 10 weeks of treatments (Figure 5). Although HFD resulted in a significant increase in corticosterone levels compared to ND, cotreatments using HFD + TBP prevented such effect. Also, Testofen could not prevent a significant increase in HFD-dependent production of corticosterone.

Serum concentrations of DHEA were also determined after 10 weeks of treatments (Figure 6). DHEA may come from synthesis and conversion of DHEA-S from different sources, including adrenals, testis and peripheral tissues. TBP treatment for 10 weeks contributed to maintain serum DHEA levels under a normal diet, whereas Testofen supplementation resulted in a decrease in serum DHEA levels by more than 50%. The HFD also resulted in a decrease in DHEA serum levels, whereas these were restored non-significantly by co-treatments with TBP and Testofen. Importantly, ELISA assays for DHEA have a minimum of 30% cross reactivity with DHEA-S, which may interfere with current results as DHEA-S is predominant within serum.

To determine if changes in steroidogenic genes expression in response to treatments with TBP were a consequence of alterations in the pituitary LH release, we measured serum LH levels following 10 weeks of treatments (Figure 7). TBP and Testofen treatments of mice under ND had no effect on LH serum levels. Numerical values for measurements following treatments of male mice are presented in Table 3.

4. Discussion

Our results indicate that TBP supplementation may have multiple health benefits, such as weight gain prevention, inhibition of HFD-dependent blood glucose increase, improvement of total testosterone levels and inhibition of the increase in corticosterone levels caused by HFD. Coming from the same family (*Fabaceae*) as fenugreek (*Trigonella foenum-graecum*), TBP has several health properties already documented in literature for this plant. Indeed, regarding the action of TBP to maintain normal body weight, similar results were obtained using daily supplementation of fenugreek with 8 weeks of resistance training, leading to reduced body fat percentage and improved overall body composition (Poole et al., 2010). Moreover, tamarind extract has been shown to have anti-obesity effects in high-fat-induced rats (Azman et al., 2012). After six months of daily treatment of rats with tamarind pulp extract, there was no significant change in body and testes weights, hematological and clinical biochemistry profiles (Iskandar et al., 2017). Interestingly, tamarind contains important amounts of flavonoids such as luteolin and apigenin (Sudjaroen et al., 2005). We and others have shown that these compounds can improve steroidogenesis in Leydig cells in vitro (Cormier et al., 2018; Li et al., 2011). Our results from cell line treatments confirmed that TBP has stimulatory effects on mitochondrial activity and anabolic hormones productions. Indeed, TBP increases the mitochondrial mass and oxygen respiration in C2C12 myoblast cells. TBP also promoted

