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Original Article

The Impact of Liraglutide on Urogenital and Reproductive System in Mice

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HIGHLIGHTS

- This study aims to explore the effect of Liraglutide on sperm characteristics, weight, and the histology of the reproductive system in mice with high fat-induced obesity.
- Liraglutide may be able to reverse the effect of a high-fat diet on the total number of sperms.
- Liraglutide can somewhat help improve the DFI of sperm.

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ABSTRACT

Introduction
We aimed to evaluate whether Liraglutide can remove the deteriorating impact of a high-fat diet (HF) diet on the genitourinary system.
Methods
Fifty-six C57 mice were recruited of which received an HF as the intervention group, and a chow diet (CH) as a control group. After two months the case group was sub-grouped into three groups; 1: group HF with a daily injection of 0.4mg/kg liraglutide (HF+Lir), 2: CH with a daily injection of 0.4mg/kg liraglutide (CH+Lir), and 3: HF diet with an infusion of normal saline (HF+NS). After two months, all mice were sacrificed for additional testing.
Results
Serological analysis showed no significant difference for lipid profile (P-value>0.05) and hormones except for PSA in males (P-value=0.019). This significance was seen when comparing CH+Lir and HF+NS to the control group (P-values=0.014 and 0.025, respectively). Liraglutide decreases testosterone in both genders and adiponectin and insulin in males. Total sperm count was decreased, and the difference between the groups was significant (P-value=0.013).
Conclusions
Liraglutide cannot recover the deteriorating impact of an HF diet on sperm morphology and motility but can improve the sperm DFI and the total number of sperm.

Keywords: Liraglutide; Urogenital System; Reproductive System; Sperm Count

Introduction

Obesity is one of the most common problems around the world. Its rate has multiplied by three in the past two decades (1). Obesity increases the risk of cardiovascular diseases such as hypertension and atherosclerosis. It can also lead to type two diabetes, arthritis, sleep apnea, and hypogonadism (2-4). It can lead to infertility via poor oocyte quality and altered endometrial structure by impairing the hypothalamic-pituitary-ovarian axis (HPO) (3). In addition, causing hyperinsulinemia can lead to PCOS (3). Women with BMI ≥25 kg/m2 had significantly higher odds of miscarriage (3). L-cells of the ileum secrete glucagon-like peptide-1 (GLP-1) after food consumption (5). It regulates the activity of pancreatic islets by increasing the concentration of insulin and reducing the concentration of glucagon; thus, it has a vital role in metabolic homeostasis (5). It also slows GI motility, reducing food consumption (5). The anorexigenic effects of GLP1 are mediated via the vagus nerve (5).

Liraglutide is a GLP1 receptor agonist (GLP1RA), a long-acting GLP1 analog with 97% similarity to human GLP1(6). It has a half-life of 13 hours (7). Its effects include increasing glucose-dependent insulin secretion, reducing glucagon secretion, slowing gastric motility, and increasing satiety.

GLP1RAs like Liraglutide can significantly improve glycemic control, body composition, and weight loss, lowering the risk of CVDs (1, 3). Liraglutide can also reduce prediabetes and obesity-related risk factors (1). In patients with PCOS, Liraglutide can lead to weight loss and reduce insulin resistance (3). Thus it can somewhat reverse the effect of PCOS on reproductive organs (8). Liraglutide can increase fertility in females with PCOS and males with obesity-induced hypogonadism (8, 9).

There are many studies on the beneficial effects of Liraglutide in hypogonadism patients of both genders (8, 9). However, there are a few on the impact of Liraglutide on the histology of the reproductive system. We aim to explore Liraglutide's effect on the histology of the reproductive system in high-fat-induced obese mice and explore the effects of a high-fat diet on the morphology, motility, and genetic integrity of sperm and see whether Liraglutide can be beneficial or not.

Methods

Fifty-six three to four weeks of C56BL/6J mice were obtained for this study: 28 males and 28 females. Females and males were separated and put in eight cages with seven mice. We gave them one-week period for acclimatization. The settings were a temperature of 22 ±2 C, relative humidity of 65.45%, and a 12-hour day-night cycle. We gave them a standard CHOW diet (Royan laboratory animal feed, Royan Institute, Iran). We kept the cages in the animal facility at the Urology Research center of Sina Hospital. This study was carried out under approved institutional animal care and used committee protocol (Ethical code: IR.TUMS.SINAHOSPITAL.REC.139 9.014).

Ketamine, xylazine 5-10 mg/kg and acipromazine 1-2 mg/kg were used to anesthetize the rats intraperitoneally in the amount of 75-90 mg/kg of their weight. 5 mg/kg of meloxicam was used to reduce pain, and carbon dioxide box (sweet sleep) was also used for their death. After adapting to the mentioned settings, they were weighed (baseline). Afterward, the control group, seven males and seven females continued receiving the CHOW diet throughout the study. At the same time, we gave the rest of them a high-fat diet (Royan laboratory animal feed, Royan Institute, Iran) to gain weight for eight weeks, and they were all weighed for a second time (weight month 2). Afterward, we divided the mice who gained weighed into three groups (each having seven males and seven females). The first group (HF+Lir) continued having an HF diet with a daily injection of 0.4 mg/kg liraglutide (Novo Nordisk, Denmark). The second group (CH+Lir) switched its diet from HF to CHOW and received a daily injection of 0.4 mg/kg of Liraglutide, the exact dosage of the first group. The final group (HF+NS) continued receiving an HF diet and a daily injection of normal saline. This course was continued for eight weeks. Afterward, they were weighed for the last time (weight month 4) and were sent to a laboratory for anesthesia after taking blood samples. We sacrificed all the mice at the end of the period. Internal organs, including the kidney, urinary bladder, uterus, ovaries, testis, and prostate, were prepared for histopathological examination. We used a 10% neutral-buffered formalin as a fixative for this assessment, and tissue sections were immersed in it. After fixing the tissues, paraffin embedding in paraffin wax and slide preparation were done before H&E (Hematoxylin and Eosin) staining. Then, all tissues were stained with hematoxylin and eosin and were monitored using an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan) in the comparative pathology laboratory at the Urology Research Center, Sina Hospital. We used the Olympus DP12 camera (Olympus Corporation, Tokyo, Japan) to take pictures. Olysia Bioreport imaging software was used for analysis.

We obtained blood samples from each mouse at the end of the study. They fasted overnight for 10-12 hours before we took the samples. Lipid profile was measured using ELISA kits purchased from Biorex Fars, Iran (triacylglycerol cat no. BXC0271, cholesterol cat no. BXC0261, LDL cat no. BXC0431, and HDL cat no. BXC0421). Insulin was measured using RayBio® Human Insulin ELISA Kit (Raybiotech, USA, cat no. ELH-Insulin). All hormones were measured using ELISA kits purchased from MyBioSource, USA (adiponectin cat no. MBS068220, mouse PSA cat. No MBS161513, testosterone cat no. KGE010, mouse prolactin cat no.

