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Experimental obesity and diabetes reduce male fertility: Potential involvement of hypothalamic Kiss-1, pituitary nitric oxide, serum vaspin and visfatin

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ABSTRACT

Reproductive dysfunction is a common consequence of both obesity and diabetes. This study investigated the impact of obesity and diabetes, alone or combined, on physiological reproductive parameters in male rats. Twenty-four male Wistar Albino rats were divided into four groups: Control; obese non-diabetic; diabetic; and obese diabetic. Obesity was provoked by consumption of a high-fat diet (HFD) consisting of 40% energy from fat for 90 days. Diabetes was induced by an intraperitoneal injection of streptozotocin at a dose of 40 mg/kg/day for three consecutive days. Semen, histopathological, and morphometric analyses were carried out. Serum testosterone, luteinizing hormone (LH), and vaspin and visfatin were measured using ELISA kits. Hypothalamic $Kiss^{-1}$ mRNA was detected using qPCR and pituitary nitric oxide (NO) was determined using Griess reagent. Our results showed a decrease in semen quality parameters, testosterone, and LH levels with degenerative changes in the testes in experimental groups when compared to control group. This had a positive correlation with hypothalamic $Kiss^{-1}$ and a negative correlation with pituitary NO and serum vaspin and visfatin. In addition, adverse effects were more pronounced in animals with obesity and diabetes combined compared to rats who were either diabetic or obese. In conclusion, obesity and diabetes, alone or combined, had a negative impact on male rat fertility. Moreover, obesity and diabetes combined had more harmful effects on male fertility when compared with obesity alone. Hypothalamic $Kiss^{-1}$, pituitary NO, and serum vaspin and visfatin may play a role in the pathophysiology of male infertility-associated with obesity and diabetes.

1. Introduction

Obesity is a substantial health hazard. Many studies have been conducted in an attempt to find ways to reduce obesity and its complications; however, its prevalence is still increasing [1]. Modern sedentary lifestyles and the consumption of a diet with more than 40% of its energy derived from animal fat for several weeks results in obesity [2]. Obesity is accompanied by a group of complications including type 2 diabetes mellitus (T2DM), cardiovascular diseases, and infertility [3,4], all of which have a negative impact on psychology and well-being. For every 9 kg increase in body weight, the incidence of infertility increases by 10% [5]. Numerous mechanisms have been determined that link obesity with male infertility. For example, reduced levels of plasma sex hormone binding globulin (SHBG) and reduced levels of free and total testosterone, with a concurrent increase in estrogen levels [6], have been associated with disruption of the hypothalamic-pituitary-gonadal (HPG) axis, which is directly proportional to the increase in body weight. Hyperestrogenemia and increased peripheral aromatization of androgens resulted in disruption of the pulses of gonadotropin releasing hormone (GnRH) and subsequently reduced LH and FSH secretions [6]. In addition to these previously mentioned mechanisms, insulin resistance, hyperinsulinemia, hyperglycemia, and increased levels of proinflammatory cytokines, all of which are observed in combined obesity and dia-

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betes, contributed to reduced male fertility through the reduction of SHBG and testosterone levels [7].

Kiss-1 performs a crucial role in the control of the hypothalamic-pituitary-gonadal (HPG) axis [8]. Kiss-1 codes an amino acid precursor that is cleaved to kisspeptins [9]. Kiss-1 mRNA has been found in arcuate nucleus, where GnRH neurons are expressed [10]. Kisspeptin regulates GnRH release and injection of kisspeptin stimulates LH release in rat [11], mice [10], and humans [12]. Kiss-1 mutation results in reduced gonadotropins and testosterone levels and impaired sexual maturation [13]. Mounting evidence indicates that Kiss-1 downregulation is associated with infertility both in animals and in humans [14]. Additionally, NO plays a pivotal role in reproduction, both centrally and peripherally. Centrally, it controls the pulsatile release of LHRH and LH [15]. Peripherally, it stimulates steroidogenesis and spermatogenesis [16]; however, the precise effects of NO on LHRH and LH release are still a subject of controversy. Adipose tissue is an active endocrine organ that produces diverse kinds of adipokines, including vaspin and visfatin. They play a crucial role in carbohydrate and lipid metabolism and affect feeding behavior [17]. Vaspin and visfatin are expressed from both the adipose tissue and from the peripheral reproductive tissues [17,18]; however, little is known about their role in male fertility.

In response to the prevalence of obesity and its complications, including diabetes and infertility, this study aimed to determine the impact of obesity and diabetes, alone or combined, on reproductive parameters in male rats. Hypothalamic Kiss-1 and pituitary NO levels were investigated as central regulators of GnRH [19,20], while serum vaspin and visfatin levels were determined as markers of impaired male fertility [21].

2. Materials and methods

2.1. Animals

Adult male Wistar Albino rats, weighing about 115–150 *g*, were used in the study. Animals were housed in clean, appropriately ventilated cages (4 rats per cage) in the animal house of the Faculty of Medicine, Assiut University. Their quarters maintained a standard light-dark cycle, at room temperature, with access to food and water *ad libitum* throughout the entire study period. The rats were left for a week to acclimatize before the experimental procedures began. The research procedures were in accordance with the 'Guidelines of Experiments on Animals' and were accepted by the Ethics Committee at the Faculty of Medicine, Assiut University, Egypt (approval number #17200214#). The rats were cared for in accordance with the *Guide for the Care and Use of Laboratory Animals* [22].

2.2. Experimental design

Twenty-four rats were arbitrarily allocated into one of the four following groups (six rats per group):

- **Control group:** fed a standard laboratory diet consisting of 14% energy from fat and received the vehicle (saline) via intraperitoneal (IP) injection;
- **Obese non-diabetic (OND) group:** fed a high-fat diet (HFD) consisting of 40% energy from fat for 90 days [23];
- **Diabetic group:** fed a standard laboratory diet consisting of 14% energy from fat from the first day of the experiment until the end. At day 67, overnight-fasted rats received a freshly prepared streptozotocin (STZ, Sigma-Aldrich, St. Louis, MO, USA), dissolved in ice-cold sterile saline (0.9%) IP injection at a dose of 40 mg/kg/day for three consecutive days [24];
- **Obese diabetic (OD) group:** fed a high-fat diet consisting of 40% energy from fat for 90 days from the first day of the experiment until the end. At day 67, overnight-fasted rats received an IP injection of freshly prepared STZ at a dosage of 40 mg/kg/day for three successive days. The reason for choosing the 67th day resulted from our preliminary data, which showed that obesity was well established by this time.

Induction of diabetes was confirmed by measuring fasting blood glucose levels from tail vein using an On-Call Ez glucometer (ACON Laboratories, Inc., USA). Rats with a fasting blood glucose level exceeding 300 mg/dL were considered diabetic [25].

2.3. Anthropometrical measures

Body weight was measured at the beginning of the experiment (initial body weight), at regular two week intervals throughout the entire study period, and on the day of euthanasia (final body weight). Abdominal circumference, measured just anterior to the forefoot, was used as a guide for abdominal and intra-abdominal fat content. Finally, the Lee index was calculated as the cube root of body weight (g) divided by the naso-anal length (cm). Rats were considered obese when the abdominal circumference >14.9 ± 2 cm and the Lee index >0.33 [26].