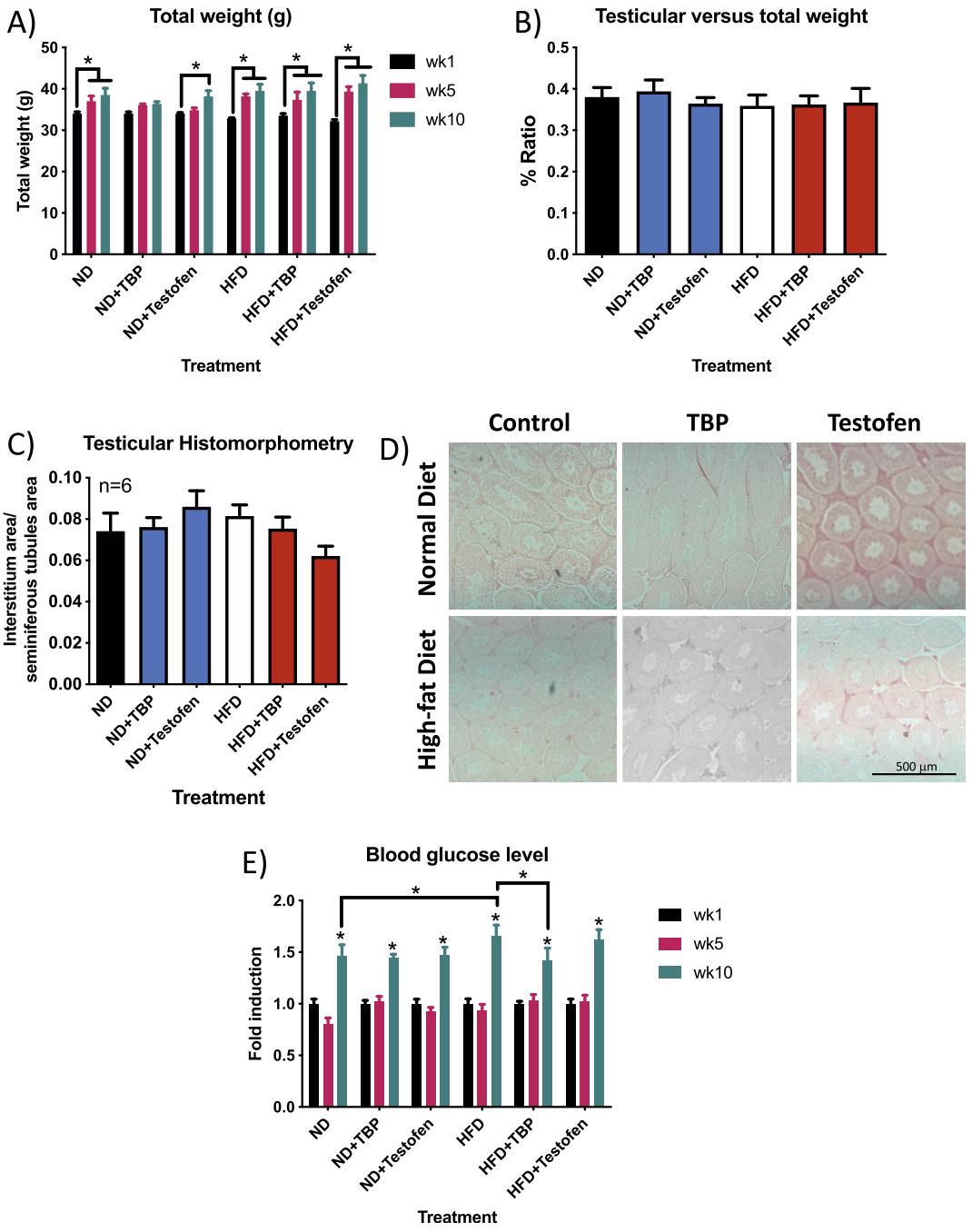

Figure 2. Total weight, testicular measurements and blood glucose level of mice following treatments of TBP or Testofen with or without high fat diet. Mice total weight measurements (A) were collected at weeks 1, 5 and 10 of treatments. Testicular weight compared to total weight (B) and testicular histomorphometric measurements from histology microscopic observations (C), where the average of interstitium area was divided by the average of seminiferous area, were collected for each mouse at week 10 of treatment. Representative microscopic images of testis slides are presented (D). Blood glucose measurements were collected at weeks 1, 5 and 10 of treatments and presented as fold induction for individuals of weeks 5 or 10 compared to results from week 1 (E). Statistical analyses were performed using two-way ANOVA followed by Fisher's LSD's multiple comparisons test. Asterisk (*: $P < 0.05$) indicates a statistically significant difference.

the production of IGF-1 in C2C12 cells and testosterone from TM3 Leydig cells.

The use of HFD in mice is well known to result in increased body weight, higher circulating glucose levels and decreased metabolic efficiency (Ikemoto et al., 1995; Winzell and Ahrén, 2004). Following 10 weeks of treatment with the HFD, blood glucose levels were, indeed, significantly increased compared to ND. However, supplementation with TBP prevented such increase, leading to glucose levels' increases comparable to ND. As fenugreek seed extract, TBP may promote glucose uptake by increasing insulin release from the pancreatic beta cells (Ajabnoor and Tilmisany, 1988; Vijayakumar et al., 2005). In addition, others have shown that aqueous extracts from tamarind seeds have antidiabetic effects in streptozotocin-induced diabetic rats (Maiti et al., 2004). Although insulin stimulates testosterone production from Leydig cells in culture (Lin et al., 1986), steroidogenesis is compromised in insulin-resistant states (Pitteloud et al., 2005) and may contribute to the lack of increase in testosterone production following 10 weeks of TBP treatments of mice under a high-fat diet.

