

Fatty acid metabolic pathway analysis in epididymal adipose and testis in obese mice

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Abstract: It had been proved obesity was one of the main causes of low fertility or infertility in males. In this paper, the fatty acid content of mice epididymal adipose and testis induced by high fat diet was detected by gas chromatography-hydrogen ion flame detector method. Then, the effects of obesity on fatty acid metabolism of mouse epididymal adipose and testis were analyzed by principal component analysis and T-test combined with KEGG pathway. In this way the mechanism of obesity on reproductive system was expounded from the level of fatty acid metabolism. The results were as follows. After feeding the 45% fat diet for 12 weeks, the body weight, BMI, serum TG and CHOL of high fat diet mice were significantly higher than the control group, which meant high fat diet successfully induced obesity and the obese mice developed metabolic abnormalities. Although the weight and the organ coefficient of epididymal adipose and testis in obese mice did not change significantly, principal component analysis showed that there were significant differences in the fatty acid patterns in epididymal adipose of the two groups. Further data mining revealed that the total fatty acids in the epididymal adipose of obese mice decreased significantly. Not only the saturated and monounsaturated fatty acids synthesized by mice their own decreased significantly, but also the n3n6 series polyunsaturated fatty acids caused by exogenous foods or tissue decomposition reduced obviously, while the n6/n3 ratio remained stable. That indicated the epididymal adipose in mice developed fat remodeling with the occurrence of obesity. The C10:0, C12:0 and C16:1n7 contents in testis in obese mice were significantly increased, which might effect the reproductive function of males.

1. Introduction

Overweight and obese people over the age of 20 accounted for 42.3% in recent China, showing a trend of younger age[1]. Among them 80% were simple obesity induced by diet. Obesity could not only lead to metabolic syndrome such as diabetes, hypertension and cardiovascular disease, but also had adverse effects on reproductive function. At present, the number of infertility couples was about 15%, and male and female factors accounted for half. Obesity could lead to a series of risks such as decreased testosterone levels, impaired sperm production, erectile dysfunction and low libido[2]. Obesity and overweight had become one of the main causes of low fertility or infertility in males, but the specific regulatory mechanism was still not clear. Therefore, in this paper, the fatty acid content of mice epididymal adipose and testis induced by high fat diet was detected by gas chromatography-hydrogen ion flame detector method (GC-FID). Then, the effects of obesity on fatty acid metabolism of mouse testis and epididymal adipose were analyzed by principal component analysis

(PCA)and T-test combined with KEGG pathway. In this way the mechanism of obesity on reproductive system was expounded from the level of fatty acid metabolism.

2. MATERIALS AND METHODS

2.1 Materials and instruments

C57BL/6 male mice aged 4 weeks were purchased from Hunan Silaikejingda Experimental Animal Co., LTD and fed in Hubei food and drug safety evaluation center with the experimental animal license of SCXK(E)2015-0018. Control feed TP23302 and high fat feed TP23000 which contented 45% fat and calories were 4.50kcal/g were bought in Nantong Trophic animal feed High-Tech Co. Ltd.

GC8890-FID/MS equipped with DB-225 capillary column which was used to fatty acid detection were purchased from Agilent Co., USA. Related reagents including acetyl chloride (analytically pure), heptadecane methyl ester (chromatographically pure), and 37 fatty acid methyl esters standard (chromatographically pure),

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purchased from Sigma-Aldrich, USA. Methane, n-hexane, chloroform, potassium carbonate and BHT (analytical pure) were purchased from Sinopharm Group. Pyrex glass centrifugal tubes were purchased from Corning Co., USA.

2.2 Animal Experiments

The animal experiments were performed in accordance with the principles of laboratory animal welfare and ethics. The mice were fed for total 14 weeks, with the first 2 weeks as the adaptation period and the last 12 weeks as the experimental period. They were fed in the SPF experiment platform at a temperature of 20-25°C and a humidity of 40-60%. The light/dark time was rotated every 12 hours, and the animals were free to eat and drink. During the adaptation period, the mice were randomly divided into obesity group induced by high fat diet (HF) and control group (Con), with 8 mice in each group. During the experimental period, HF group was given high-fat diet while Con group control feed. Physiological parameters such as body weight, body length, food intake and water intake were measured weekly during the experimental period. After 12 weeks, the mice were sacrificed. About 60uL orbital serum was taken for blood biochemical. Glucose (GLU), total cholesterol (CHOL), high density lipoprotein (HDL) and triglyceride (TG) were examined by Chemray 800 automatic biochemical analyzer, which were conducted in Wuhan Sevicebio Co., LTD. And the testis and epididymal adipose of mice were weighed and stored in liquid nitrogen for fatty acid detection.

