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Effect of Ketogenic Diet on the Hypothalamic-Pituitary-Gonadal Axis and Weight loss in induced Metabolic Syndrome Rat mode

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***ABSTRACT*: Background: Metabolic syndrome (MetS) is a metabolic disorderknown for abdominal obesity, hyperglycemia, dyslipidemia, and hypertension and is a condition that is commonly induced in lab animals by high-fat diet (HFD). Hypogonadism, or low total testosterone, has been linked to an increase in the risk of MetS. change. Aim: The purpose of this study was to examine the impact of ketogenic diet (KD) in induced MetS caused by a HFD on the hypothalamic-pituitary-gonadal (HPG) axis and testosterone secretion from Leydig cells in male rats. Materials& methods: For 24 weeks, 40 male rats were divided into four groups: those fed a conventional chow diet (the control group), those provided with KD (KD group), group provided with HFD for 8 weeks (the Mets induced group), and MetS+KD treated group received the diet for 16 weeks. Results: The Mets induced group had lower serum levels of testosterone, FSH, and LH. KD group and MetS+KD treated group restored the levels of HPG. They also both had decreases in body mass index (BMI), while the Mets induced group saw significant increases. Conclusion: Our findings suggest that the KD diet is beneficial for metabolic processes and helps with weight gain and dyslipidemia associated with MetS, also helps in restoring a normal regulation of (HPG) and testosterone secretion.**

***KEYWORDS:* Ketogenic diet; hypothalamic-pituitary-gonadal axis; metabolic syndrome; high-fat diet; Leydig cells.**

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I. INTRODUCTION

Obesity is defined as "an abnormal or excessive accumulation of fat that may impair health" by the World Health Organization. The Western Diet (WD), or modern diet, has exacerbated the problem because it is high in processed foods, sugar, salt, and preservatives. Adipose tissue fat storage is stimulated after WD ingestion because of the increased blood sugar and insulin production. Obesity and overweight develop as a result of adipocyte hypertrophy in the subcutaneous and visceral tissues, brought on by a chronic oversupply of calories. Increases in plasma triacylglycerol (TAG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) have all been linked to obesity. Dyslipidemia, a key risk factor for cardiovascular disease, results from these alterations (Ahmed et al., 2021).

There has been a clear increase in the prevalence of obesity over the past two decades, with estimates ranging from 20% to 30% (Pasquali et al., 2020). Several diseases and conditions, including essential hypertension, type 2 diabetes mellitus, cerebro-cardiovascular disease, obstructive sleep apnea, hypogonadism, polycystic ovary syndrome, and several malignancies (De Lorenzo et al., 2019; Matsubayashi et al., 2022), are frequently co-occurring with obesity. Losing 5–10% of one's body weight is associated with notable improvements in obesity-related comorbidities, according to the research; nonetheless, treating obesity and its comorbidities presents a significant problem for doctors (Jastreboff et al., 2019).

When it comes to reproductive function, the hypothalamic-pituitary-gonadal (HPG) axis is a key player. Hypothalamic gonadotropin-releasing hormone (GnRH) increases anterior pituitary LH production, which in turn increases androgen synthesis and secretion by Leydig cells. The development of sperm and secondary

sexual traits depends on androgen. About 7% of males have infertility, most commonly due to azoospermia or changes in the HPG axis (Gibson et al., 2015).

The MetS-related low androgen may contribute to the development of further illnesses. For instance, hyperlipidemia generated by reduced testosterone levels (Hsia et al., 2022; Van Hulsteijn et al., 2020) may account for the increased risk of cardiovascular mortality seen in male hypogonadism with diabetes (Foresta et al., 2015). Hypogonadism is linked to diabetes and its main complication, chronic kidney disease (Foresta et al., 2015). As a result, it is crucial to investigate diabetes-related reproductive abnormalities as this condition may be linked to the impairment of other bodily functions. Diabetes has been linked to a decrease in male fertility in the past. Additionally, this impact is linked to HPG axis disruption (Dimopoulou et al., 2018).

Animals at research facilities are often induced to acquire MetS by feeding them a high-fat diet. Animals have been artificially fattened by the use of high-fat diets for a long time. Many studies have used high-fat diets, with the percentage of calories from fat ranging from 20% to 60% of the total. Plant-based fats include corn, safflower, and olive oils, whereas animal-based fats include tallow and lard (Fuchs et al., 2018; Wong et al., 2016).