A promising action of TBP is its potency to improve testosterone production by stimulating testicular Leydig cells directly, as suggested by

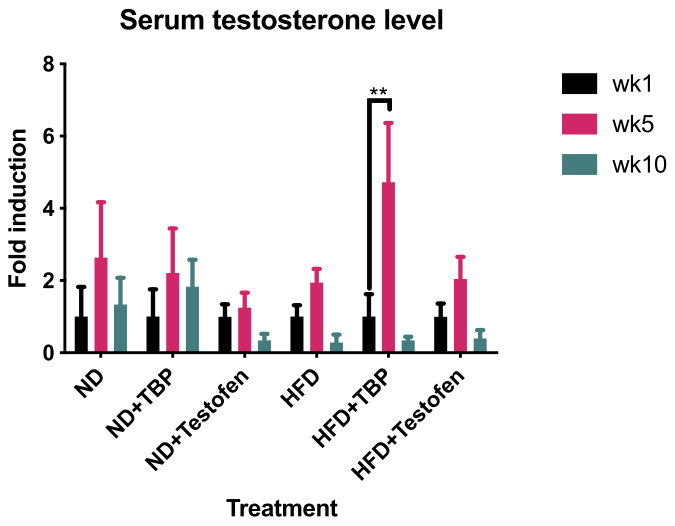

Figure 3. Serum testosterone levels of mice following treatments of TBP or Testofen with or without high fat diet. Testosterone measurements were performed on serum samples collected from mice at weeks 1, 5 and 10 of treatments. Statistical analyses were performed using two-way ANOVA followed by Fisher's LSD's multiple comparisons test. Asterisk (**: P < 0.01) indicates a statistically significant difference.

treatment of TM3 Leydig cells. Here we confirmed that after 5 weeks of co-treatment with the HFD, TBP contributed to increase testosterone production by 4.7 folds. Such improvement in testosterone production may be attributed to an accumulation of adipose tissue leading to an increase in serum leptin to a level stimulating testosterone production (Landry et al., 2013). However, after 10 weeks of HFD + TBP treatment, serum leptin levels may have reached pathological concentrations leading to repression of steroidogenesis as reported previously (Landry et al., 2017; Tena-Sempere et al., 1999; Wang et al., 2011).

Here we confirmed that TBP stimulated the expression of genes encoding the mitochondrial transporter of cholesterol (*Star*) and steroidogenic enzymes (*Cyp11a1*, *Hsd3b1*, *Cyp17a1* and *Hsd17b3*) after 10 weeks of treatment. However, such improvement in androgen production did not result in significant increase in serum levels of testosterone. This may be attributed to the negative feedback control of testosterone on the hypothalamus and pituitary, resulting in modulation of LH production and secretion. Indeed, under normal physiological condition, testosterone serum levels are consistently maintained in a normal range by retro-inhibition and/or retro-activation on gonadotrophin secretion. However, such regulatory mechanism may be disrupted under pathophysiological conditions such as chronic stress, obesity or aging (Matsumoto, 2002) and may be compensated by TBP supplementation. Interestingly, TBP tended to increase LH serum levels after 10 weeks of supplementation. This suggests that TBP may not only have a stimulatory effect on steroidogenic genes expression from testis Leydig cells, but also on pituitary LH production and/or secretion, further contributing to stimulate testis testosterone production. The mechanism of action of TBP to promote steroidogenesis may be different from fenugreek. Indeed, fenugreek extract increased total testosterone levels in men by inhibiting aromatase and 5α-reductase (Wilborn et al., 2010), thus reducing the conversion of testosterone into other metabolites.