DY1445, estradiol cat no. KGE014, mouse insulin cat co. ab2777390, LH cat. No. ab277390, FSH cat no. LS-F9659). For examining the methylation of genes related to bladder cancer, DNA of the bladder tissue samples of mice of all studied groups was extracted by Qiagen Dneasy Blood & Tissue kit (Cat.No. 69504) and then with the help of Qiagen Epitect Bisulfite kit (Cat.No. 59104), the extracted samples were bisulfited. Finally, methylation in MMP14, TERT and RASSF1 genes were evaluated through MSP (Methylation-specific PCR) method. The following table shows the sequence of primers used (Table 1 supplementary).

Statistical analysis

The continuous variables were reported using mean (standard deviation (SD)) or median (interquartile range (IQR)), depending on whether the distribution is concordant with the normal distribution or not, respectively. The repeated measure ANOVA test measures the gradual changes of the response variables over the replications. Furthermore, a non-parametric test for comparing the medians was used in these two scenarios. We also ran a post-doc test for statistically significant parameters. We used Stata 13 (Stata Corp. 2013. Stata Statistical Software: Release 13. College Station, TX: Stata Corp LP) to perform the analyses. We set the statistical significance at 0.05.

DNA Fragmentation index

Sperm samples were taken from the cross-section of the epididymis. We first diluted the sperm to a maximum count of 20 million per milliliter in a sperm extender. We then transferred 50 ml of sperm to an Eppendorf tube and mixed it gently. We then placed 30 microliters of cell suspension onto the center of the sample well and covered the wells with a coverslip. Afterward, slides were placed in the fridge at four°C for five minutes. We removed the coverslips, put the slides into a staining tray, and applied solution A (denaturant agent) to the well. We left the wells for seven minutes for incubation. After removing solution, A, we used solution B (lysis agent) in the well and let it incubate for 15 minutes. After the slides were dried, we washed them for five minutes with abundant distilled water. Afterward, they were dehydrated with ethanol and incubated for two minutes. We then applied solution C (fixing solution) and let it incubate for 75 seconds. After that, the wells were immersed in solution D (staining solution) and incubated for three minutes. After removing solution D, we added solution E (staining solution) to the slides and let it stay for two minutes. Afterward, we removed the excess stain with water and let them dry at room temperature. We visualized the wells using a bright field microscope and the formula below to determine DFI.

SDF=(fragmented+degraded)/(total sperm count) ×100

DIFF-Quick staining protocol

We used DIFF quick kit (Ideh Varzan Farda, Tehran, Iran) for rapid staining of sperm. First, we let the semen liquify, then we made the smear and incubated it for ten minutes to dry. We then applied solution A (fixative solution) to the smear on the slide and incubated it for 75 seconds. After removing solution, A, we used solution B (staining solution) to the smear and let it incubate for 60 seconds. We then added solution C (staining solution) and let it incubate for 15 seconds. Lastly, we washed the slides using distilled water and let them dry. We visualized the slides using a bright field microscope.

Sperm count

We measured the sperm count of each mouse twice during the study, at the baseline (after two months), and the end of the study (after four months). We also measured the number of rapid progressives, slow progressive, non-progressive and immotile sperm for each group.

Results

Serological parameters

We reported each group's lipid profile (Table 1) and hormone panel (Table 2) at the end of the study for both male and female mice. The changes for lipid profile were non-significant (P-value>0.05). The changes in the hormone panel were also non-significant, except for the changes in PSA in male mice (P-value=0.019). Post-Hoc analysis revealed a significant difference between the PSA of CH+Lir vs. control group (P-value=0.025) and HF+NS vs. control group (P-value=0.025). Other comparisons can be seen in Table 2 supplementary.

Weight

We weighed the mice three times (at the baseline, after two months, and at the end of the study), as seen in Table 3. After two months, all the groups in both genders gained weight; however, weight gain was more in groups that received a high-fat diet. After liraglutide administration, we detected a decrease in the weight of all the intervention groups. However, the reduction in the Liraglutide receiving groups was more noticeable. Table 3 shows a significant difference between the groups for both males and females (P-value<0.001). Post hoc analysis showed that in males comparing the weight changes of HF+Lir vs. HF+NS, HF+Lir vs. Control, CH+Lir vs. HF+NS, CH+Lir vs. Control, and HF+NS vs. Control were all significant (P-value<0.001). In females, post hoc analysis revealed that comparing the weight changes of HF+Lir vs. CH+Lir, HF+Lir vs. HF+NS, HF+Lir vs. Control, CH+Lir vs. Control, and HF+NS vs. Control were all significant (P-values<0.05). Post hoc analysis comparisons can be

Table 1. Lipid profile of the mice at the end of the study (median with (IQR)

	Females				P-value	Males				P-value
Group	**HF+Lir (n=3)**	**CH+Lir (n=3)**	**HF+NS (n=2)**	**Control (n=2)**		**HF+Lir (n=2)**	**CH+Lir (n=3)**	**HF+NS (n=2)**	**Control (n=3)**	
TG (mg/ml)	69.5 (59-77.5)	49 (46-59)	76 (61.5-90.5)	65.8 (64.5-67)	0.149	96.7 (61.5-132)	63 (60-64)	82.5 (73-92)	54 (41.5-56)	0.149
CHL (mg/ml)	72.5 (60-81.5)	70.5 (61-71)	73.2 (72.5-74)	65 (61.5-68.5)	0.198	68.7 (52-85.5)	83.5 (83-89)	70.5 (69.5-71.5)	61 (53-61.5)	0.112
HDL (mg/ml)	95.5 (60-117)	110 (90.5-120)	105 (104-106)	86.5 (86-87)	0.198	73.9 (11.8-136)	128 (126-139)	106.5 (106-107)	79 (73.5-104)	0.112
LDL (mg/ml)	11.8 (6.8-13.2)	9.7 (8.3-11.6)	9.5 (9.4-9.5)	7.5 (7.4-7.5)	0.446	5.7 (0-11.4)	9.8 (9.5-11.5)	8.6 (7.9-9.4)	9.6 (9.6-10.9)	0.501

*P-value < 0.05 defines as the significant ones

Table 2. Hormone panel of the mice at the end of the study (median with (IQR))