In-depth research was conducted to determine the long-term effects of dietary fats with varying fat contents (10, 32, and 45%) on body adiposity and metabolic rate in rats. Energy intake, weight gain, fat mass, plasma glucose, cholesterol, triglycerides, free fatty acid, leptin, and insulin levels were all found to increase in a dose-dependent manner when dietary fat was increased (Ghibaudi et al., 2002). Another recent study found that compared to mice on a normal diet, high-fat-diet mice were much heavier, had higher plasma lipid levels, plasma insulin, and insulin resistance. The liver's overproduction of triglycerides is facilitated by an increase in very low-density lipoprotein (VLDL) production in response to a high-fat diet (Li et al., 2015).

Changing one's lifestyle (through diet and exercise) is one option, although pharmaceutical intervention and bariatric surgery are also viable options for facilitating weight loss (Castellana et al., 2020). The KDs are an attractive alternative among the several methods available for achieving substantial weight loss (Mongioì et al., 2020; Via & Mechanick, 2023). Academic and public health sectors have taken an interest in KD due to its purported health benefits, such as improved glycemic control and weight loss/maintenance. In addition, they are gaining popularity among health and fitness buffs (Basolo et al., 2022). Because of their shared low carbohydrate content, all KD have the potential to trigger physiological responses similar to those experienced during times of calorie restriction. When the mitochondria in the liver metabolize fatty acids and a few specific amino acids inefficiently or in the absence of insulin, ketones are the end product. Ketogenesis is a metabolic process that uses fat instead of glucose to fuel the body's peripheral tissues (such as the heart, brain, muscles, etc.). It is crucial to understand how a KD impacts hepatic metabolic pathways and how this kind of diet influences exercise adaptations because the liver is vital for both gluconeogenesis and ketogenesis (Huang et al., 2020).

However, the methods through which obesity affects the HPG axis remain unclear. The current investigation set out to determine whether a KD exacerbates metabolic syndrome in rats.

II. MATERIALS AND METHODS

2.1 Diets

The typical KD (SKD) consisted of the following macronutrient: 5% vitamin combination; 5% fibers; 15% protein; 70% fat; 5% carbohydrates. El-Gomhoria Company in Cairo, Egypt supplied the supplies for both diets.

2.2 Ethical approval

Zagazig University's Experimental animal house provided us with 40 male albino adult rats weighing 165-170 g. ZU-IACUC granted approval for this study (approval number: ZU-IACUC/1F/130/2021).

2.3 Animals and study protocol

Ten animals each were randomly assigned to one of four groups. The control group received no treatment and served as a baseline for the other groups. For the Mets induced group, the HFD was maintained for a full eight weeks. For 16 weeks, rats in the KD group were fed an SKD consisting of 70% fat, 15% protein, and only 5% carbohydrates. Before the meals were given to the rats, the animals fasted for 24 hours. In the MetS + KD group, rats developed Mets after 8 weeks of induction similar to group 2, and then they were given SKD similar to group 3 for an additional 16 weeks.

2.4 Sampling and tissue processing

Pentobarbital (100 mg/kg) was used to euthanize the rats, and 0.1M phosphate buffered saline was infused trans-cardiacally. Following collection of blood samples at the time of sacrifice, centrifugation at 3000 rpm for 15 minutes was used to isolate serum, which was then pipetted into dry clean tubes and stored at -20°C until biochemical and hormonal analysis. After carefully removing the skin on the back of the neck, after exposing the abdominal contents, we weighed the testicles and neighboring epididymis, and then tissues were immediately preserved in a buffered solution of 3% formaldehyde (pH 7.4, 48oC) containing 54 mM NaH2PO4 and 28 mM Na2HPO4. We performed histology on these specimens.

2.5 Anthropometric measurements

Body weight, Animal length L and abdominal circumference AC was taken on weekly basis using the electronic weighing scale and the measuring tape. Body mass index were measured three times at baseline, at the middle and at the end of the experiment. The BMI was calculated by dividing the weight (g) by the square length (cm^2) (W/L^2). The length of the rats was measured between nasal and anal region. Following the guidelines laid out by Novelli et al, we determined each animal's body mass and Lee index (Novelli et al., 2007).
BMI = body weight (g) divided by the square of the nose-to-anus length (cm).