2.3 The processing and detection of fatty acid samples

According to the reference[3], about 25mg of testis and epididymal adipose were weighed. 500uL of chloroform-methanol solution was added, then broken and mixed. After mixing 20uL 1mg/mL internal standard (heptadecane methyl ester), 2mg/mL BHT and 1mL methanol-hexane, 200uL acetyl chloride was added for methyl ester reaction in liquid nitrogen bath. 24h later, 2mL 6% K₂CO₃ was added in the ice bath, then extracted with hexane. The supernatant was dried to be prepare for GC detection.

The qualitative analysis of fatty acid was mainly determined through comparison with gas chromatography-mass spectrometry (GC-MS) database and the retention time of gas chromatography-hydrogen ion flame detector (GC-FID) standard. Fatty acid content was determined by internal standard method. Experimental conditions referred to the literature[3]. The temperature of GC inlet and FID detector were both 250°C. The heating program were as follows. Firstly held 115°C for 0.5min, then raised to 205°C for 3min at the speed of 30°C/min. At last the temperature was heated at 5°C/min to 240°C for 1.5min. The sample volume was 2uL while split ratio was 10:1.

2.4 Analysis and statistics of experimental data

The formula for calculating fatty acid in mice tissue was:

$$Cx(\text{umol/g}) = \frac{100 \times 1000 \times Sx}{S_{17} \times Mx \times mx}$$

where Cx was the content of fatty acid to be measured, Sx was the integral area of corresponding fatty acid methyl ester, S₁₇ was the integral area of the internal standard 17-carbon fatty acid methyl ester, Mx was the molar mass of the fatty acid methyl ester, mx was the mass of the sample. Every fatty acid in each group had five samples, so the fatty acids content was expressed as mean±SD.

Principal component analysis (PCA) of tissue fatty acids was performed by SIMCA13.0, and the data were processed by UV mode. At the same time, SPSS22.0 independent samples T-test was used to test the significant difference between HF and Con groups, with the 5% significance level. In the chart * meant *p*<0.05, ** meant *p*<0.01, and *** meant *p*<0.001. The fatty acid metabolic pathways were based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, and the species was selected as "Mus musculus". In addition, the body weight and BMI between HF and Con group were analysis by two-factor analysis of variance using SPSS 22.0.

3. RESULTS AND ANALYSIS

3.1 Body weight and BMI increased significantly of obese mice induced by high-fat diet

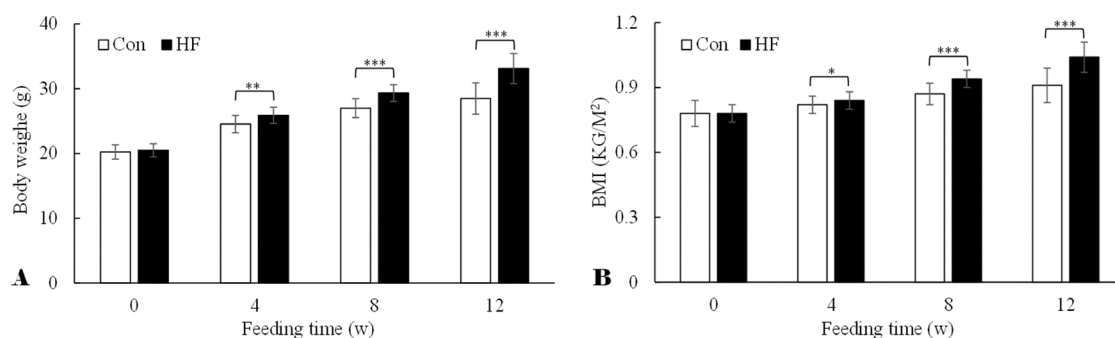


Figure 1: Effect of high-fat diet on body weight (A) and BMI (B) of mice.