	Female				P-value	Males				P-value
Group	**HF+Lir (n=3)**	**CH+Lir (n=3)**	**HF+NS (n=2)**	**Control (n=2)**		**HF+Lir (n=2)**	**CH+Lir (n=3)**	**HF+NS (n=2)**	**Control (n=3)**	
PSA (mg/ml)	0.11 (0.09-0.37)	0.07 (0.04-0.15)	0.24 (0.01-0.48)	0.43 (0.42-0.44)	0.446	0.63 (0.62-0.64)	0.71 (0.69-0.74)	0.7 (0.68-0.72)	0.55 (0.47-0.62)	0.019*
Testosterone (mg/ml)	0.51 (0.28-0.87)	0.22 (0.04-0.26)	0.29 (0.18-0.4)	0.625 (0.43-0.82)	0.149	0.765 (0.54-0.99)	2.92 (2.37-4.42)	1.625 (1.42-1.83)	5.56 (3.24-6.49)	0.062
Prolactin (mg/ml)	3.15 (2.84-4.47)	3.43 (3.26-3.88)	4.105 (3.87-4.34)	3.26 (2.51-4.01)	0.446	3.505 (3.28-3.73)	3.43 (2.75-3.67)	2.9 (2.16-3.64)	2.85 (2.36-3.28)	0.383
LH (mg/ml)	1.16 (0.77-1.61)	0.96 (0.75-1.9)	0.84 (0.31-1.37)	0.665 (0.32-1.01)	0.881	0.67 (0.35-0.99)	0.52 (0.47-0.59)	0.655 (0.27-1.04)	0.53 (0.34-0.93)	0.881
FSH (mg/ml)	2.28 (1.42-3.42)	1.57 (0.71-2.14)	2.78 (1.14-4.42)	3.355 (1.71-5)	0.881	4.21 (1.85-6.57)	4 (3.14-4.28)	1.92 (1.42-2.42)	3.71 (2.28-9.14)	0.446
Estradiol (mg/ml)	48 (38-70)	25 (24-64)	20.5 (14-27)	36.5 (35-38)	0.149	30 (13-47)	44 (19-44)	20.5 (17-24)	37 (29-44)	0.446
Adiponectin (mg/ml)	25.3 (24-59.6)	23.7 (19.3-37)	23 (19-27)	35.65 (16.3-55)	0.881	41.3 (21-61.6)	28.3 (17.6-60.6)	39.5 (30-49)	32 (26.6-65)	0.881
Insulin (mg/ml)	4.5 (4.2-4.5)	3.8 (3.7-4.3)	2.4 (0.3-4.5)	3.6 (3.0-4.2)	0.501	3.15 (2.9-3.4)	4.9 (4.6-5)	4.45 (2.7-6.2)	5.8 (5.3-7.1)	0.149

Table 3. Weight Changes (Mean ± SD)

Gender	Group	Weight baseline (gr)	Weight months 2 (gr)	Weight month 4 (gr)	P-value
Females	HF+Lir (n=7)	18.79 ± 1.78	25.07 ± 0.73	20.43 ± 0.45	<0.001*
	CH+Lir (n=7)	18.57 ± 1.27	24.79 ± 1.73	22.71 ± 1.22	
	HF+NS (n=7)	17.57 ± 1.43	23.29 ± 1.11	22.29 ± 0.99	
	Control (n=7)	16.57 ± 1.51	23.00 ± 1.19	26.36 ± 0.85	
Males	HF+Lir (n=7)	19.81 ± 1.02	27.14 ± 1.46	23.43 ± 1.59	<0.001*
	CH+Lir (n=7)	20.14 ± 1.07	27.14 ± 1.07	25.14 ± 0.63	
	HF+NS (n=7)	19.07 ± 1.69	28.86 ± 1.57	28.79 ± 1.41	
	Control (n=7)	18.00 ± 1.15	19.00 ± 0.82	25.79 ± 1.29	

*P-value < 0.05

seen in Table 3a. and 3b supplementary.

Histopathological analysis

Testis

The testes of all groups except the control had interstitial edema. The control group had interstitial tissue hyperplasia seen in Leydig cells (Figure 1).

Ovaries

The control group had atretic follicles. However, the other three groups showed no sign of atretic follicles (AF). Corpus luteum was seen in all groups; however, the HF+Lir group had two corpus locums while others had one. Healthy follicles were seen in all four groups. (Figure 2).

Table 4. DNA Fragmentation Index and Motility of sperms*

Group	DFI	Motility			
		Rapid Progressive	Slow Progressive	Non Progressive	Immotile
HF+Lir	25	3.8	3.5	9.2	84
CH+Lir	7-10	2.7	2.7	7.1	87
HF+NS	20-25	4.1	4.8	12.1	76.7
Control	5	8	3	4	60

Table 5a. Total Sperm Count (median ± Standard deviation)

Group	Baseline sperm count (million/ml)	Final sperm count (million/ml)	P-value
HF+LIR	100.857 ± 23.878	57.286 ± 22.089	0.013*
CH+LIR	200.714 ± 56.305	162.571 ± 57.523	
HF+NS	212.857 ± 54.380	118.571 ± 64.402	
Control	151.714 ± 61.432	122.143 ± 53.841	

*P-value < 0.05

Table 5b. Post-Hoc analysis of Total Sperm Count

Groups	P-value
HF+LIR Vs CH+LIR	0.781
HF+LIR Vs HF+NS	0.009*
HF+LIR Vs CONTROL	0.474
CH+LIR Vs HF+NS	0.004*
CH+LIR Vs CONTROL	0.661
HF+NS Vs CONTROL	0.001*

*P-value < 0.05

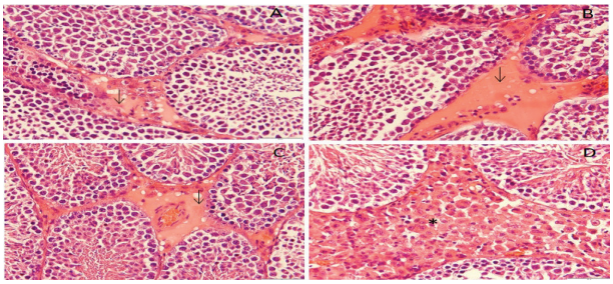

Figure 1. Testis (x400). A. HF+Lir with interstitial edema. B. CH+Lir with interstitial edema C. HF+NS with interstitial edema D. control group with interstitial tissue hyperplasia (Leydig cells). Arrow= interstitial edema. *Leydig cell hyperplasia

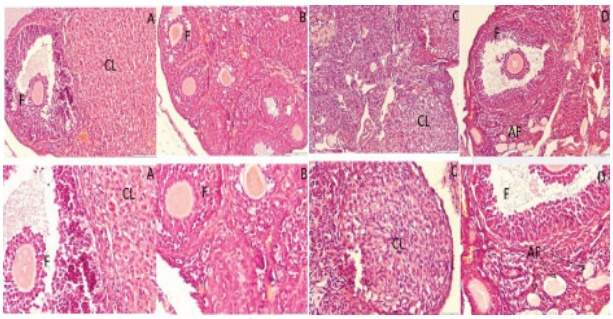

Figure 2. Ovaries (upper x200, lower x400). A. HF+Lir. B. CH+Lir C. HF+NS D. Control group with atretic follicules. CL= corpus loteum. AF=atretic follicules. F=normal follicules

Uterus

The uterus showed no significant changes except for infiltration of mononuclear inflammatory cells in the HF+NS group and dilated tubular glands in the control group (Figure 3).

Prostate

The prostate of all groups was normal and showed no abnormal changes (Figure 4).

Urinary Bladder

In males, the bladders were normal except for congestion seen in CH+Lir and HF+NS. Female urinary bladders showed cholesterol clefts and congestion in all groups (Figures 5a and 5b).

Kidney

In male mice, hypertrophy of the vascular wall, edema, and hyperemia was seen in CH+Lir and HF+NS groups. Accumulation of lymphatic cells was seen in the HF+NS group. HF+Lir group showed tubular necrosis and interstitial nephritis. The control group had congestion and hemorrhage.