2.6 Hormonal immunoassay

We determined the serum levels of testosterone, luteinizing (LH), and follicle stimulating (FSH) hormones using ELISA kits designed for each hormone (Adebayo et al., 2020). Testosterone was measured using the DS-EIA-STERIOD-TESTOSTERONE-RT kit (Interco Diagnostic Ltd, UK), luteinizing hormone (DS-EIA-GONADOTROPIN-LH; Interco Diagnostic Ltd, UK), and follicle-stimulating hormone (DS-EIA-GONADOTROPIN-FSH; Interco Diagnostic Ltd, UK) were utilized. Each kit's testing protocol was carried out in accordance with the accompanying user manual.
In brief, 25 ul of calibrators (serum reference for the hormone at graded concentrations), controls (serum without the hormone), and samples (serum from individual cane rats) were pipetted into Anti-hormone-coated the wells that had been properly labelled. After adding 10 µl of the Conjugate (monoclonal anti-hormone-antibodies conjugated with horse radish peroxidase), mixing for 20-30 seconds by swirling the plate, then covering and incubating at room temperature for 60 minutes, the plates were read. The contents of the microtitre were drained and blotted dry using paper towels. The concentrated Washing Solution was diluted with distilled water at a 1:25 ratio in a separate container, and then 300 µl of the reconstituted washing solution was added to each well before being decanted and blot-dried. After four more washes, 100 µl of TMB-Substrate was pipetted into each well at regular intervals, and the plates were incubated at room temperature in a dark cabinet for 15-20 minutes. After adding 150 µl of the Stopping reagent (0.2M sulphuric acid solution) to each well at predetermined intervals, the wells were examined using an ELISA reader (Elx 800, BioTek, England).
Using a 4-parameter calibrator curve with optic densities/Absorbance on the Y-axis and calibrator concentration on the X-axis, the serum concentration of the hormone in each sample was determined. In accordance with the guidelines provided by the kit manufacturers, all of the test validation criteria for each assay were achieved in this work. After homogenization, coefficients of variation (% CV) were determined from duplicates and from numerous test plates. We then compared these results to the CVs calculated by the kit manufacturer, which were less than 6% intra-assay and 9% inter-assay. were evaluated following the manufacturer's instructions.

2.7 Histopathological investigations

Paraffin-embedded, formaldehyde-fixed testicles and neighboring epididymis sections were cut (3-4 mm thick) and placed on slides that had been treated with saline. After xylol deparaffinization and hematoxylin-eosin staining, histological examinations were done (Bancroft & Gamble, 2008).

2.8 Statistical investigation

The results are shown as a mean ± SD. One-way analysis of variance (ANOVA) was used for the statistical analyses, with Tukey's post hoc test serving as a follow-up. Significant results were found at the p 0.05 level. SPSS was used to run the statistical tests and find the associations (Dawson & Trapp, 2004).

III. RESULTS

Effect of KD on BMI in HFD induced MetS rat model

Anthropometric and body composition measurements at day zero there were non-significant change in BMI in all groups, while within the 13-week study period, rats in the control group and KD group demonstrated significant reductions of BMI (0.690±0.054, 0.618±0.048 versus Mets induced group 0.804 ± 0.079^{a}; $p < 0.005$) and Mets induced group (0.804±0.079) non significantly changes in comparison with MetS+KD group (0.804±0.037). At the 24-week study period the BMI of rats in-between the control group, MetS+KD groups and KD group non-significant to each other, on the other hand BMI at Mets induced group was significantly increased in comparison with other 3 groups. Anthropometric result is presented as mean ± SD in Fig. 1.