2.4. Blood and tissue sampling

2.4.1. Blood sampling

Fasting blood samples were obtained at the end of the experiment and centrifuged for 15 min at 3000 RPM. The clear supernatants (sera) were taken and distributed into small aliquots and kept at −20 °C until use. After euthanasia of all fasting rats, the hypothalamus and pituitary glands were rapidly dissected, frozen in liquid nitrogen, and stored at −80 °C until further use. Testes were rapidly dissected and processed for histological examinations.

2.4.2. Tissue sampling

Pituitary glands were homogenized in a cold phosphate buffer and the homogenates centrifuged at 4000 RPM for 10 min at 4 °C. Pituitary homogenates were used for nitric oxide estimation. The hypothalamus was used for determination of Kiss-1 mRNA by qPCR.

2.5. Biochemical examinations

2.5.1. Determination of serum luteinizing hormone (LH) and vaspin and visfatin levels

Levels of serum LH and vaspin and visfatin were determined using commercially available enzyme-linked immunosorbent assay kits (ELISA) (Weka Med Supplies Corp., Changchun, China). All analyses were conducted according to the manufacturer's instructions.

2.5.2. Determination of serum testosterone level

Serum testosterone levels were determined using a commercially available ELISA kit bought from BioCheck (California, USA) following the manufacturer's instructions.

2.5.3. Determination of nitric oxide (NO) in the pituitary homogenate

Nitric oxide (NO) levels were determined in the pituitary homogenates using the Biodiagnostic Nitrite Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's instructions. Briefly, endogenous nitrite concentration, a marker of nitric oxide production, was colorimetrically measured in the pituitary homogenate by the addition of Griess Reagents, which transformed nitrite into a dark purple azo compound. NO levels

Table 1
Anthropometric measures and fasting blood glucose in all groups studied.

	Control	OND	Diabetic (D)	OD
Initial body weight (g)	114.6 ± 4.5	119.8 ± 5.1	124.3 ± 6.6	118.6 ± 6.4
Final body weight (g)	216.3 ± 13.9	402.3 ± 14.6[a]	169 ± 11.9[b]	302.2 ± 16.7 [a,b,c]
Lee index	0.33 ± 0.01	0.41 ± 0.01[a]	0.32 ± 0.01[b]	0.37 ± 0.01 [a,b,c]
Abdominal circumference (cm)	14.2 ± 0.8	17.1 ± 0.8[a]	14.8 ± 0.36 [b]	16.3 ± 0.5[a,c]
Fasting blood glucose (mg/dL)	70.6 ± 1.5	71.2 ± 1.3	366.8 ± 25.5 [a,b]	388.5 ± 28.6[a,b]

Mean ± SEM (n = 6) shown.
OD, obese diabetic; OND, obese non-diabetic.
The Kruskal-Wallis H test followed by the Mann-Whitney U test was used for statistical analysis.
[a] Significant as compared with the control group, $P < 0.05$.
[b] Significant as compared with the obese non-diabetic group, $P < 0.05$.
[c] Significant as compared with the diabetic group, $P < 0.05$.

were expressed relative to the protein content in each sample. Protein contents were measured according to the methods of Lowry et al. [27].

2.6. Semen analyses

Semen was obtained as formerly described by Gomaa et al. [28]. Briefly, a longitudinal cut was made in the cauda epididymis and the contents released into a sterile glass. To disperse spermatozoa, the sperm suspension was diluted to 1:100 with Tyrode's albumin lactate pyruvate (Sp-TALP) and analyzed immediately after collection. Using a light microscope (Olympus CH, Japan) at x10, the motile sperm (those showing flagellar oscillation for more than 10 s) and the non-motile sperm were counted in five different fields [29]. The percentage ratio of motile sperm to the sum of motile and non-motile sperm was calculated. Sperm concentrations (millions/mL) were assessed using a hemocytometer [30] with the improved Neubauer chamber (Deep 1/10 mm, LABART, Germany). Briefly, counting was performed in two chambers using a light microscope at x200 magnification. Counting was done in each chamber's central area, which contains 25 large squares; each 'large square' is bounded by a triple line on all sides. The average number of sperm counted in both chambers divided by the volume obtained was calculated and expressed as $x10^6$/mL [31].

After application of alkaline Methyl Violet, the sperm morphology was evaluated using the light microscope at x40 objective magnification; the sperm appeared violet in color. The percentage ratio of morphologically normal sperm to the total sperm examined (200 sperm examined from each rat) was calculated. The viability was assessed using Eosin & Nigrosine stain; non-viable sperm absorbed the stain and appeared red, while viable sperm did not absorb the dye and appeared colorless. Viability was calculated as the percentage of the number of colorless sperm compared to the total number of sperm examined [32].

2.7. Histological and morphometrical analyses of testes

After euthanasia, one testis was removed from each rat, fixed in Bouin's solution overnight, dehydrated in increasing concentrations of ethanol, and set in paraffin. Coronal sections, 5 μm in thickness, were cut and stained using Haematoxylin and Eosin (H&E) and examined under the light microscope. For morphometric analysis, the diameter of the seminiferous tubule and the epithelial height was evaluated in 10 non-overlapping fields from ten randomly chosen sections per rat. For seminiferous tubule diameter measurement, the most circular seminiferous tubules were identified in each section and captured at x400 magnification with the assistance of a digital camera located on a Leica microscope using image-analyzing system software (Leica Q 500 MCO) at the Histology Department, Faculty of Medicine, Assiut University.

2.8. Quantitative PCR

Total RNA was extracted from the hypothalamic samples using a QIAamp RNA Mini Kit (Qiagen, USA), according to the manufacturer's instructions. RNA (100 ng) was reverse transcribed into cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, USA) following the manufacturer's instructions. SYBR Green-based quantitative polymerase reaction (qPCR) was carried out using a QuantiTect SYBR Green PCR Kit (Qiagen, USA) and Mx3000 P qPCR detection system (Agilent Technologies, USA). The PCR protocol consisted of 5 min at 95 °C; 40 cycles of denaturation (30 s at 95 °C); annealing (30 s at 55 °C for Kiss-1 and 53 °C for RP-S 11); and polymerization (extension) at 72 °C for 1 min. Product purity was confirmed by a melting dissociation curve, which was generated by heating to 95 °C for 15 s, 60 °C for 15 s, and 95 °C for another 15 s, followed by agarose gel electrophoresis. Each sample was run and analyzed in duplicate. The sequences of primers used are as follows: for Kiss-1 (forward, 5′- TGGCACCTGTGGTGAACCCTGAAC-3′, reverse 5′- ATCAGGCGACTGCGGGTGGCACAC-3′) and for RP-S11, the housekeeping gene (forward, 5′-CATTCAGACGGAGCGTGCTTAC-3′, reverse 5′-TGCATCTTCATCTTCGTCAC-3′) [33]. The relative expression level of the Kiss-1 gene versus the housekeeping reference gene (ribosomal protein S11; RPS11) was calculated using the equation of $2^{-\Delta\Delta Ct}$ as described by Navarro et al. [33]. The relative expression level of the Kiss-1 gene is represented as the fold change relative to the control group; its value is 100.