In females, hypertrophy of vessel walls was seen only in the CH+Lir group. CH+Lir also had perivascular cuffing and hydropic degeneration. HF+NS showed signs of hyperemia and perivascular coughing. The control group had hydropic degeneration, hyperemia, and atrophic glomerulus. HF+Lir group had no abnormal histological changes.

Sperm morphology

The control group had typical hook shape sperm heads with long tails. HF+NS group showed detached heads with short or broken tails. CH+Lir group had detached heads with bent tails. The last group, being HF+Lir, had detached heads and tails. (Figure 6a. 6b).

DFI and Sperm Motility

The control group had a DFI of five percent. CH+Lir group had a DFI of 7-10 percent, while the other two, HF+Lir and HF+NS, had a DFI of 25 and 20-25 percent, respectively. Sixty percent of sperms in control groups were reported immotile. In HF+Lir and CH+Lir, sperm immotility was reported at 84 and 87 percent, respectively. HF+NS group had an immotility of 76.7 percent. Percent regarding the type of motility and DFI of each group can be seen in Table 4.

Table 6. Rapid-Progressive Sperm count (median ± Standard deviation)

Group	Baseline Rapid progressive count (million/ml)	Final Rapid progressive count (million/ml)	P-value
HF+LIR	5 ± 0	2.286 ± 2.059	0.029
CH+LIR	5 ± 0	2.286 ± 2.059	
HF+NS	5 ± 0	3.857 ± 2.035	
CONTROL	5 ± 0	6.000 ± 3.416	

Table 7. Slow-Progressive Sperm Count (median ± Standard deviation)

Group	Baseline Slow progressive count (million/ml)	Final slow progressive count (million/ml)	P-value
HF+LIR	64.286 ± 6.726	5.000 ± 2.887	0.336
CH+LIR	68.571 ± 7.48	3.000 ± 2.000	
HF+NS	67.857 ± 6.362	5.000 ± 2.708	
CONTROL	63.214 ± 6.244	2.857 ± 1.464	

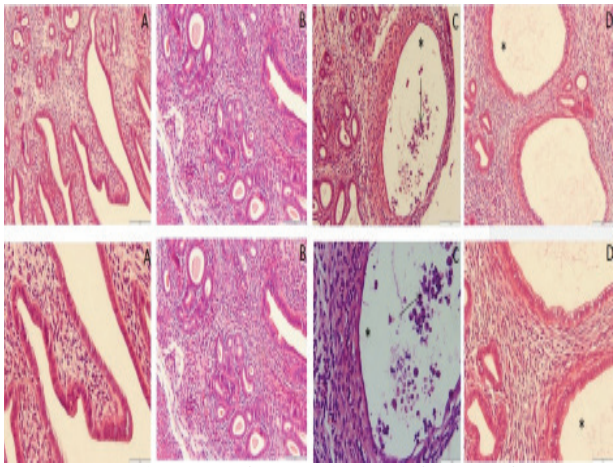

Figure 3. Uterus (upper x200, lower x400). A. HF+Lir B. CH+Lir. C. HF+NS with infiltration of inflammatory cells shown by arrow D. control. *dialated tubular gland

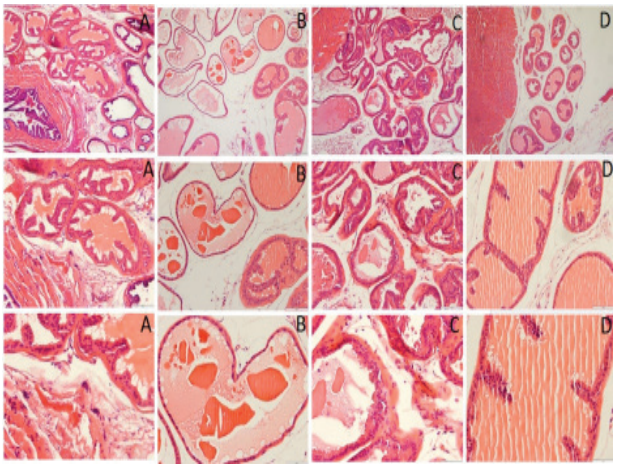

Figure 4. Prostate (upper x100, middle x200, lower x400). A. HF+Lir. B. CH+Lir. C.HF+NS D. control. All groups showed normal histology

Total sperm count

Two months of a high-fat diet raised the total sperm count in two groups (HF+NS and CH+Lir) and lowered it in one (HF+Lir), as seen in Table 5a. Also, at the end of the study, the sperm counts of all the groups decreased. We report a significant difference between the groups (P-value=0.013). This significant difference was seen when comparing sperm count changes of HF+Lir vs. HF+NS (P-value=0.009), CH+Lir vs HF+NS (P-value =0.004) and HF+NS vs Control (P-value=0.001), as seen in Table 5b. Hence, the reduction of sperm counts in HF+NS is significant compared to the other groups. We detected no significant difference comparing Liraglutide receiving groups (Figure 7).

Rapid-progressive sperm count

The rapid progressive sperm count shown in Table 6 indicates a significant difference when comparing the groups (P-value=0.029). All the intervention groups that received a high-fat diet for two months had a reduced number of rapid progressive sperms; however, the control group had an increased number of the mentioned sperms. Further comparison revealed that the significance was caused by CH+Lir vs. Control and HF+Lir vs. control (P-value=0.005), as seen in Table 4 supplementary.

Slow-progressive sperm count

Comparing the count of slow progressive sperm revealed no significant difference among the groups (P-value =0.336). As seen in Table 7, compared to the baseline,

Table 8. Non-Progressive Sperm Count (median ±Standard deviation)

Group	Baseline non-progressive count (million/ml)	Final non-progressive count (million/ml)	P-value
HF+LIR	18.571 ± 4.756	11.429 ± 3.780	0.034
CH+LIR	15.714 ± 4.499	5.143 ± 3.579	
HF+NS	15.714 ± 4.499	12.429 ± 2.507	
Control	17.857 ± 4.88	4.000 ± 1.414	

Table 9. Immotile Sperm Count (median ± Standard deviation)

Group	Baseline Immotile count (million/ml)	Final Immotile count (million/ml)	P-value
HF+LIR	12.143 ± 2.673	80.429 ± 11.603	<0.001
CH+LIR	10.714 ± 3.450	66.429 ± 8.997	
HF+NS	11.429 ± 2.440	67.000 ± 8.583	
Control	13.929 ± 1.967	36.429 ± 9.880	

the number of slow-progressive sperms was lowered in all the groups; however, there was no difference between the reduction of the groups.

Non-progressive sperm count

Comparing the number of non-progressive sperms showed a significant difference (P-value=0.034), as seen in Table 8. Overall, we saw a reduction in non-progressive sperms among all the groups; however, the highest reduction was in the control group. Further analysis (Table 5 Supplementary) revealed that the significant difference was caused by HF+Lir vs Control (P-value=0.023), CH+Lir vs HF+NS (0.014) and HF+NS vs Control (P-value<0.001).

Immotile sperm count

As seen in Table 9, the number of immotile sperms increased. The highest and lowest increase was seen in HF+Lir and Control groups, respectively. The differences between the groups were significant (P-value<0.001). Further investigation revealed that only CH+Lir vs. HF+NS was non-significant (P-value=0.978). Other comparisons are in Table 6 supplementary.