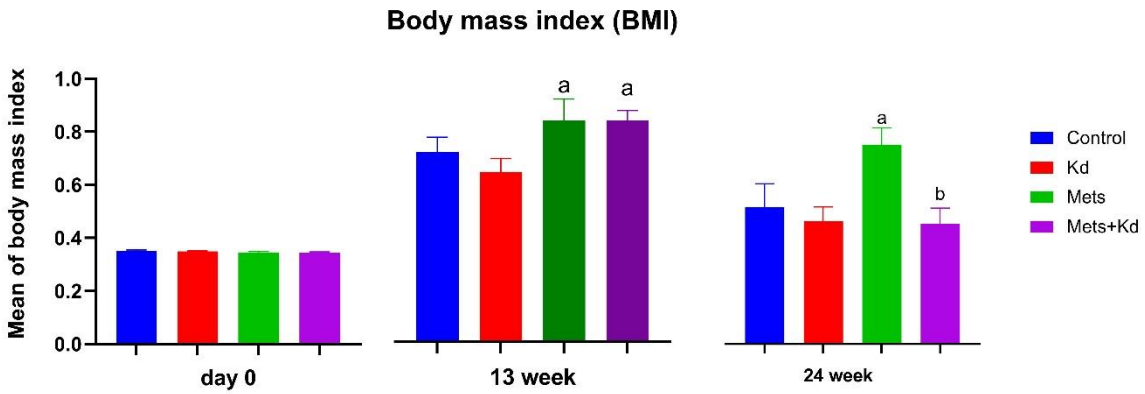

Fig. 1. Effect of KD on BMI in HFD rats at day zero, 13-week and 24-week. Results are presented as Mean ±SEM (n=3); **a**: significant vs control group, **b**: significant vs Mets induced group. P < 0.05 by one-way ANOVA followed by Tukey's post-hoc test.

Effect of KD on the serum levels of hypothalamic-pituitary-gonadal axis in HFD induced MetS rat model

Figure 2 illustrated that the serum levels of FSH (miu/ml), and testosterone (ng/dl) were significantly decreased in Mets induced group compared with the control group, Mets+ KD group and KD group. Control group was non-significant with KD group but significant with Mets+ KD group. While the levels of LH (miu/ml) were increased in control group, Mets+ KD group and KD group compared with Mets induced group.

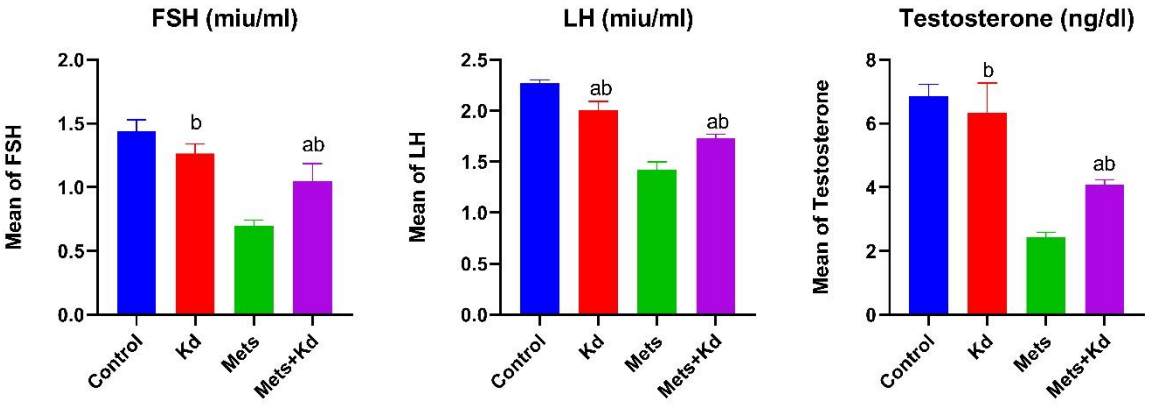

Fig. 2. Effect of KD on the levels of serum FSH (miu/ml), LH (miu/ml) and testosterone(ng/dl) in HFD induced MetS rat model. Results are presented as Mean ±SEM (n=3); **a**: significant vs control group, **b**: significant vs Mets induced group. P < 0.05 by one-way ANOVA followed by Tukey's post-hoc test.