Mice under a HFD release more corticosterone and aldosterone from the adrenal glands, resulting in greater plasma levels of these hormones (McManus et al., 2015; Swierczynska et al., 2015). Following 10 weeks under HFD, our results also indicate that serum corticosterone levels were increased. However, such increase was moderated with TBP

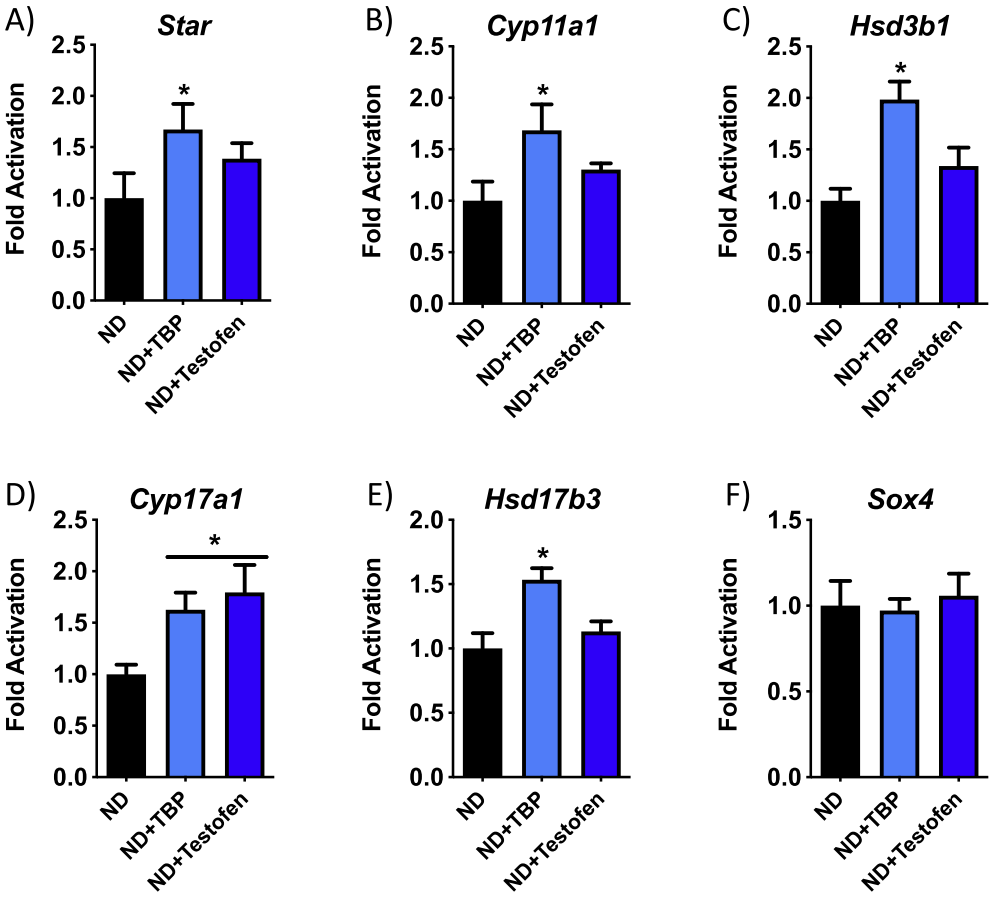

Figure 4. Steroidogenic genes expressions in testes of mice following treatments with TBP or Testofen. Mice were treated with TBP or Testofen for 10 weeks, followed by sacrifice, testes removal, mRNA extractions and quantitative real-time PCR assays for *Star* (A), *Cyp11a1* (B), *Hsd3b1* (C), *Cyp17a1* (D), *Hsd17b3* (E) and *Sox4* (F) genes expressions. Results were normalized using the geometric average of *Rpl19* and *Eif4h* reference genes expressions and reported to control (normal diet, ND) as fold activations (±SEM). Statistical analyses were performed using one-way ANOVA followed by Fisher's LSD's multiple comparisons test. Asterisk (*: P < 0.05) indicate statistically significant difference compared to ND alone.