There was no statistical difference in water intake and food intake between Con and HF groups at the same time in the experimental period (the data was not given), but the body weight (Figure 1A) and BMI (Figure 1B) of HF group was significantly higher than Con mice from the 4th weeks then on. At the 12th week, the mean weight of HF group was 33.1g, compared with 28.5g in Con group (Figure 1A). By weight and BMI analysis, high-fat diet successfully induced obesity in mice.

3.2 Influence of obesity on blood biochemistry of mice

Table 1: Effect of high-fat diet on blood biochemistry in mice

	Con	HF
GLU (μmol/L)	4.31±0.98	5.01±1.00
TG (μmol/L)	0.45±0.05	0.57±0.05*
CHO (μmol/L)	2.31±0.71	3.83±0.22*

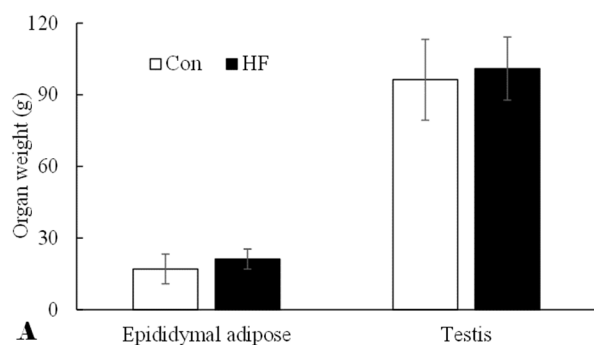


Figure 2: Effects of obesity on epididymal adipose and testis weight (A) and organ coefficient (B) of mice.

As shown in Figure 2, obese mice had slightly heavier epididymal fat and testis than the control group, but there was no statistically significant difference between groups. Their organ coefficient also had no statistically significant difference between two groups. There were literature showed that high fat diet could lead to a significant reduction in the weight and organ coefficients in testis and epididymal of rats[6], and also lead to testicular structural abnormalities, including vacuolation of spermatogenic epithelium and loose intercellular connections[1].

3.4 Effects of obesity on the metabolic pathway of fatty acid in mice epididymal adipose and testis

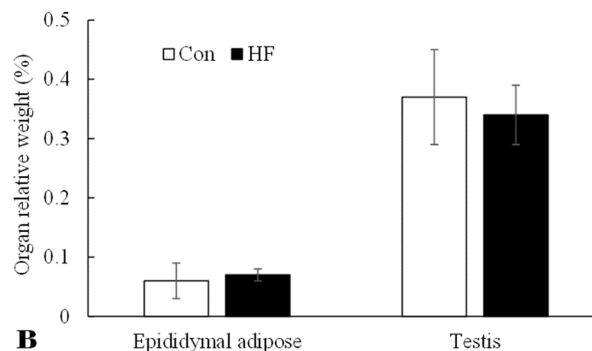
3.4.1 Total ion chromatogram of fatty acids in mice epididymal adipose and testis

By GC-MS/FID detection, 11 kinds of fatty acids were examined in testis of the control mice (Figure 3C). The

HDL (μmol/L)	1.14±0.13	1.84±0.06**
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Blood biochemistry results (Table 1) showed that there was no significant difference in GLU between two mice groups, but TG, CHO and HDL of obese mice were significantly higher than those of control group. It was generally believed that obesity was accompanied by abnormal lipid metabolism in the whole body, which often led to hyperlipidemia. Hyperlipidemia referred to high levels of TG, CHO and LDL and low levels of HDL in the serum[4]. Current studies showed that HDL had a variety of biological functions, such as mediating cholesterol efflux, anti-inflammatory and antithrombus, etc. And its function was affected by its components[5]. In the experiment, the higher content of serum HDL in obese mice was worthy of further study.

3.3 Effects of obesity on the weight of mice epididymal adipose and testis



most abundant fatty acids were C16:0, C18:0, C8:0 and C18:1n9 while there was no n3 polyunsaturated fatty acids detected in the testes of normal mice. What is more, one more fatty acid C18:1n9 was detected in testis of obese mice. Thirteen fatty acids were analyzed in the epididymal adipose of mice (Figure 3B), and C18:1n9 was the most abundant fatty acid, followed by C8:0 and C16:0 in the epididymal adipose. Different from the fatty acid composition of testis, the proportion of C18:0 in epididymal fat was very small, while the content of C18:2n6 was larger, accounting for 14.5% of the total fatty acids.