2.9. Statistical analyses

Data are presented as the mean ± standard error of the mean (SEM). Statistical differences between groups were analyzed using the non-parametric Kruskal-Wallis H test followed by the Mann–Whitney U test. A value of $P \leq 0.05$ was considered statistically significant. Spearman's rank correlations were used according to Knapp and Miller (1992). All statistical analyses were carried out with SPSS software version 20 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Anthropometric measures and fasting blood glucose

There were no significant differences in the initial body weight among the studied groups. Final body weight, abdominal circumference, and the Lee index significantly increased in rats fed a HFD (the OND and OD groups) compared to those parameters in the controls, confirming the induction of obesity (Table 1). Fasting blood glucose levels increased significantly in the diabetic and OD rats compared to those in the control group and OND rats, confirming the induction of diabetes (Table 1). Combined obesity and diabetes in the OD rats resulted in a significant increase in final body

Table 2
Changes in semen quality parameters and serum levels of LH and testosterone in all groups studied.

	Control	OND	Diabetic	OD
Sperm concentration (x10^6/mL)	**39 ± 1.86**	**27 ± 1.59**[a]	**29 ± 1.3**[a]	**17.8 ± 1.37**[a,b,c]
Motility (%)	**71.6 ± 2.47**	**48.3 ± 4.59**[a]	**48.3 ± 4.2**[a]	**31.6 1.6**[a,b,c]
Normal Morphology (%)	**89.5 ± 2.43**	**66.6 ± 4.77**[a]	**66.6 ± 1.05**[a]	**50.83 ± 3**[a,b,c]
Viability (%)	**83.3 ± 3.07**	**66.2 ± 4.58**[a]	**63.3 ± 2.1**[a]	**45.8 ± 2**[a,b,c]
LH (ng/mL)	**2.2 ± 0.07**	**2 ± 0.01**[a]	**1.7 ± 0.05**[a,b]	**1.8 ± 0.03**[a,b]
Testosterone (ng/mL)	**8.5 ± 0.27**	**3.6 ± 0.38**[a]	**2.6 ± 0.41**[a]	**2.3 ± 0.18**[a,b]

Mean ± SEM (n = 6) shown.
OD, obese diabetic; OND, obese non-diabetic.
The Kruskal-Wallis H test followed by the Mann-Whitney U test was used for statistical analysis.
[a] Significant as compared with the control group, $P < 0.05$.
[b] Significant as compared with the obese non-diabetic group, $P < 0.05$.
[c] Significant as compared with the diabetic group, $P < 0.05$.

Table 3
Seminiferous tubule diameter (mm) and epithelial height (mm) in all of the experimental groups.

	Control	OND	Diabetic	OD
Tubular diameter (mm)	**331.4 ± 4.6**	**304.8 ± 5.0**[a]	**254.2 ± 6.9**[a,b]	**285.1 ± 4.1**[a,b,c]
Epithelial Height (mm)	**51.8 ± 1.4**	**36 ± 1**[a]	**23.5 ± 0.9**[a,b]	**30.1 ± 0.9**[a,b,c]

Mean ± SEM (n = 6) shown.
OD, obese diabetic; OND, obese non-diabetic.
The Kruskal-Wallis H test followed by the Mann-Whitney U test was used for statistical analysis.
[a] Significant as compared with the control group, $P < 0.05$.
[b] Significant as compared with the obese non-diabetic group, $P < 0.05$.
[c] Significant as compared with the diabetic group, $P < 0.05$.

weight, abdominal circumference, and Lee index as compared to those parameters in non-obese diabetic rats.

3.2. Semen quality parameters and levels of reproductive hormones

A significant reduction in semen quality parameters was noted in the experimental groups (OND, diabetic, and OD) compared to those in the control group in the form of reduced sperm concentration and a reduced percentage of motile, viable, and morphologically normal sperm (Table 2). Additionally, these parameters were significantly lower in the combined obesity and diabetes group (OD) as compared to the groups with obesity alone (OND) or diabetes alone (diabetic group), with no significant differences observed between the OND and diabetic groups.

LH and testosterone levels were significantly lower in experimental groups (OND, diabetic, and OD) as compared to those in the control group. Combined obesity and diabetes in the OD group resulted in a significantly lower level of LH compared to that in the obesity alone group (OND) and a significantly lower level of testosterone compared to those in both the obesity alone and diabetes alone groups (Table 2).

3.3. Testicular morphological structure

Microscopic examination of the testes from the control group showed firmly packed seminiferous tubules regular in form and contour (Fig. 1A). The seminiferous tubules were lined with many layers of spermatogenic cells. The interstitial tissue in between contained Leydig cells near blood capillaries (Fig. 1B).

In the rats with obesity alone (OND), testicular sections revealed irregularly shaped seminiferous tubules with acidophilic degenerated hyalinized exudates in the interstitium (Fig. 1C), which made it difficult to recognize the Leydig cells. Moreover, the basement membranes of the seminiferous tubules were thickened, with an interrupted outline, and expulsion of their contents was noted (Fig. 1D). The nuclei of the spermatogonia and primary spermatocytes were darkly stained (Fig. 1D).

Diabetic rats showed degenerative changes in the form of distorted seminiferous tubules, the presence of sloughed cells in the lumen of them, and the presence of acidophilic exudate in the interstitial space (Fig. 1E). Seminiferous tubules had an irregular basement membrane with darkly stained apoptotic nuclei of the spermatogenic cells and Leydig cells (Fig. 1F).

The testes of rats with combined obesity and diabetes (OD) showed marked morphological alterations such as distorted architecture of the seminiferous tubules, some of which exhibited an acidophilic hyalinized center with depletion of the spermatogenic cells (Fig. 1G&H). The interstitial compartment showed thickened interstitial capillaries surrounded by Leydig cells and an acidophilic exudate (Fig. 1H).

Morphometric analysis of the seminiferous tubules revealed that the diameter and the epithelial height in the lining of the seminiferous tubules were reduced significantly in the experimental rats (OND, diabetic, and OD) as compared with the controls (Table 3).

3.4. Expression of the hypothalamic Kiss-1 mRNA

Quantitative PCR showed that the fold expression of Kiss-1 mRNA in the hypothalamus was significantly lower in the experimental rats (OND, diabetic, and OD) compared with the control rats (Fig. 2A). Also, expression of the hypothalamic Kiss-1 mRNA was significantly lower in the rats with combined obesity and diabetes as compared with rats with obesity alone (OND) but not significant compared with expression in rats with diabetes alone. Moreover, there was no significant change in the expression of the hypothalamic Kiss-1 mRNA between the OND and diabetic rats (Fig. 2A).