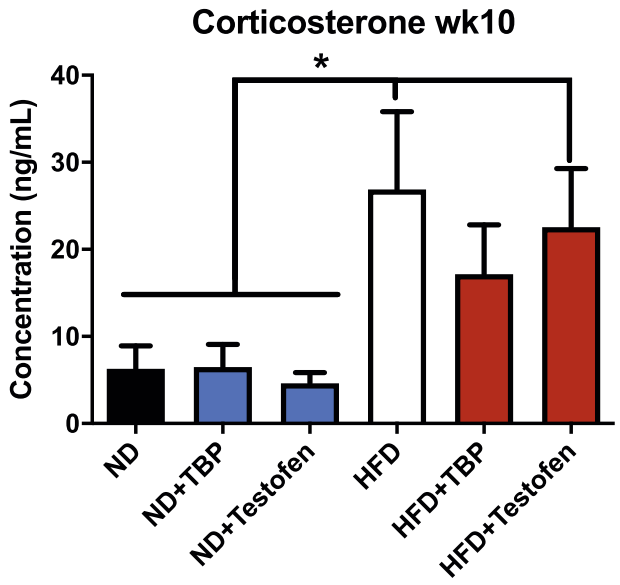

Figure 5. Serum corticosterone levels of mice following treatments of TBP or Testofen with or without high fat diet. Corticosterone measurements were performed on serum samples collected from mice at week 10 of treatments. Statistical analyses were performed using two-way ANOVA followed by Fisher's LSD's multiple comparisons test. Asterisk (*: $P < 0.05$) indicates a statistically significant difference.

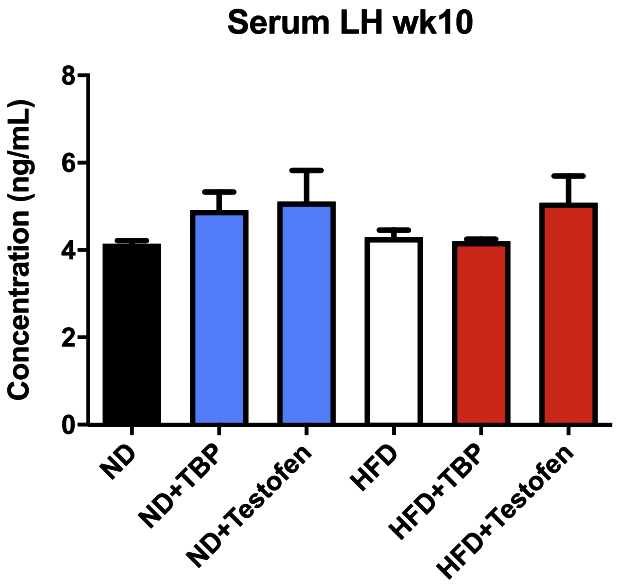

Figure 7. Serum LH levels of mice following treatments of TBP or Testofen with or without high fat diet. LH measurements were performed on serum samples collected from mice at week 10 of treatments. Statistical analyses were performed using two-way ANOVA followed by Fisher's LSD's multiple comparisons test.

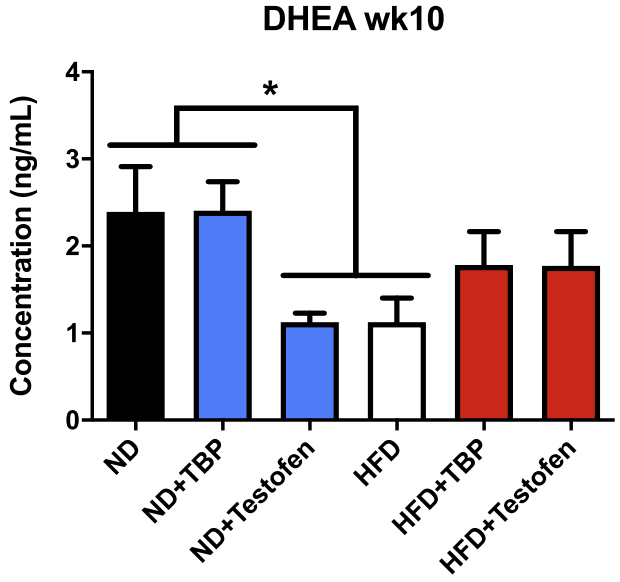

Figure 6. Serum DHEA levels of mice following treatments of TBP or Testofen with or without high fat diet. DHEA measurements were performed on serum samples collected from mice at week 10 of treatments. Statistical analyses were performed using two-way ANOVA followed by Fisher's LSD's multiple comparisons test. Asterisk (*: $P < 0.05$) indicates a statistically significant difference.

co-treatment. Interestingly, steroidal saponins, found in ginseng, fenugreek and tamarind extracts, have been reported to have a similar effect on morphine-induced serum corticosterone levels (Kim et al., 1999) and may be responsible for such action of TBP.