Methylation detection

Although the results of MMP14 methylation were not significant, it can be seen that liraglutide consumption in obese male and female mice can correct MMP14 hypomethylation caused by obesity. Also, the consumption of liraglutide in obese mice leads to an increase in hypomethylation of the TERT gene, although this result is more observed in male mice (P-value=0.03). Regarding the RASSF1 gene, no difference promoter methylation status was observed in the studied groups (Figures 1-4 Supplementary).

Discussion

GLP1 can increase levels of gonadotropin-releasing hormone (GnRH), thus increasing LH and circulating gonadal steroids. Glp1 has neuroendocrine effects, increasing the hypothalamic-pituitary-adrenal axis activity in rodents and humans, regardless of the metabolic state (10). It seems that GLP1 can modify thyrotropin levels. In female rats, GLP1 increases preovulatory LH surge (10). Acute GLP-1 infusion in men resulted in an inhibitory effect on testosterone pulses, making them last longer. Acute intracerebral injection of GLP-1 promoted an immediate increase in preovulatory LH (6). This increase provoked a significant rise in the level of estrogen and progesterone and the number of mature follicles (6). GLP-1 also significantly suppressed progesterone levels with no effect on estrogen synthesis (6). GLP-1 facilitates glucose disposal in an insulin-independent fashion (6). There is evidence suggesting that GLP-1 possesses anti-inflammatory properties (6). Treatment with glp1 doubles the LH surge and results in increased progesterone in the luteal phase, and these changes increase the number of mature Graafian follicles resulting in increased fertility (10). Knockout of GLP1R in mice can lead to fewer ovarian follicles, reduction in the weight of adrenal, testis, and seminal vesicles, and pubertal delay, despite having normal steroid hormones (8, 11, 12).

Many tissues express GLP1R. These include the lung, pancreas, intestines, kidney, heart, testis, ovaries, and smooth muscles of the vessel wall (8, 12-14). It is also expressed in CNS, for example, the hypothalamus, hippocampus, and cortex (10). Liraglutide can directly affect the mentioned organs, such as the reproductive system (12). In our study, Liraglutide did not affect the histological architecture of the prostate or urinary bladder.

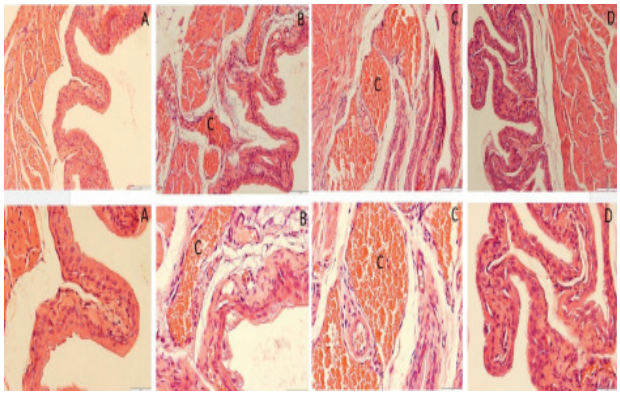

Figure 5a. Male urinary bladder (upper x200, lower x400). A. HF+Lir B. CH+Lir. C. HF+NS. D. control. Normal structure with congestion in HF+NS and CH+Lir

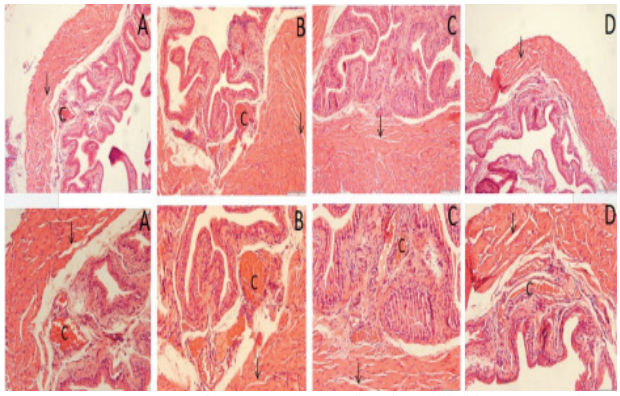

Figure 5b. (upper x100, lower x200). Female urinary bladder. A. HF+Lir. B. CH+Lir C. HF+NS D. Control. Congestion (c) and cholesterol clef (arrow) are shown in all groups

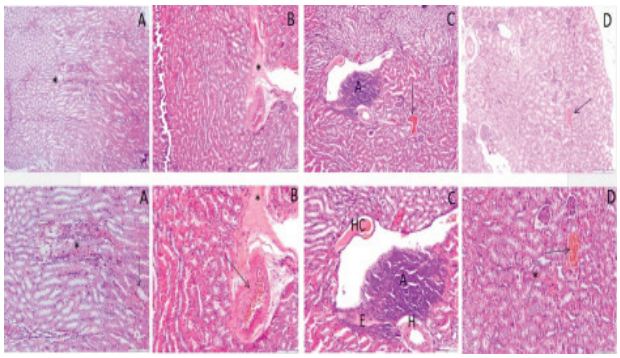

Figure 6a. Male kidney (upper x100, lower x200). A. HF+Lir (* = interstitial nephritis, arrow = tubular necrosis), B. CH+Lir (* = edema, arrow = hyperemia and severe hypertrophy of vascular wall), C. HF+NS (HC = hyaline cyst, E = edema, H = hypertrophy of smooth muscle of vascular wall, A = lymphatic cells, arrow = hyperemia), D. Control (* = hemorrhage, arrow = congestion)

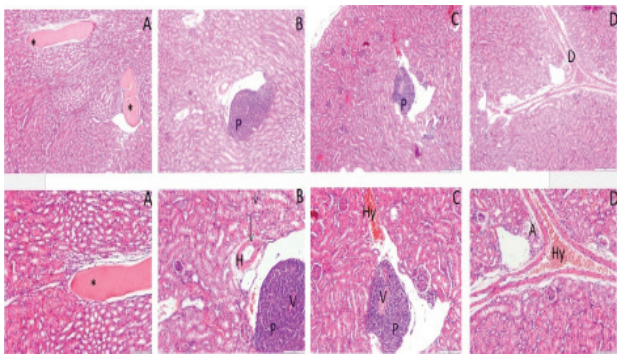

Figure 6b. Female kidney (upper x100, lower x200) A. HF+Lir with a large hyaline cast, B. CH+Lir with perivascular cuffing, hypertrophy of smooth muscle of vascular wall and hydropic degeneration, C. HF+NS with perivascular cuffing and hyperemia D. Control with a dilated vessel, hyperemia, and atrophic glomerulus. *=large hyaline cast, P = perivascular cuffing, H = hypertrophy of smooth muscle of vascular wall, arrow = hydropic degeneration, Hy = hyperemia, D = dilated vessel, A = atrophic glomerulus

This finding may suggest that GLP1R is not expressed in these two organs; however, further studies should be conducted due to changed PSA levels. Expression of GLP1R changes during the estrous cycle in the CNS and ovaries, increasing in the hypothalamus during the proestrus phase (10). Also, during the estrous cycle, responsiveness to GLP1 and GLP1RA, like liraglutide, changes (10).