3.5. Pituitary NO

Pituitary NO levels were significantly higher in the OND, diabetic, and OD groups compared to levels in the controls. In addition, the diabetic group had significantly higher pituitary NO levels compared to those in OND rats (Fig. 2B). Rats with combined obesity and diabetes showed no significant change in pituitary NO levels compared to rats with obesity alone or diabetes alone.

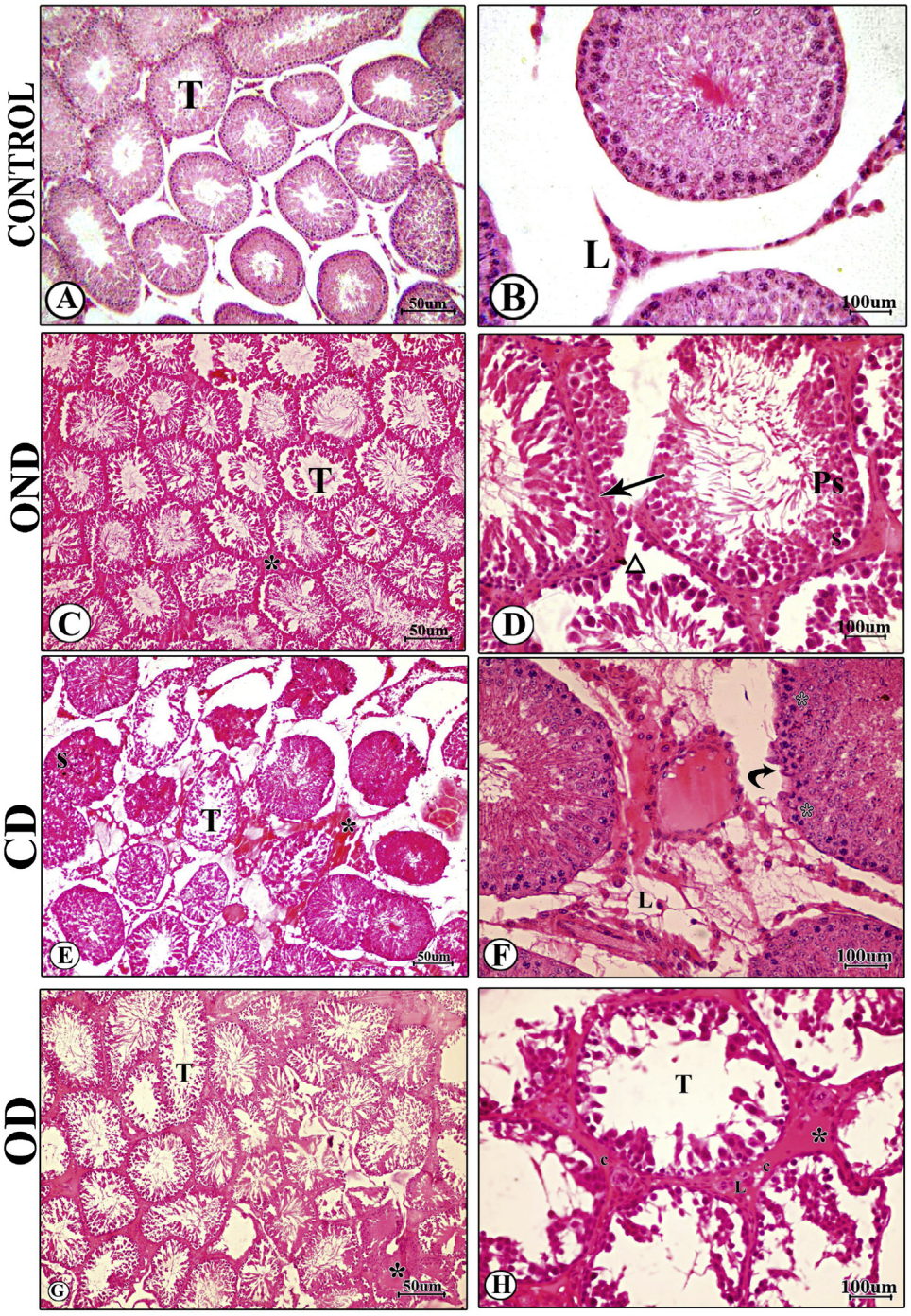

Fig. 1. (A–H) Photomicrographs of H&E-stained testicular sections from all experimental groups. A: Control rat testis showed normal architecture in the form of closely packed seminiferous tubules (T) regular in form and contour (x100). B: Seminiferous tubules from control rats were lined with healthy multi-layers of spermatogenic cells and separated with interstitial tissue containing Leydig cells (L) (x400). C: Obese non-diabetic rat testis (OND) showed irregularly shaped seminiferous tubules (T) with acidophilic degenerated exudates in the interstitium (*) (x100). D: Seminiferous tubules from the OND rats had thickened basement membranes (arrow) with interrupted outlines and expulsion of their contents (Δ). Darkly stained nuclei were also observed in spermatogonia (s) and primary spermatocytes (Ps) (x400). E: Diabetic (CD) rat testis showed distorted seminiferous tubules (T), the presence of sloughed cells in the lumen of the seminiferous tubules (s) and the presence of acidophilic exudate in the interstitial space (*) (x100). F: Higher magnification of the diabetic rat testis showed irregular basement membrane (curved arrow) and darkly stained apoptotic nuclei of the spermatogenic cells (white star) and Leydig cells (L) (x400). G: Obese diabetic rat testis (OD) had seminiferous tubules with distorted architecture (T) that exhibited acidophilic hyalinized centers (*) (x100). H: Seminiferous tubules of OD rats were irregular and atrophied (T) with depletion of the spermatogenic cells in most of them. Notice the thickened interstitial capillaries (c) surrounded by atrophied Leydig cells (L) and acidophilic exudate (*) (x400).

3.6. Serum vaspin and visfatin

Fig. 3 shows that serum vaspin and visfatin levels were significantly higher in OND, diabetic, and OD rats compared to those in the controls. Also, combined obesity and diabetes and diabetes alone resulted in significantly higher levels of both serum vaspin and visfatin as compared to those in obesity alone as demonstrated in OND rats.