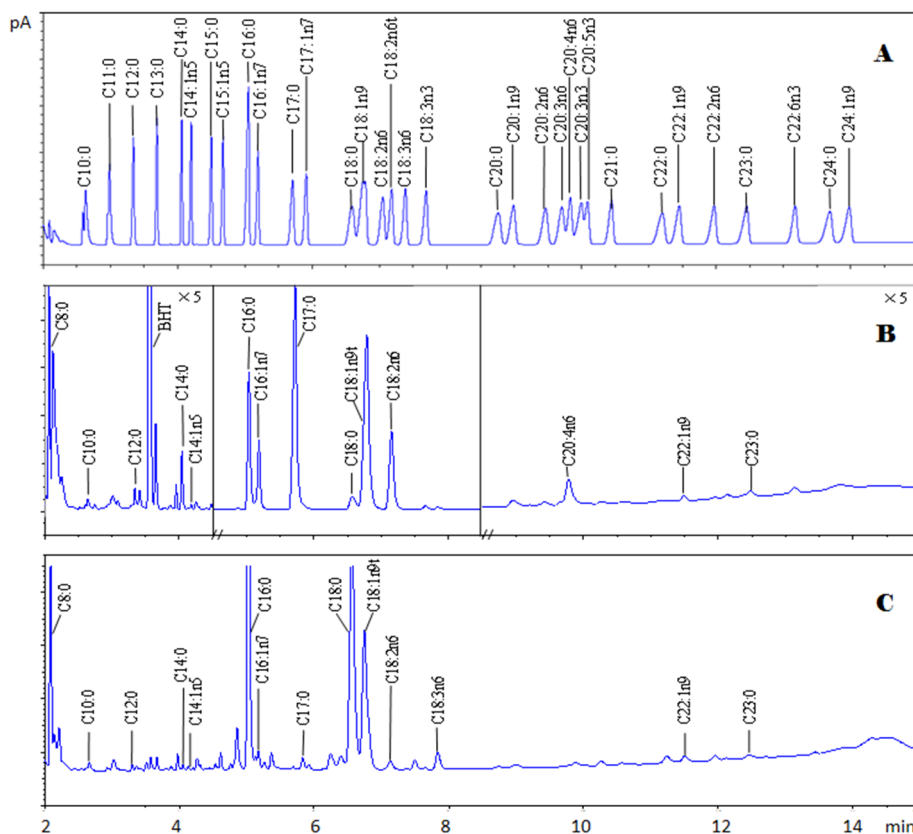


Figure 3: Total ion chromatogram of fatty acid standards (A) and mice epididymal adipose (B) and testis(C).

3.4.2 PCA of fatty acids content in mice epididymal fat and testis

The principal component analysis of fatty acids in mice epididymal adipose and testis was performed by SIMCA13.0, and the results were shown in Figure 4. The fatty acid composition pattern of epididymal fat and testis

was grouped obviously, with the model parameters R2X0.947 and Q20.865. Meanwhile, PCA of epididymal fat in Con and HF groups was shown in Figure 5, and the model parameter were R2X0.681. These indicated that there was significantly difference between the fatty acid composition of epididymal fat epididymal fat in Con and HF groups, which was worth further data mining and in-depth analysis.

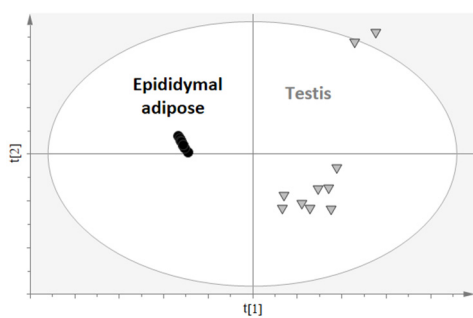


Figure 4:PCA of fatty acids in mice epididymal adipose and testis .