GLP1R activation can reduce inflammation and fibrosis in organs expressing GLP1R (8). This anti-inflammatory effect can result in weight loss, improved glucose control, targeting immune cells, or targeting GLP1R in specific organs (8). Also, GLP1 could have anti-fibrotic effects in gonads and endometrium as well. Our study suggested that Liraglutide may lead to reduced inflammation because inflammatory changes were not seen in the Liraglutide receiving groups in the uterus.

Infusion of Liraglutide did not affect FSH or FSH's area under the curve (AUC) (15). Chronic infusion of Liraglutide increased the overall testosterone level (15). We did not record the food intake of the mice, but we detected an overall non-significant reduction in FSH and testosterone.

According to Sha et al., who investigated the effect of GLP1 on systemic inflammation in mice, GLP1 can change CD4+ T subsets and levels of cytokines in high-fat included mice (16). They suggested that altering IL17, INF gamma, and IL22 may be responsible for this change (16). They also found that GLP1 can regulate metabolic disorders (16). Decreased secretion of GLP1 may contribute to obesity (16). Interestingly, along with the change of CD4 + T subsets, GLP-1 altered the function of CD4+ CD25+ Foxp3+ T cells as well (16). GLP-1 could regulate the immune subsets independent of the influence of diet (16). These findings may implicate that GLP1-RA, like Liraglutide, may have the same effect on high fat-induced mice. In our study, Liraglutide prevented the infiltration of inflammatory cells in the uterus, partly due

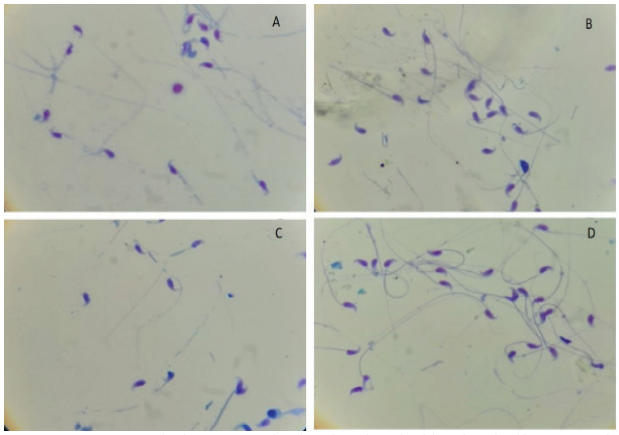

Figure 7. Sperm analysis. A. HF+Lir, showing detached head and tail. B. CH+Lir group, showing detached head and tail with bent tails. C. HF+NS, showing detached head and tail, with short-broken tail D. Control group typical hook shape sperm with intact head and long tail

to its impact on immune cells.

In a meta-analysis by Niafar et al., it was seen that Liraglutide can significantly lower BMI (P-value= 0.0005) and reduce waist circumference non-significantly (P-value=0.142)(17). In another study by Frøssing et al., a significant reduction was seen in the BMI of PCOS patients treated with Liraglutide compared to the placebo group (P-value<0.001) (18). In another study by Jenstrele exploring the effects of Liraglutide on obesity-related functioning hypogonadism, the authors' stated that Liraglutide resulted in a significant decrease in BMI (P-value=0.001). They also noted that Liraglutide could significantly lower BMI compared to testosterone (P-value<0.001).

Regarding the weight changes between different female groups, we can say that in a high-fat diet setting, liraglutide can cause significant weight loss (HF+Lir vs. HF+NS P-value=0.001). The same can also be noted by comparing the control group and HF+Lir group (control vs. HF+Lir, P-value<0.001) and by comparing CH+Lir and the control group (CH+Lir vs. control, P-value<0.001). We also observed that in females, Liraglutide could cause a more significant weight loss in a high-fat diet setting compared to a regular diet (HF+Lir vs. CH+Lir, P-value =0.019). Another thing that we saw was that Liraglutide and the change in diet did not cause a significant reduction in weight (CH+Lir vs. HF+NS, P-value=0.525).

In males, the weight changes revealed that Liraglutide's effect on weight loss was independent of the diet since there was no difference between the weight changes of HF+Lir and CH+Lir (P-value=0.175). In females, we saw that Liraglutide significantly reduced weight in a high-fat diet, and the same was seen in males (HF+Lir vs. HF+NS, P-value<0.001). Also, by comparing CH+Lir and the control group, we can say that liraglutide use combined with a normal diet after a high-fat diet period can significantly reduce the weight compared to the control mice who only received a chow diet (CH+Lir vs. control, P-value<0.001). The same results were seen with HF+Lir and control, meaning that Liraglutide can result in a lesser weight than the control group, even in a high-fat diet setting (P-value<0.001). According to Yan et al., Liraglutide added to metformin significantly reduced body weight, visceral adipose tissue, BMI, and magnetic resonance imaging-estimated proton delay fat fraction (19).

In obese adolescents, a 3.0mg liraglutide with lifestyle changes can significantly reduce body weight and BMI compared to a placebo. Interestingly, the same study observed a more significant increase in BMI in the liraglutide group after discontinuation (20). Weight loss in Liraglutide is dose-dependent 3. 2.4 – 3.0mg of daily Liraglutide can result in a more significant weight loss when compared to orlistat (21).

Liraglutide is contraindicated in patients with a family history of medullary thyroid carcinoma or patients with multiple endocrine neoplasia syndrome 2 (MEN 2). Common side effects of Liraglutide include nausea, diarrhea, vomiting, and other gastrointestinal symptoms. To reduce these side effects, start the treatment with 0.6mg of daily Liraglutide and increase the dose weekly by 0.6mg until it reaches the desired dosage. Acute gall bladder disease risk is related to acute weight loss and is not directly associated with liraglutide (21). According to an RCT by Lundgren et al., combined exercise therapy with Liraglutide is significantly more effective in maintaining weight loss than either method alone (22).

Overall, our results on the effectiveness of Liraglutide on weight reduction were in line with the literature, meaning that the use of Liraglutide can significantly reduce body weight.

It has been said that Liraglutide's impact on weight reduction is independent of food intake (6).

The effect of obesity on sperm quality is controversial in the literature; however, in two studies, it was seen that a high BMI (higher than 30) is significantly associated with lower sperm count, lower progressive and total motility, and a higher percentage of head defects (23, 24). However, high BMI is not associated with impaired DNA fragmentation, pretermination, and apoptosis (24). Higher BMI was, however, associated with increased mitochondrial damage (24).

In another study on mice, it was seen that mice with a high-fat diet have a significantly lower number of sperm with normal morphology than mice with a regular diet (25). Also, switching from a high-fat to a regular diet can significantly lower tail defects (25). On motility, it was seen that a high-fat diet significantly lowers the motility of sperm, and a diet switch cannot reverse this effect (25). Interestingly, no changes were seen when comparing sperm count (25). In a study conducted in

Washington, it was seen that obese men have significantly lower sperm motility (26). Also, an increase in male BMI can significantly lower sperm count and sperm motility (26). According to Demirci et al., obesity can lead to a significant reduction in sperm concentration and motility and an increase in abnormal morphology (27). Also, the testicular structure deteriorated compared to the control group (27). Moreover, GAT1 expression and apoptosis were prominent, and a significant decrease in spermatogenesis quality was seen (27).