Spearman's correlation was carried out within the combined groups of the control, OND, diabetic, and OD. Kiss-1 had sig-

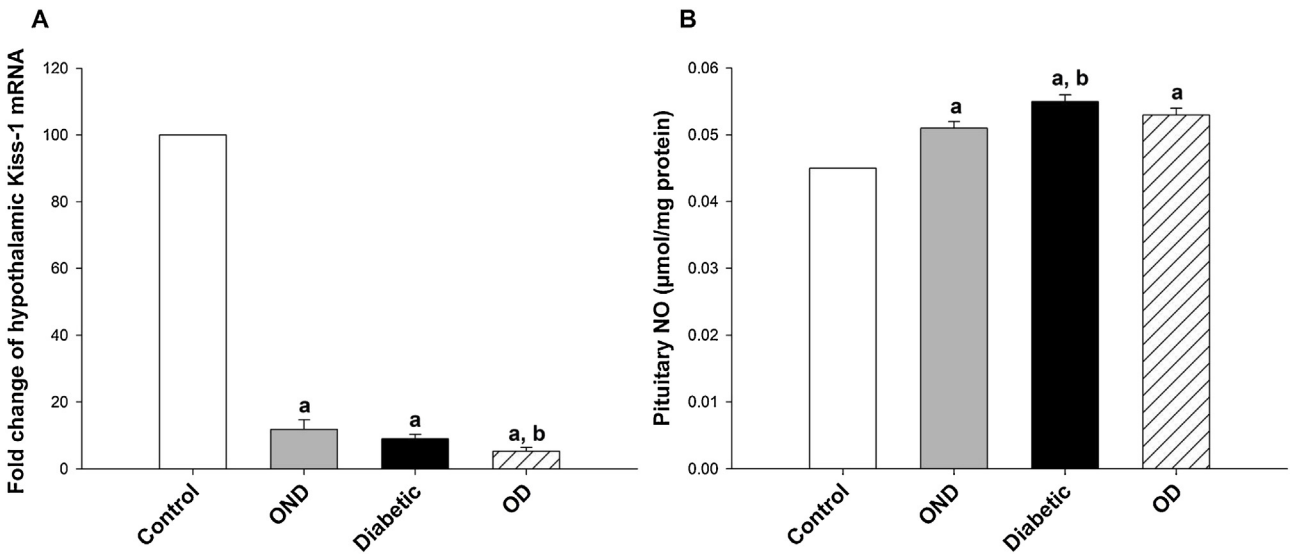

Fig. 2. (A) The fold expression of the hypothalamic Kiss^{-1} mRNA and (B) pituitary nitric oxide (NO) levels in the different experimental groups. Mean ± SEM (n = 6) are shown. The relative expression of the Kiss^{-1} gene versus a housekeeping reference gene (ribosomal protein S11; RPS11) was calculated using the equation of $2^{-\Delta\Delta Ct}$ and represented as fold change relative to the control group. OD, obese diabetic; OND, obese non-diabetic.
[a] Significant as compared with the control group $P < 0.05$. [b] Significant as compared with obese non-diabetic group $P < 0.05$. The Kruskal-Wallis H test followed by the Mann-Whitney U test were used for statistical analysis.

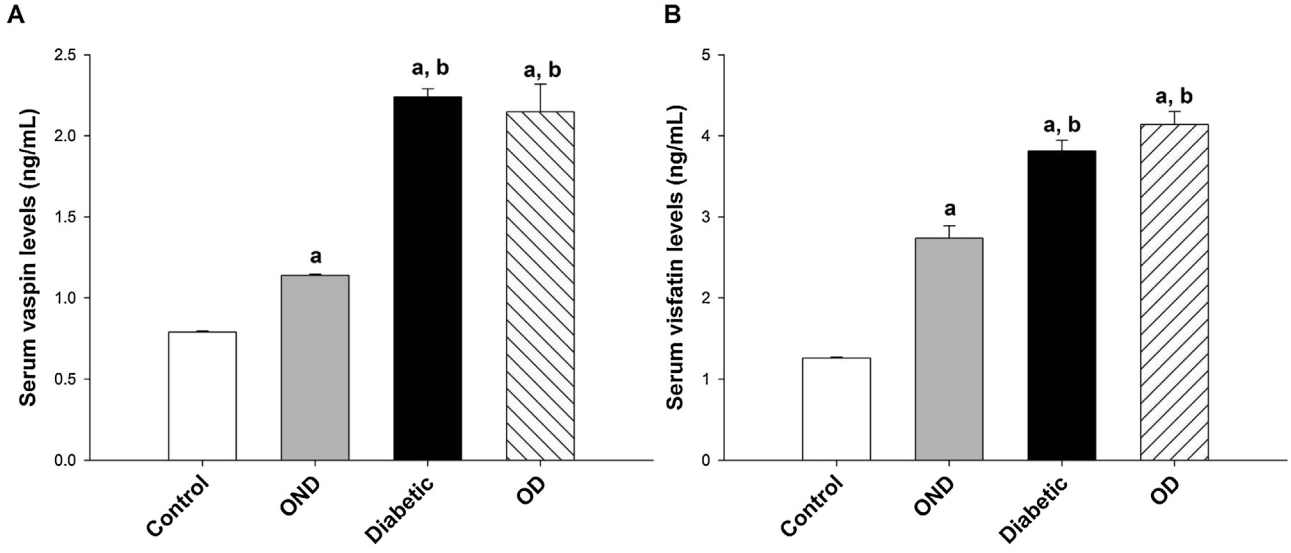

Fig. 3. (A) Serum vaspin and (B) visfatin levels (ng/mL) in all experimental groups. Data are presented as Mean ± SEM (n = 6). OD, obese diabetic; OND, obese non-diabetic.
[a] Significantly different vs the control group $P < 0.05$. [b] Significantly different vs the obese non-diabetic group $P < 0.05$. The Kruskal-Wallis H test followed by the Mann-Whitney U test were used for statistical analysis.

Table 4
Correlations between the hypothalamic Kiss^{-1} mRNA, pituitary nitric oxide (NO), and serum levels of vaspin and visfatin and semen quality parameters and reproductive hormones (LH and testosterone).

	Sperm concentration (x10^6/ml)	Motility (%)	Normal Morphology (%)	Viability (%)	LH (ng/mL)	Testosterone (ng/mL)
Kiss-1	r: 0.766 p: 0.000	r: 0.749 p: 0.000	r: 0.778 p: 0.000	r: 0.791 p: 0.000	r: 0.652 p: 0.001	r: 0.724 p: 0.000
NO (µmoL/mg protein)	r: -0.472 p: 0.02	r: -0.571 p: 0.004	r: -0.623 p: 0.001	r: -0.566 p: 0.004	r: -0.664 p: 0.000	r: -0.607 p: 0.002
Vaspin (ng/mL)	r: -0.578 p: 0.003	r: -0.736 p: 0.000	r: -0.656 p: 0.001	r: -0.664 p: 0.000	r: -0.928 p: 0.000	r: -0.902 p: 0.000
Visfatin (ng/mL)	r: -0.593 p: 0.002	r: -0.766 p: 0.000	r: -0.645 p: 0.001	r: -0.691 p: 0.000	r: -0.947 p: 0.000	r: -0.954 p: 0.000

Spearman's correlation was carried out within the combined groups of the control, OND, diabetic, and OD.

Table 5
Correlations between the hypothalamic Kiss^{-1} mRNA and pituitary nitric oxide (NO) and serum levels of vaspin and visfatin.

	NO (μmoL/mg protein)	Vaspin	Visfatin
Kiss-1	r: -0.59 p: 0.01	r: -0.73 p: 0.000	r: -0.75 p: 0.000

Spearman's correlation was carried out within the combined groups of the control, OND, diabetic, and OD.

nificant positive correlations with reproductive hormones (LH, testosterone) and semen quality parameters, suggesting that Kiss-1 plays a pivotal role in the regulation of reproductive function in the male rat (Table 4).