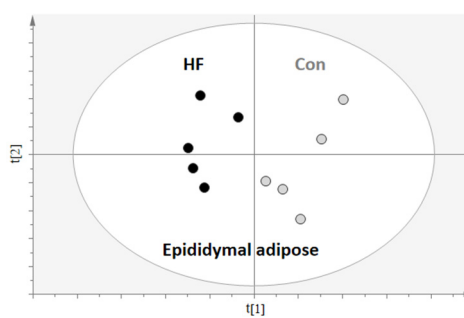


Figure 5:PCA of epididymal adipose in Con and HF groups.

3.4.3 Effects of obesity on fatty acids in epididymal adipose and testis of mice

Table 2: Fatty acid content in epididymal adipose and testis of mice

Retention time (min)	Fatty acid	Testis (umol/g wet weight)		Epididymal adipose (umol/gwet weight)	
		Con	HF	Con	HF
1.98	C8:0	14.34±2.72	15.37±1.59	27.62±10.34	22.63±2.41
2.64	C10:0	0.16±0.03	0.26±0.07*	2.13±0.34	1.03±0.26***
3.34	C12:0	0.15±0.04	0.39±0.19*	1.13±0.37	1.30±0.21
4.06	C14:0	0.23±0.07	0.23±0.06	2.01±0.53	1.14±0.34*
4.20	C14:1n5	0.06±0.01	0.06±0.01	0.16±0.02	0.27±0.08*

5.05	C16:0	24.97±3.59	30.15±3.89	37.18±8.32	20.78±4.52**
5.20	C16:1n7	0.97±0.33	1.83±0.44**	13.08±3.38	5.37±1.73**
6.60	C18:0	19.55±3.36	21.59±3.65	6.90±0.79	6.64±1.63
6.80	C18:1n9t	12.79±3.58	16.91±5.78	69.04±16.90	40.89±13.44*
7.18	C18:2n6	1.86±0.8	2.93±1.33	28.22±5.31	14.71±4.86**
7.39	C18:3n6	ND	0.20±0.03	ND	ND
7.70	C18:3n3	ND	ND	1.52±0.46	0.81±0.14*
9.83	C20:4n6	ND	ND	3.31±0.98	2.83±0.37
11.50	C22:1n9	0.49±0.1	0.53±0.05	0.88±0.19	0.69±0.12
Total fatty acids		75.55±13.17	89.93±11.33	193.18±40.79	119.10±26.66**
Saturated fatty acids		59.39±8.90	67.99±7.31	76.97±17.21	53.51±6.73*
Unsaturated fatty acids		16.16±4.78	21.95±7.26	116.21±24.48	65.58±20.00**
Monounsaturated fatty acids		14.30±3.98	19.32±5.97	83.16±19.30	47.22±14.74*
Polyunsaturated fatty acids		1.86±0.80	2.62±1.54	33.05±5.33	18.36±5.29**
n3 polyunsaturated fatty acids		ND	ND	1.52±0.46	0.81±0.14*
n6 polyunsaturated fatty acids		1.86±0.80	2.62±1.54	31.53±5.39	17.55±5.17**
n6/n3		ND	ND	22.13±6.24	21.48±3.35

ND meant not detected in the sample. n6/n3, the ratio of n6 series to n3 series polyunsaturated fatty acids.

C10:0, C12:0 and C16:1n7 levels were significantly increased in the testes of obese mice, and C18:3n6 were also present in the testes of obese mice induced by high fat diet (Table 2). Obesity was always thought to cause a decline in male reproductive function by inducing oxidative stress, inflammation and apoptosis in the testis[7]. It had been reported that free saturated fatty acids could cause apoptosis of rat testicular cells cultured in vitro, and the mechanism might be through stimulating the production of sphingosphain, increasing the production of nitric oxide, up-regulating the expression of apoptotic gene Bax and inhibiting the decrease of apoptotic gene Bcl-2[8].