Using Liraglutide in otherwise healthy males can inhibit spermatogenesis (28). In one case report, where the patient had diabetes, Liraglutide hindered sperm production (11). Drug withdrawal was able to reverse this effect (11).

According to the world health organization, the total motility (progressive and non-progressive) of sperm should be 40 percent (95% CI 38-42), with progressive motility of 32 percent (95% CI 31-34). In our study, the motility of all groups was lower than the mentioned range. HF+NS had the highest motility among the intervention groups, while the two groups receiving Liraglutide had lower sperm motility. It may be explained by the detrimental effects of Liraglutide on sperm motility in healthy and diabetic mice, as said by Pourheydar et al., (28). DNA fragmentation index or DFI is a crucial measure for assessing male fertility, which shows the damage to sperm's genetic integrity. It is proven to be very useful in male fertility evaluation. Fragmentation of nuclear DNA in sperm depends on many factors, including oxidative stress, radiation, lifestyle, endogenous caspases, and errors in spermiogenesis, like poor chromatin compaction (29). In one study by Li et al., DFI was inversely correlated with total and progressive motility and normal head and tail morphology (29). Also, decreased sperm count is significantly associated with higher DFI (29). An inverse correlation between DFI and fertilization rates was only seen in poor-quality sperms (29). However, according to the Canadian fertility and andrology society, DFI lacks the high-grade evidence needed to be included in practice (30). Liraglutide seems to be able to improve DNA fragmentation in diabetic mice15. Otherwise, in healthy mice, Liraglutide can worsen DNA fragmentation 15. According to Pourheydar et al., 1.8 and 1.2 mg/kg of daily Liraglutide significantly improved chromatin condensation among diabetic mice. DNA integrity was significantly lower in all groups compared to the control group. They stated that the lowest percentage of DNA integrity was seen in the diabetic group, which was significantly improved in the 1.2mg/kg liraglutide receiving group (28). In our study, as expected, the control group had the lowest DFI (5%), highest DFI was reported in groups with a continued high-fat diet (HF+Lir and HF+NS, 25 and 20-25%, respectively). The CH+Lir group showed an improvement in DFI (7-10%). Our results indicate that high-fat-induced obesity can result in increased DFI, and a switch to regular along with the use of Liraglutide can reverse this deteriorating effect. However, comparing HF+Lir and HF+NS, a 0.4mg/kg injection of Liraglutide may be ineffective in restoring DFI in an HF diet setting.

In our study, a high-fat diet reduced the total number of sperms. Also, the reduction of sperms in HF+NS was significant when compared to the other groups (HF+Lir vs. HF+NS (P-value=0.009), CH+Lir vs. HF+NS (P-value =0.004), and HF+NS vs. Control (P-value=0.001), meaning that the reduction of sperms in HF+NS was more significant compared to the control group as well as HF+Lir and CH+Lir groups). There was no significant difference between the total sperm count of Liraglutide receiving groups. It may indicate that Liraglutide may help reverse the detrimental effect of a high-fat diet on total sperm count. A high-fat diet also caused a reduction in the number of rapid progressive sperms.

Furthermore, comparing the groups that received Liraglutide with the control group, there was a significant reduction in the number of rapid progressive sperms. It may indicate that Liraglutide can significantly reduce the number of rapid progressive sperms. We also detected a decrease in the number of slow progressive sperms; however, all these differences were non-significant (P-value=0.336), meaning a high-fat diet with or without Liraglutide does not affect the number of slow progressive sperms.

Comparing the difference in non-progressive sperms, the significance between HF+NS vs. control (P-value<0.001) shows that a high-fat diet may cause a lesser reduction in the number of non-progressive sperms. Liraglutide can help reverse this effect, especially when it is accompanied by a diet switch (CH+Lir vs. HF+NS, P-value=0.014). Thus, Liraglutide can reverse the impact of high fat on the number of non-progressive sperms. We saw a significant increase in immotile sperms among all groups (P-value<0.001). Comparing HF+Lir vs. CH+Lir (P-value=0.014) indicates that a diet switch can cause a significantly less increase in the number of immotile sperms. Comparing HF+Lir vs. HF+NS shows that Liraglutide can dramatically increase the number of immotile sperms in a high-fat diet setting. Lastly, we can conclude that a high-fat diet can significantly increase the number of immotile sperms (HF+NS vs. Control, P-value<0.001).

Based on our findings, a high-fat diet causes ineffective spermatogenesis leading to the detachment of the sperm's head and an abnormal tail. 0.4mg/kg of Liraglutide seems inadequate in restoring the detrimental effect of a high-fat diet on sperm morphology, even if the diet is discontinued. In a case report by Fortuna et al., 0.6mg/kg of daily Liraglutide in a non-diabetic overweight man led to a significant reduction in sperm

count, sperm motility, and sperm morphology (11). Improved DFI is seen when Liraglutide is taken with a regular diet in obese mice. Liraglutide's beneficial effects on spermatogenesis seem to be visible when the patient has morbidities such as obesity with hypogonadism and diabetes. Under physiological conditions, Liraglutide negatively affects the metabolic system of germ cells. Attention to sperm count and motility is warranted when prescribing Liraglutide to young, healthy male patients who wish to reproduce.

Overall, Liraglutide benefits the structure of the kidney in a high-fat diet setting and in diabetic patients. According to Mann et al., Liraglutide has beneficial effects on kidneys, mediated partly by lowering HbA1c, body weight, and systolic blood pressure. Also, GLP1RAs like Liraglutide can directly affect the kidney (31). However, in one case report in 2012, the authors' stated that 1.8 mg of daily Liraglutide could result in acute tubular necrosis, an effect which was reversed after drug withdrawal (32). In diabetic nephropathy, Liraglutide can reduce oxidative stress and decrease albumin secretion and preserve GFR (33). Liraglutide in diabetic nephropathy rats can significantly reduce the 24-hour urine microalbumin content (34). Based on SUSTAIN 6 and LEADER studies, Liraglutide's protective effect is more pronounced in patients with preexisting chronic kidney disease (35). Treatment with Liraglutide in DN kidneys led to a more regular morphology of the glomerulus, and Bowman sac cavity and a reduction in tubular edema (36). HF diet and obesity can lead to tubular hypertrophy and glomerular hypertrophy, thickening of the glomerular basement membrane (13). These changes can lead to albuminuria, glomerulosclerosis, and tubulointerstitial fibrosis (13). Since GLP1R is expressed in proximal tubular cells and smooth muscles of the vascular wall, Liraglutide can impact kidneys. Liraglutide can improve the albumin-to-creatinine ratio (ACR), alleviate kidney weight, and improve the morphology of obesity-induced kidney damage by enhancing the thickened glomerular basement membrane (GBM), glomerulomegaly, and vacuolated tubules. Liraglutide can also decrease the ratio of apoptotic cells in the kidney of mice fed with a high-fat diet (13). In our study, high fat may have led to edema, hyperemia, accumulation of lymphatic cells, hypertrophy of vessel walls, and inflammation around the vessel walls. CH+Lir did not show any sign of inflammation or inflammatory cells, suggesting that a diet switch combined with Liraglutide can be beneficial in preventing inflammation. It seems that Liraglutide could not prevent the hypertrophy of smooth muscles of the vascular wall in a high-fat diet setting. HF+Lir showed tubular necrosis and inflammatory cells. Tubular necrosis was also recorded in a case report (32). Our female control group showed atrophic glomerulus and hydropic degeneration; we cannot explain why these changes occurred. Cignarelli et al., reported that Liraglutide improves erection in diabetes-induced ED and leads to weight loss (12, 37). However, it is still unknown if the same beneficial effects of Liraglutide on the reproductive system can be seen without significant weight loss (15).