Combination of TBP with other plant extracts may improve its stimulatory effect on testosterone production. Indeed, use of a mixed extract of *Trigonella foenum-graecum* seed and *Lespedeza cuneate* for 8 weeks in men significantly increased serum total testosterone by more than 25% (Park et al., 2018). Similarly to what we observed for TBP, Furosap™, a patented fenugreek seed extract enriched in 20% protodioscin, has been shown to significantly increase free testosterone levels, whereas total serum testosterone concentrations were not altered, in males of 35–65 years of age over a period of 12 weeks (Maheshwari et al., 2017). In addition, no significant changes were observed for DHEA-S and fasting blood glucose following treatments with Furosap™, as we showed in mice using TBP and ND. DHEA is produced from DHEA-S, which progressively declines with aging during adulthood. Our results indicate that Testofen accelerated such decline in DHEA serum levels after 10 weeks of supplementation, as opposed to TBP which contributed to maintain DHEA production. Interestingly, treatment with tamarind seed extracts prevented the decrease in fasting serum DHEA-S in fructose-fed rats (Shahraki et al., 2011). Importantly, DHEA can be converted to active androgen in peripheral tissues, contributing to maintain muscle mass and prevent osteoporosis.

To determine if TBP could improve free testosterone levels, Sex Hormone Binding Globulin (SHBG) could have been measured from our serum samples as a decrease in SHBG content is highly correlated with increased free testosterone levels. Interestingly, others have shown that a daily dose of 600 mg of fenugreek extract resulted in an increase of both free and total testosterone levels in men aged 43–70 years after 12 weeks of treatment (Rao et al., 2016). However, such difference between our results from TBP on mice and fenugreek on men may be attributed to the size of cohorts as well as the more consistent sampling procedure in men compared to mice.

Others have also investigated the effects of fenugreek seed extract on sex hormones of menstruating women aged 20–49 years and showed that after 8 weeks of treatment, there was an increase in free testosterone and estradiol levels (Rao et al., 2015). Hence, it would be interesting to evaluate if TBP has a similar effect, which may be attributed to steroidal saponins binding to E2 receptors and activation E2 responsive genes as demonstrated for fenugreek extracts (Sreeja et al., 2010).

Importantly, TBP seems to have no toxicity for animal and human consumption. Indeed, it has been shown that daily intake of tamarind seed extracts by rats for two weeks under similar conditions as ours had no toxicological effects (Sundaram et al., 2014). Others have shown that daily ingestion of tamarind pulp extract for up to six months was considered safe and well tolerated for up to 1000 mg/kg bw in rats

Table 3. Measurements following treatments of male CD-1 mice with the indicated compounds.