Pituitary NO and serum levels of vaspin and visfatin correlated negatively with reproductive hormones (LH and testosterone) as well as with semen quality parameters (Table 4).

Lastly, negative correlations were observed between hypothalamic Kiss-1 and pituitary NO and serum vaspin and visfatin (Table 5).

4. Discussion

Obesity, diabetes, and their consequences are increasing worldwide [34]. Reproductive dysfunction is one of the common consequences of both obesity [6,35] and uncontrolled diabetes [36]. Several pathophysiological mechanisms have been suggested; however, the exact mechanism remains unclear. This study assessed the effects of obesity, diabetes, alone or combined, on physiological and mechanistic reproductive parameters in male rats.

Our results showed that obesity and diabetes, alone or combined, had negative effects on semen quality parameters, testosterone, and LH levels with degenerative changes in the testes in the experimental groups when compared to the control group. These results are in agreement with data from previous studies in obese [37] and diabetic rats [38,39], which demonstrated a reduced sperm concentration, increased percentages of non-motile and dead sperm and reduced LH and testosterone levels with obesity and diabetes alone. These histopathological changes could be the result of impaired Leydig cell steroidogenesis [19], oxidative stress [39], and increased testicular cell apoptosis [37]. Obesity is associated with chronic inflammatory status, both systemic and locally in the testes. Inflammation activates the production of reactive oxygen species, induces testicular apotosis, and disrupts the blood testicular barrier, resulting in degenerative changes in the germ cells and impaired spermatogenesis [40]. In diabetes, hyperglycemia boosts the production of reactive oxygen species resulting in impaired Leydig cell function and subsequent reduction in testosterone biosynthesis [41]. Since testosterone is essential for germinal cell mitotic division [42], reduced testosterone could be responsible for the degenerative changes in the seminiferous tubules and the reduction in tubular diameter and epithelial height observed in this study. Recently, it has been shown that T2DM reduces the production of vascular endothelial growth factor resulting in impaired testicular microcirculation and subsequent degeneration of the testis [43]. In our study, the reduction in semen quality and degenerative testicular changes were more pronounced in rats with combined obesity and diabetes than in the rats with obesity or diabetes alone. This could be due to all of the previously mentioned mechanisms occurring together.

Diabetes and obesity are associated with reduced GnRH and the downstream secretion of basal and pulsatile LH secretion and testosterone [36,44]. Similarly, our results showed a significant reduction of LH levels in all experimental groups compared to those of the controls, suggesting a central impact of obesity and/or diabetes. To investigate the cause of the reduced LH levels noted in this study, we measured the expression level of hypothalamic Kiss-1 mRNA, which performs a vital role in regulating GnRH release [45]. Our results showed that hypothalamic Kiss-1 mRNA was substantially lower in animals with obesity and diabetes, alone or combined (OND, diabetic, and OD), than in the controls, which correlated positively with semen quality, testosterone, and the levels of LH hormones, suggesting that impaired semen quality and reduced reproductive hormones may be a consequence of reduced Kiss-1 mRNA. Dhillo et al. [46] reported that infusion of kisspeptins to male volunteers significantly increased LH and testosterone levels. Central administration of Kisspeptin to diabetic rats also increased the LH level [36]. Additionally, Dedes [47] reported that mutation of Kiss-1 resulted in arrested spermatogenesis at the early spermatid stage. Taken together, these observations suggest that reduced hypothalamic Kiss-1 mRNA could be responsible, in part, for the reduced LH levels and thereby reduced levels of testosterone hormone in OND, diabetic, and OD rats. Obese diabetic rats had significantly lower LH and testosterone levels and semen quality compared to rats with obesity alone, which could be attributed to the further reduction in hypothalamic Kiss-1 observed in rats with combined obesity and diabetes.

Since both Kiss-1 and NO play a role in GnRH and LH release [20], we investigated the role of pituitary NO in obesity and diabetes-induced male infertility. NO acts as a regulator of gonadotropin release [15,48]. NOS is expressed from GnRH secreting cells [49] and LH gonadotropes [50,51]. Yu et al. [48] demonstrated that NOS inhibition, and thereby NO reduction, suppresses leptin-induced GnRH and LH release. In contrast, our results showed that obesity and diabetes, alone and combined, resulted in a subtle significant increase in pituitary NO as compared with the controls, which was negatively correlated with semen quality parameters and the levels of testosterone and LH hormones. This increase in the pituitary NO level could be a compensation for the reduced LH level, either as an attempt to increase the LH level and reset the reproductive homeostasis [48] or due to NO formation being freed from the usual inhibitory effect of testosterone. Testosterone inhibits NO production, *in vivo*, and exerts a negative feedback mechanism on GnRH and NOS activity [52].

Additionally, this study was conducted to clarify the interplay between novel adipokines such as vaspin and visfatin and the HPG axis in obese, diabetic, and obese diabetic male rats.

Vaspin is an insulin-sensitizing adipokine [53], while visfatin is an insulin-mimicking agent [54]. Although the role of vaspin and visfatin as insulin sensitizers in obesity and diabetes is well established, their role in male fertility is still unclear. Our results showed that levels of vaspin and visfatin were significantly higher in rats with obesity and diabetes, alone or combined (OND, diabetic, and OD), as compared to those in the controls. Their levels also correlated negatively with all semen quality parameters and the levels of LH and testosterone hormones, suggesting that they do play a role in male fertility, both centrally and peripherally. Similarly, Thomas et al. [17] reported higher levels of vaspin and visfatin in human seminal plasma than in the serum, with vaspin correlated negatively with semen volume and positively with sperm DNA fragmentation. Riammer et al. [21] detected visfatin in different cell types in human testes, with a higher level in immature than in mature spermatozoa. These findings identify vaspin and visfatin as markers of impaired male fertility. Tu et al. [55] demonstrated that visfatin increases testosterone levels in cultured Leydig cells, suggesting that it plays a role in steroidogenesis, which seems to contradict the negative correlation between visfatin and testosterone observed in our study. The high visfatin levels observed in our study could be compensation for the reduced testosterone levels, which was prevented by the degenerative changes in Leydig cells. In addition, negative correlations were observed between

levels of vaspin and visfatin and semen quality, levels of LH and testosterone, and hypothalamic Kiss-1, suggesting that reduced hypothalamic Kiss-1 could be a consequence of high levels of vaspin and visfatin. However, further investigations need be conducted to localize the receptor for vaspin and visfatin in the brain and to determine their impact on GnRH and LH secretion and their interactions with the Kiss-1 system.

To summarize, obesity and diabetes have negative effects on semen quality and reproductive hormones, both centrally and locally. Reduced hypothalamic Kiss-1 mRNA, higher pituitary nitric oxide (NO), and higher serum vaspin and visfatin levels could be involved in the pathophysiology of male infertility associated with obesity and diabetes. Centrally, reduced Kiss-1 mRNA could be the main contributor to the reduced levels of LH and impaired reproductive function. Obesity and diabetes reduce hypothalamic Kiss-1 either directly or indirectly via increasing serum levels of vaspin and visfatin; however, further studies are necessary to elucidate these effects.