In one study comparing the effects of 3 mg of daily Liraglutide vs. testosterone in obese hypogonadism men, the authors concluded that Liraglutide caused a significant increase in LH and FSH (9). It also caused a non-significant increase in total testosterone and SHBG (9). Liraglutide caused a significant increase in libido, morning erections, and ejaculation (9, 38). Liraglutide did not affect FBS, glucose, insulin, OGTT, or total cholesterol; however, it did increase triacylglycerol significantly (9, 39-41). The authors noted that psychosocial factors related to improved body image could be responsible for enhancing sexual symptoms (9). In our study, we did not find any significant effect of Liraglutide on the male reproductive hormones; however, in males, it did seem to have a reductive effect on testosterone levels and an increase in adiponectin. We detected a significant rise in the PSA of CH+Lir and HF+NS compared to the control group. As mentioned earlier, we did not find any effect on cholesterol or insulin.

In a study by saber et al., investigating the effect of Liraglutide on ovarian and uterine tissues in healthy albino rats, liraglutide exposure significantly reduces body, ovary, and uterine weight (5). Liraglutide caused a significant decrease in reproductive female hormone concentration and an increase in testosterone (5). Also, Liraglutide significantly changed the level of some oxidative stress markers, leading to ovarian follicle injury and ovarian follicle atresia (5). Liraglutide reduced the developing follicles in ovarian tissue and increased atretic and disorganized ones (5). An increase in fibrous tissue and apoptotic cells and severe vacuolar changes in granulosa cells were seen as well (5). Liraglutide can also change the expression of progesterone and estrogen receptors in the uterus (5). Liraglutide destroys the luminal epithelium in the uterine tissue, causing edema, vacuolar degeneration, and severe inflammatory cell infiltration. It also caused shrinkage in muscle fibers (5). The changes mentioned were all restored after Liraglutide withdrawal (5). Suppression in follicle development and hormones can explain the weight reduction in ovaries (5). A lack of circulating estrogen can explain the weight reduction in the uterus since Liraglutide inhibits the aromatase activity (5). We conducted our study on mice with high-diet fat, and Liraglutide seemed to be somewhat effective in preventing the detrimental effect of the high diet on uterine tissue, that is, preventing the infiltration of inflammatory cells in the uterus. Our findings on Liraglutide's effect on ovaries may be incidental; therefore, we decided not to make any conclusions. A study by Paschou et al., showed that a daily dose of 1.8mg/kg of Liraglutide in women with

PCOS could lead to weight loss, reduction in NAFLD, fat liver content, and visceral adipose tissue (42).

In PCOS, weight loss can recover folliculogenesis (43). Liraglutide may result in reduced ovarian androgen production (43). Liraglutide resulted in significant suppression of androstenedione and free testosterone and a significant increase of SHBG when prescribed to PCOS patients (8). Two randomized studies reported that glp1ra in the preconception period had higher pregnancy rates after glp1ra withdrawal in women with PCOS (8). GLP1RA could have reverse effects on PCOS morphology and can reduce the levels of androgen (8). Treatment with this class of drugs effectively increases fertility and improves menstrual regularity in obese women with PCOS (8).

According to a meta-analysis by Niafar et al., three months of treatment for women with PCOS resulted in a significant reduction in body mass and testosterone levels (17). Waist circumference, fasting insulin levels, insulin sensitivity, and SHBG did not change significantly (17). One-year liraglutide treatment did not affect substantially hirsutism or menstrual cycles per year (17).

Treatment of PCOS with Liraglutide causes a significant improvement in the social health, physical and psychological components (6). In a randomized clinical trial, Salamun et al., concluded that metformin plus 1.2mg QD s.c. Liraglutide vs. metformin alone was associated with significantly higher cumulative pregnancy and pregnancy per embryo transfer (44). Interestingly, both groups had comparable weight loss (44). Also, There was no difference between the groups comparing the number of retrieved oocytes, the respective number of mature, fertilized, and degenerated oocytes, the number of embryos, and the number of blastocysts on day 5 (44).

In one study investigating the effect of 0.2 mg/kg BD s.c. Liraglutide on PCOS-induced rats, Liraglutide caused a significant reduction in body weight, abdominal adipose tissue, and cholesterol levels (45). They concluded that Liraglutide could improve metabolic perturbations and hypertension in the rat model of PCOS (45). In an RCT by Frøssing et al., where they worked on the effect of 1.8 mg of daily Liraglutide on ectopic fat in women with PCOS, they concluded that this drug significantly reduced liver fat content and weight VAT, and prevalence of NAFLD (18). It also caused a significant increase in SHBG and a non-significant decrease in free testosterone. HBA1C, FBG, and leptin were reduced significantly (18). No changes were seen in eGFR, TAG, total cholesterol, HDL, or LDL (18).

Livadas et al., suggested that Liraglutide could effectively treat women with HAIR-AN syndrome by improving insulin resistance, fat deposition pattern, regulation of menstrual cycles, and hirsutism (7). It also led to spontaneous pregnancy (7).

Our data suggest that Liraglutide does not cause a significant change in hormone levels in females with a high-fat diet; however, it does seem to reduce testosterone, FSH, and adiponectin levels, increasing the level of LH. Overall, it appears that Liraglutide is a promising drug for treating PCOS. It can lead to weight loss and effectively increase fertility and the chance of pregnancy, no matter the method of conception.

Conclusions

We conclude that 0.4mg/kg of Liraglutide seems ineffective in preventing the detrimental effects of a high-fat diet on sperm morphology and motility; however, it seems that it can help restore the genetic integrity of sperm and the total sperm count and it also can have a significant impact on weight reduction. Our histological analysis shows that Liraglutide with this dosage may be able to prevent inflammation, but other than that, our data remain inconclusive.

Authors' contributions

ANSH, ME and NA were responsible for project development and mmanuscript writing, SFH, PZ, LZB, GHM, HH, RR, AKH, RM, and MN data collecting and processing and analysis, JRS and MKH manuscript editing, and ME and MRN was the principal investigator.

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Conflict of interest

All authors declare that there is no conflict of interest.

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There was no funding.

Ethics statement

This study was carried out under approved institutional animal care and used committee protocol (IR.TUMS.SINAHOSPITAL.REC.139 9.014).

Data availability

Data will be provided on request.

Abbreviations

HPO	Hypothalamic-pituitary-ovarian axis
HF	High-fat diet
GnRH	Gonadotropin-releasing hormone

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