	Wk	ND	ND + TBP	ND + Testofen	HFD	HFD + TBP	HFD + Testofen
Food consumption (g/day)	1	5.5 ± 1.5	7.2 ± 1.8	5.3 ± 0.6	5.0 ± 0.6	5.9 ± 1.2	7.4 ± 3.3
	5	6.5 ± 2.1	6.6 ± 2.3	6.3 ± 3.2	8.0 ± 1.1	7.2 ± 2.4	8.2 ± 2.8
	10	5.8 ± 1.7	6.3 ± 1.0	6.9 ± 2.3	7.9 ± 1.4	7.1 ± 2.2	7.8 ± 1.9
Water consumption (mL/day)	1	5.7 ± 1.5	6.3 ± 1.4	5.0 ± 1.6	7.1 ± 2.5	7.4 ± 2.7	6.9 ± 2.2
	5	5.8 ± 1.6	6.2 ± 1.5	5.5 ± 1.7	6.1 ± 1.9	6.5 ± 1.4	5.7 ± 2.4
	10	8.2 ± 1.2	9.3 ± 0.8	7.2 ± 1.5	9.1 ± 1.7	9.2 ± 2.2	8.9 ± 1.2
Body weight (g)	1	34.00 ± 1.09	34.00 ± 1.09	34.00 ± 0.63	32.83 ± 0.41	33.50 ± 1.22	32.17 ± 0.98
	5	37.00 ± 3.09	36 ± 0.89	34.83 ± 1.33	38.17 ± 1.47	37.33 ± 4.63	39.33 ± 2.94
	10	38.50 ± 3.98	36.33 ± 1.36	38.17 ± 3.37	39.50 ± 3.93	39.50 ± 4.72	41.33 ± 4.63
Testis weight (mg)	1	-	-	-	-	-	-
	5	-	-	-	-	-	-
	10	145.17 ± 16.04	142.67 ± 22.88	138.50 ± 12.00	140.42 ± 16.44	142.17 ± 17.36	150.67 ± 34.54
Serum glucose level	1	7.35 ± 0.82	8.02 ± 0.63	7.72 ± 0.84	7.08 ± 0.83	7.78 ± 0.47	7.37 ± 0.82
	5	5.92 ± 1.02	8.22 ± 0.89	7.16 ± 0.69	6.65 ± 0.94	8.05 ± 1.03	7.53 ± 1.07
	10	10.78 ± 1.88	11.57 ± 0.51	11.35 ± 1.44	11.77 ± 1.75	11.03 ± 2.31	11.93 ± 1.77
Serum total testosterone level (ng/mL)	1	1.44 ± 2.90	1.71 ± 3.17	5.93 ± 4.95	4.37 ± 3.37	1.07 ± 1.63	5.07 ± 4.51
	5	3.80 ± 5.40	3.78 ± 5.14	7.42 ± 5.96	8.49 ± 4.08	5.07 ± 4.29	10.37 ± 7.56
	10	1.93 ± 2.60	3.13 ± 3.09	2.04 ± 2.60	1.23 ± 2.36	0.37 ± 0.26	2.03 ± 2.84
Serum DHEA level (ng/mL)	1	1.10 ± 1.04	4.03 ± 2.98	4.59 ± 3.89	3.37 ± 2.01	2.47 ± 1.58	1.80 ± 0.80
	5	3.03 ± 1.62	2.00 ± 0.47	2.20 ± 0.56	2.07 ± 0.62	1.77 ± 0.30	1.89 ± 0.29
	10	0.33 ± 0.17	0.34 ± 0.11	0.16 ± 0.35	0.16 ± 0.09	0.25 ± 0.13	0.25 ± 0.13
Serum corticosterone level (ng/mL)	1	84.56 ± 67.67	27.09 ± 34.47	29.92 ± 34.08	9.08 ± 9.24	36.98 ± 33.82	44.17 ± 44.27
	5	4.80 ± 3.43	2.15 ± 1.27	8.03 ± 10.20	15.60 ± 17.19	42.58 ± 35.33	21.40 ± 12.73
	10	1.14 ± 1.17	1.18 ± 1.06	2.04 ± 2.97	4.89 ± 3.97	3.12 ± 2.52	4.10 ± 2.98
Serum LH level (ng/mL)	1	-	-	-	-	-	-
	5	18.27 ± 0.73	17.21 ± 0.24	17.51 ± 0.88	17.62 ± 0.52	16.92 ± 0.51	18.02 ± 0.94
	10	4.14 ± 0.17	4.91 ± 1.01	5.11 ± 1.74	4.30 ± 0.39	4.21 ± 0.11	5.08 ± 1.50

(Iskandar et al., 2017; Silva et al., 2009). In male Wistar rats, daily intake of 250 mg/kg bw for 54 days resulted in increased sperm production and absence of testicular toxicity (Rai et al., 2018).

In conclusion, we have shown that TBP contributes to maintain total body weight in mice under a normal diet, to lower blood glucose level and corticosterone production in mice under a high fat diet and to improve total testosterone production after 5 weeks of treatment with a high fat diet. In addition, TBP has a stimulatory effect on the expression of genes being important for testosterone production. Hence, TBP may prevent the detrimental effects of long-term consumption of a high fat diet and may have health benefits when consumed with appropriate nutrition and physical activity.

Declarations

Author contribution statement

Frank Cloutier: Performed the experiments; Analyzed and interpreted the data.

Pauline Roumaud, Sabrina Ayoub-Charette: Performed the experiments.

Subrata Chowdhury: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data.

Luc J. Martin: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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