Raised levels of pituitary NO could be a mechanism used to restore the regular release of gonadotropins and thereby reestablish normal reproductive function, while raised levels of vaspin and visfatin mainly reset normal metabolism, insulin sensitivity, and testosterone levels.

Reduction in semen quality and reproductive hormones was more pronounced in animals with obesity and diabetes combined compared to that in obese rats. In combined obesity and diabetes, further elevation of serum levels of vaspin and visfatin in combined pathology might cause a further reduction in hypothalamic Kiss-1 and thereby in LH and testosterone levels and semen quality parameters. Taken together, data from rats with combined obesity and diabetes demonstrate that the combination has a more deleterious effect on male fertility than obesity alone through the reduction of hypothalamic Kiss-1 mRNA, elevation of pituitary NO levels, and elevation of serum vaspin and visfatin levels.

Conflict of interest

The authors have no conflicts of interest to disclose.

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References

[1] C. Marques, M. Meireles, S. Norberto, J. Leite, J. Freitas, D. Pestana, et al., High-fat diet-induced obesity Rat model: a comparison between Wistar and Sprague-Dawley Rat, Adipocyte 5 (2016) 11–21.
[2] R. Buettner, J. Scholmerich, L.C. Bollheimer, High-fat diets: modeling the metabolic disorders of human obesity in rodents, Obesity (Silver Spring) 15 (2007) 798–808.
[3] S. Cabler, A. Agarwal, M. Flint, S.S. du Plessis, Obesity: modern man's fertility nemesis, Asian J. Androl. 12 (2010) 480–489.
[4] J.F. Davis, D.L. Choi, J.D. Schurdak, M.F. Fitzgerald, D.J. Clegg, J.W. Lipton, et al., Leptin regulates energy balance and motivation through action at distinct neural circuits, Biol. Psychiatry 69 (2011) 668–674.
[5] M. Sallmen, D.P. Sandler, J.A. Hoppin, A. Blair, D.D. Baird, Reduced fertility among overweight and obese men, Epidemiology 17 (2006) 520–523.
[6] A. Katib, Mechanisms linking obesity to male infertility, Cent. Eur. J. Urol. 68 (2015) 79–85.
[7] E.C. Tsai, A.M. Matsumoto, W.Y. Fujimoto, E.J. Boyko, Association of bioavailable, free, and total testosterone with insulin resistance: influence of sex hormone-binding globulin and body fat, Diabetes Care 27 (2004) 861–868.
[8] Y.F. Jia, Y. Guo, F. Zhou, K.S. Zhang, X.W. Wang, W.H. Lu, et al., Expression of kisspeptin-GnRH system is down-regulated in hypothalamic arcuate nucleus of male rats with high-fat-diet, Int. J. Clin. Exp. Pathol. 10 (2017) 6099–6107.
[9] M. Kotani, M. Detheux, A. Vandenbogaerde, D. Communi, J.M. Vanderwinden, E. Le Poul, et al., The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54, J. Biol. Chem. 276 (2001) 34631–34636.
[10] M.L. Gottsch, M.J. Cunningham, J.T. Smith, S.M. Popa, B.V. Acohido, W.F. Crowley, et al., A role for kisspeptins in the regulation of gonadotropin secretion in the mouse, Endocrinology 145 (2004) 4073–4077.
[11] H. Matsui, Y. Takatsu, S. Kumano, H. Matsumoto, T. Ohtaki, Peripheral administration of metastin induces marked gonadotropin release and ovulation in the rat, Biochem. Biophys. Res. Commun. 320 (2004) 383–388.
[12] W.S. Dhillo, Kisspeptin: a novel regulator of reproductive function, J. Neuroendocrinol. 20 (2008) 963–970.
[13] H. Migaud, R. Ismail, M. Cowan, A. Davie, Kisspeptin and seasonal control of reproduction in male European sea bass (Dicentrarchus labrax), Gen. Comp. Endocrinol. 179 (2012) 384–399.
[14] A.K. Topaloglu, J.A. Tello, L.D. Kotan, M.N. Ozbek, M.B. Yilmaz, S. Erdogan, et al., Inactivating KISS1 mutation and hypogonadotropic hypogonadism, N. Engl. J. Med. 366 (2012) 629–635.
[15] S.M. McCann, C. Mastronardi, A. Walczewska, S. Karanth, V. Rettori, W.H. Yu, The role of nitric oxide in reproduction, Braz. J. Med. Biol. Res. 32 (1999) 1367–1379.
[16] V.K. Singh, B. Lal, Pro-steroidogenic and pro-spermatogenic actions of nitric oxide (NO) on the catfish, Clarias batrachus: an in vivo study, Gen. Comp. Endocrinol. 242 (2017) 1–10.
[17] S. Thomas, D. Kratzsch, M. Schaab, M. Scholz, S. Grunewald, J. Thiery, et al., Seminal plasma adipokine levels are correlated with functional characteristics of spermatozoa, Fertil. Steril. 99 (2013) 1256–1263, e3.
[18] J. Dupont, X. Pollet-Villard, M. Reverchon, N. Mellouk, R. Levy, Adipokines in human reproduction, Horm. Mol. Biol. Clin. Investig. 24 (2015) 11–24.
[19] M. Caprio, A.M. Isidori, A.R. Carta, C. Moretti, M.L. Dufau, A. Fabbri, Expression of functional leptin receptors in rodent Leydig cells, Endocrinology 140 (1999) 4939–4947.
[20] V.M. Navarro, J.M. Castellano, R. Fernandez-Fernandez, S. Tovar, J. Roa, A. Mayen, et al., Effects of KiSS-1 peptide, the natural ligand of GPR54, on follicle-stimulating hormone secretion in the rat, Endocrinology 146 (2005) 1689–1697.
[21] S. Riammer, A. Garten, M. Schaab, S. Grunewald, W. Kiess, J. Kratzsch, et al., Nicotinamide phosphoribosyltransferase production in human spermatozoa is influenced by maturation stage, Andrology 4 (2016) 1045–1053.
[22] NAC Guide for the Care and Use of Laboratory Animals, 8th ed., National Academy Press, Washington, D.C, 2011.
[23] R. Buettner, K.G. Parhofer, M. Woenckhaus, C.E. Wrede, L.A. Kunz-Schughart, J. Scholmerich, et al., Defining high-fat-diet rat models: metabolic and molecular effects of different fat types, J. Mol. Endocrinol. 36 (2006) 485–501.
[24] M.D. Mythili, R. Vyas, G. Akila, S. Gunasekaran, Effect of streptozotocin on the ultrastructure of rat pancreatic islets, Microsc. Res. Technol. 63 (2004) 274–281.
[25] S.Y. Han, G.A. So, Y.H. Jee, K.H. Han, Y.S. Kang, H.K. Kim, et al., Effect of retinoic acid in experimental diabetic nephropathy, Immunol. Cell Biol. 82 (2004) 568–576.
[26] E.L. Novelli, Y.S. Diniz, C.M. Galhardi, G.M. Ebaid, H.G. Rodrigues, F. Mani, et al., Anthropometrical parameters and markers of obesity in rats, Lab. Anim. 41 (2007) 111–119.
[27] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[28] A.M. Gomaa, N.S. Abou Khalil, M.A. Abdel-Ghani, The protective role of folic acid against testicular dysfunction in lead-intoxicated rat model, Gen. Physiol. Biophys. 36 (2017) 297–308.
[29] Zemjanis collection and evaluation of semen, in: Diagnostic and Therapeutic Techniques in Animal Reproduction, William and Wilkins company, Baltimore, USA, 1977.
[30] N. Pant, S.P. Srivastava, Testicular and spermatotoxic effects of quinalphos in rats, J. Appl. Toxicol. 23 (2003) 271–274.
[31] M.J. Luthfi, A simple and practical method for rat epididymal sperm count (Rattus norvegicus), Biol. Med. Nat. Prod. Chem. 4 (2015) 1–3.
[32] L. Bjorndahl, I. Soderlund, U. Kvist, Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment, Hum. Reprod. 18 (2003) 813–816.
[33] V.M. Navarro, J.M. Castellano, R. Fernandez-Fernandez, M.L. Barreiro, J. Roa, J.E. Sanchez-Criado, et al., Developmental and hormonally regulated messenger ribonucleic acid expression of KiSS-1 and its putative receptor, GPR54, in rat hypothalamus and potent luteinizing hormone-releasing activity of KiSS-1 peptide, Endocrinology 145 (2004) 4565–4574.
[34] WHO, Obesity and Overweight, 2016.
[35] K.P. Phillips, N. Tanphaichitr, Mechanisms of obesity-induced male infertility, Expert Rev. Endocrinol. Metab. 5 (2010) 229–251.
[36] J.M. Castellano, V.M. Navarro, R. Fernandez-Fernandez, J. Roa, E. Vigo, R. Pineda, et al., Expression of hypothalamic KiSS-1 system and rescue of defective gonadotropic responses by kisspeptin in streptozotocin-induced diabetic male rats, Diabetes 55 (2006) 2602–2610.
[37] X.L. Miao, G.M. Gao, L. Jiang, R. Xu, D.P. Wan, Asiatic acid attenuates high-fat diet-induced impaired spermatogenesis, Exp. Ther. Med. 15 (2018) 2397–2403.
[38] L. Navarro-Casado, M.A. Juncos-Tobarra, M. Chafer-Rudilla, L.I. de Onzono, J.A. Blazquez-Cabrera, J.M. Miralles-Garcia, Effect of experimental diabetes and STZ on male fertility capacity. Study in rats, J. Androl. 31 (2010) 584–592.
[39] H. Sebai, S. Selmi, K. Rtibi, N. Gharbi, M. Sakly, Protective Effect of Lavandula stoechas and Rosmarinus officinalis essential oils against reproductive damage and oxidative stress in alloxan-induced diabetic rats, J. Med. Food 18 (2014) 241–249.