Histopathological results

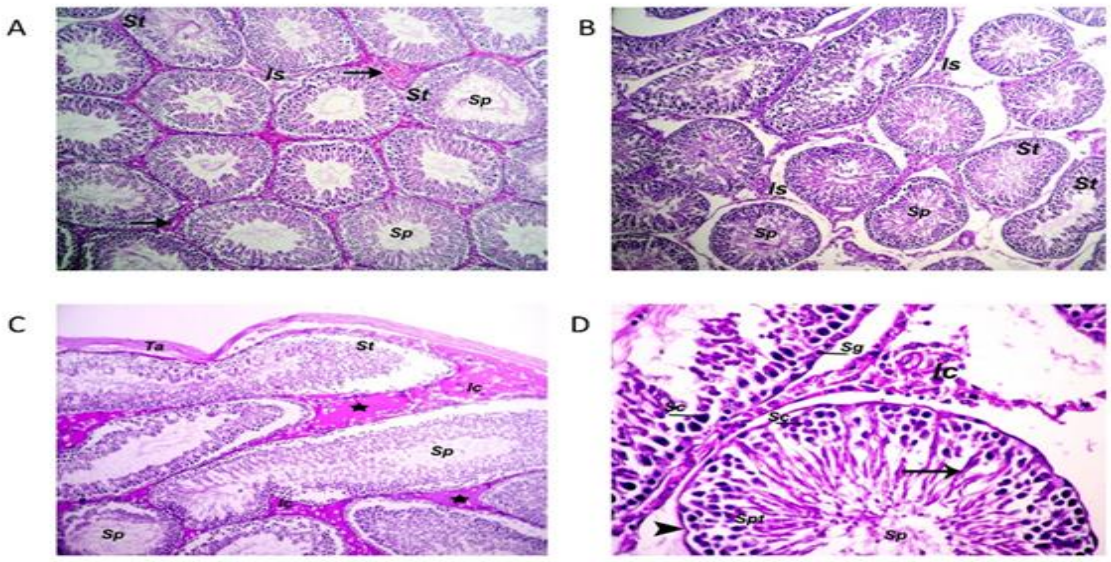

Fig. 3. Histopathological results in testicular tissue. **(A)** testicular tissue of control rat shows normal histological picture, showing closely packed seminiferous tubules (St) and narrow interstitial spaces (Is) with blood capillaries (arrow). Lumens mostly full of sperm (Sp), H&E, X 100. **(B)** testicular tissue of KD group showing seminiferous tubules with regular shapes and structures while, few tubules with irregular shape (St). They are widely separated and wide interstitium with numerous interstitial cells (Ic). Notice, lumens full of sperm (Sp), H&E, X 100. **(C)** testicular tissue of Mets induced rat showing seminiferous tubules (St) with irregular shapes and structures or forms. They are separated, wide interstitium with homogenous acidophilic materials or exudate (star) and few interstitial cells (Ic). Notice, some lumens devoid of sperms (Sp) and thick tunica albuginea (Ta), H&E, X 100. **(D)** testicular tissue of Mets+KD group showing seminiferous tubules with different stages of spermatogenic cells. Spermatogonia (Sg), primary spermatocytes (Sc), spermatids (Spt) and cytoplasmic processes of Sertoli cells (arrow) are observed. A group of interstitial Leydig cells (Ic) in the interstitium and luminal sperms (Sp) can be seen. Notice, still some irregularity in the basement membrane (arrow head), H&E, X 400.

IV. DISCUSSION

The HFD consumption is associated with metabolic syndrome and subsequent diabetes. diabetic testicular disease as metabolic syndrome, is characterized by low testosterone levels and male reproductive system failure in around 25–40% of patients. In this study, we found that FSH, LH and testosterone synthesis are linked to a HFD, an animal model of metabolic syndrome. The BMI has also been decreased by KD treatment. The testicular structure has been enhanced in the groups treated with KD.

Previous research has shown that after 16 weeks on a high-fat diet, metabolic syndrome lowers total plasma testosterone levels **(Roushandeh et al., 2015).** Increased testicular DNA damage and sperm destruction due to alterations in glucose transport and increased oxidative stress **(Aksu et al., 2021; Keyhanmanesh et al., 2019)** are just two of the many ways in which diabetes negatively impacts sperm parameters. Our research also corroborated previous findings that obesity sugar levels reduce reproductive function.

In the high fat diet-related diabetes paradigm, HFD intake is among the most dangerous behaviors **(Bugga et al., 2022)**. Reduced spermatogenesis and testicular diseases may result from prolonged inflammation and energy depletion **(Yildirim et al., 2019).** Rat testes showed a morphological problem and an increase in the rate of apoptosis after being fed fat for eight weeks **(Meydanli et al., 2018).** According to a study, testosterone levels drop and sperm mortality rises. Both the male reproductive system and the regulation of insulin secretion rely on testosterone secretion. High-fructose and fat diets have been linked to insulin secretion disruption, which in turn has been linked to aberrant sperm morphology and functioning **(Tkachenko et al., 2020).**