[40] W. Fan, Y. Xu, Y. Liu, Z. Zhang, L. Lu, Z. Ding, Obesity or Overweight, a Chronic Inflammatory Status in Male Reproductive System, Leads to Mice and Human Subfertility, Front. Physiol. 8 (2018) 1117.
[41] Z. Heidari, H. Mahmoudzadeh-Sagheb, B. Moudi, A quantitative study of sodium tungstate protective effect on pancreatic beta cells in streptozotocin-induced diabetic rats, Micron 39 (2008) 1300–1305.
[42] F. Khaneshi, O. Nasrolahi, S. Azizi, V. Nejati, Sesame effects on testicular damage in streptozotocin-induced diabetes rats, Avicenna J. Phytomed. 3 (2019) 347–355.
[43] L. Long, H. Qiu, B. Cai, N. Chen, X. Lu, S. Zheng, et al., Hyperglycemia induced testicular damage in type 2 diabetes mellitus rats exhibiting microcirculation impairments associated with vascular endothelial growth factor decreased via PI3K/Akt pathway, Oncotarget 9 (2018) 5321–5336.
[44] J.T. George, R.P. Millar, R.A. Anderson, Hypothesis: kisspeptin mediates male hypogonadism in obesity and type 2 diabetes, Neuroendocrinology 91 (2010) 302–307.
[45] I.J. Clarke, Q. Li, B.A. Henry, R.P. Millar, Continuous kisspeptin restores luteinizing hormone pulsatility following cessation by a neurokinin B antagonist in female sheep, Endocrinology 159 (2018) 639–646.
[46] W.S. Dhillo, O.B. Chaudhri, M. Patterson, E.L. Thompson, K.G. Murphy, M.K. Badman, et al., Kisspeptin-54 stimulates the hypothalamic-pituitary gonadal axis in human males, J. Clin. Endocrinol. Metab. 90 (2005) 6609–6615.
[47] I. Dedes, Kisspeptins and the control of gonadotrophin secretion, Syst. Biol. Reprod. Med. 58 (2012) 121–128.
[48] W.H. Yu, A. Walczewska, S. Karanth, S.M. McCann, Nitric oxide mediates leptin-induced luteinizing hormone-releasing hormone (LHRH) and LHRH and leptin-induced LH release from the pituitary gland, Endocrinology 138 (1997) 5055–5058.
[49] V. Rettori, M. Gimeno, K. Lyson, S.M. McCann, Nitric oxide mediates norepinephrine-induced prostaglandin E2 release from the hypothalamus, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 11543–11546.
[50] B.H. Duvilanski, C. Zambruno, A. Seilicovich, D. Pisera, M. Lasaga, M.C. Diaz, et al., Role of nitric oxide in control of prolactin release by the adenohypophysis, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 170–174.
[51] S. Ceccatelli, A.L. Hulting, X. Zhang, L. Gustafsson, M. Villar, T. Hokfelt, Nitric oxide synthase in the rat anterior pituitary gland and the role of nitric oxide in regulation of luteinizing hormone secretion, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 11292–11296.
[52] P.A. Juliet, T. Hayashi, S. Daigo, H. Matsui-Hirai, A. Miyazaki, A. Fukatsu, et al., Combined effect of testosterone and apocynin on nitric oxide and superoxide production in PMA-differentiated THP-1 cells, Biochim. Biophys. Acta 1693 (2004) 185–191.
[53] J.T. Heiker, N. Kloting, P. Kovacs, E.B. Kuettner, N. Strater, S. Schultz, et al., Vaspin inhibits kallikrein 7 by serpin mechanism, Cell. Mol. Life Sci. 70 (2013) 2569–2583.
[54] A. Fukuhara, M. Matsuda, M. Nishizawa, K. Segawa, M. Tanaka, K. Kishimoto, et al., Visfatin: a protein secreted by visceral fat that mimics the effects of insulin, Science 307 (2005) 426–430.
[55] T.H. Tu, I.S. Nam-Goong, J. Lee, S. Yang, J.G. Kim, Visfatin triggers anorexia and body weight loss through regulating the inflammatory response in the hypothalamic microglia, Mediators Inflamm. 2017 (2017), 1958947.