The HPG axis controls many aspects of male reproduction, including sexual behavior, spermatogenesis, and fertility. Activation of LH receptors, which acts on the cAMP/PKA pathway and raises the expression and activity of steroidogenic enzymes **(Tkachenko et al., 2020),** is a major regulator of testosterone synthesis. Testosterone plays an important role in controlling sexual behavior and increasing muscle mass. Reducing LH secretion by controlling extracellular signal-regulated protein kinases 1 and 2 (ERK 1/2) may account for the increase in LH receptors. Testosterone release in response to hCG was significantly increased in the fructose diet-fed group compared to the control group in the present study. In addition, 8-Br-cAMP or forskolin-enhanced testosterone release, but this effect was not statistically significant. The steroidogenic enzyme activity showed similar patterns. It was found that "when the receptors for LH/hCG are reduced by more than 30% of

their normal levels, the sensitivity of the response to LH/hCG is significantly reduced" **(Menon & Menon, 2014),** which may explain these results. We found that LH and testosterone levels were significantly lower in HFD-induced diabetic rats. On the other hand, the isolated anterior pituitary from the Mets induced group secreted more LH at a basal level. The Leydig cells' response to these upstream hormones also contributed to the dramatic increase. These findings show that diabetes can at least partially eliminate the HPG axis by breaking the negative feedback loop and increasing androgen production.

The modern diet has moved away from a focus on staple foods and towards a greater emphasis on meals derived from animals and vegetable oils. Additionally, a calorically dense dietary pattern has been developed due to rising total food consumption, increased added sugar levels, and increased intakes of fatty food and highly processed food. Previous research has linked obesity with a reduced live birth rate and lower quality sperm. Human studies have shown significant rates of hypercholesterolemia and hypertriglyceridemia in sterile men, and the amount and type of fat consumed is also linked to semen quality indicators. The KD has been shown to be effective for weight loss since the 1960s. Weight loss, serum lipids, and glucose all decreased in previous investigations of the ketogenic diet in obese patients. In addition, losing weight is an effective way for overweight and obese people to lessen DNA damage in their sperm and improve the quality of their sperm **(Liu et al., 2022).**

Males with a higher BMI have been shown to have a different testosterone and estrogen profile, as well as different levels of sex-hormone binding globulin. It has been suggested that the increased adipose tissue present at a higher BMI contributes to these hormonal anomalies by increasing the peripheral conversion of testosterone to estrogen **(Mongioì et al., 2020).** Rapid improvements in testosterone levels due to weight loss are seen in male obese non-diabetic participants with obesity-related hypogonadism after starting KD **(Cignarelli et al., 2023).**The capacity for testicular function is measured using testicular function indices. The results strengthen the case for further research into the potential of a ketogenic diet to improve sperm quality in male rats **(Kayode et al., 2020).** Given the above findings in humans, it is reasonable to assume that the canine rat's observed link between body mass index and HPG is causal **(Adebayo et al., 2020).**

Body weight increase, aberrant relative organ weights, metabolic dysfunction, and liver injury were seen in the diet-induced obesity mice model used in this investigation. Amedium-chain triacylglycerol (MCT)-based ketogenic diet significantly corrected the weight imbalance that developed in HFC-fed mice. The mechanisms discussed include decreased appetite **(Roushandeh et al., 2015)**, increased lipolysis with decreased lipogenesis, increased satiety, and a thermic effect due to protein consumption, all of which are consistent with the results of other studies demonstrating the efficacy of the ketogenic diet. Obesity has been linked, in a number of studies, to decreased sperm quality in men. Semen quality, as measured by total sperm count and total motile sperm count **(Tosi et al., 2017),** is lower in underweight and overweight persons, according to an observational study. A high fat or high cholesterol diet has been linked to negative effects on testicular shape and function. The germinal epithelium thickness decreased, spermatogenesis was hindered, and the quality of the sperm was low in this study due to the effects of HFC consumption on the testes.

However, the ketogenic diet improved sperm motility, sperm percentage with normal morphology, and spermatogenic cell maturation. Male fertility was restored in a mouse model lacking in the leptin receptor, the db/db strain, through the consumption of MCTs. Additionally, ketone bodies can fuel sperm and stimulate sperm motility **(Liu et al., 2022).** In conclusion, the present findings show that a HFD causes metabolic syndrome by eliminating the HPG axis's physiological regulatory role and KD improved this disturbance.

V –CONCLUION

The detrimental effects of obesity on health are exacerbated since the clinical condition has a domino effect on a person's lifestyle choices. KD has the potential to eliminate additional acquired risk factors that are associated to obesity, such as a sedentary lifestyle or depression, because of its greater efficiency in controlling body weight compared to the standard diet. Obese patients frequently have impaired testicular function, which, given its systemic consequences, may constitute yet another factor potential to perpetuate the obesity-related reproductive health crisis. The present study's findings lend support to the idea that KD can be used successfully to combat obesity. BMI showed considerable enhancements. Also, rats with decreased levels of FSH, LH and testosterone showed substantial increases when supplement with KD, indicating that KD could be an effective therapeutic option.

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