The content of total fatty acids in epididymal adipose in HF group decreased significantly, including C10:0, C14:0, C14:1n5, C16:0, C16:1n7, C18:1n9, C18:2n6 and C18:3n3 (Table 2). But the ratio of n6/n3 did not change. Recent literature showed that white adipose tissues displayed a remarkable ability to adapt to the dietary status. Intake of surplus metabolic energy might lead to obesity, involving both an increase in the size of

adipocytes as well as an increase in de novo differentiation of adipocyte progenitor cells. In addition, obesity led to compositional and phenotypical changes of other cell types in white adipose tissues[9]. Mouse epididymal adipose had five kinds of cells, including immune cells, endothelial cells, mesothelial cells, fibr-adipogenic progenitors and adipocytes. The proportion of immune cells increased significantly while adipocytes decreased significantly in obese mice. Adipocytes could be divided into three distinct subcell groups (lipogenic adipocytes, lipid-scavenging adipocytes, stressed lipid-scavenging adipocytes). Obesity induced by a high fat diet resulted in the near disappearance of lipid-scavenging adipocytes, while the other two subcells were significantly upregulated, accompanied by a significant increase in the expression of stress kinase signaling and immune response-related genes[10].

3.4.4 Effects of obesity on fatty acid metabolic pathway in mice epididymal adipose

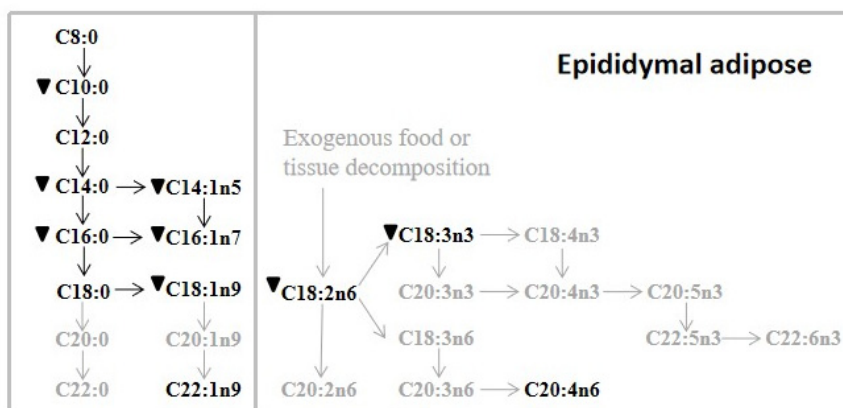


Figure 6. Effect of obesity on fatty acid metabolic pathway of mice epididymal adipose

Note: ▼ indicated a significant decrease in fatty acid content in HF group, while ▲ indicated increasing.

As shown in Figure 6, combined with KEGG fatty acid metabolic pathway in mouse, the total fatty acids per unit wet weight of epididymal adipose in obese mice were significantly decreased, and the synthesized saturated fatty acids (C10:0, C14:0, C16:0) and monounsaturated

fatty acids (C14:1n5, C16:1n7, C18:1n9) were significantly decreased. In addition, n3n6 series polyunsaturated fatty acids (C18:2n6, C18:3n3) caused by exogenous food or tissue decomposition also decreased significantly, but the n6/n3 ratio remained stable. This

indicates tissue remodeling in epididymal fat associated with obesity induced by high fat diet.

4. CONCLUSION

After feeding the 45% fat diet for 12 weeks, the body weight, BMI, serum TG and CHOL of HF group were significantly higher than Con group, which meant high fat diet successfully induced obesity and the obese mice developed metabolic abnormalities. Although the weight and the organ coefficient of epididymal adipose and testis in obese mice did not change significantly, principal component analysis showed that there were significant differences in the fatty acid patterns in mouse epididymal adipose between HF and Con group. Further data mining revealed that the total fatty acids in the epididymal adipose of obese mice decreased significantly. Not only the saturated and monounsaturated fatty acids synthesized by mice their own decreased significantly, but also the n3/n6 series polyunsaturated fatty acids caused by exogenous foods or tissue decomposition reduced obviously, while the n6/n3 ratio remained stable. That indicated the epididymal adipose in mice developed fat remodeling with the occurrence of obesity. The C10:0, C12:0 and C16:1/n7 contents in testis in obese mice were significantly increased, which might effect the reproductive function of males.

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REFERENCES

1. Weiwei Jiao, Yan Lu, Wenran Qiu, Dongdong Lu, Jie Zeng and Chuanhang Wang. 2021. Research progress of the effects of obesity on male reproductive function. *World Journal of Integrated Chinese and Western Medicine*,16(03):585-588.DOI:10.13935/j.cnki.sjzx.210338.
2. Aiping Zhang, Yonglu Hao, Yanchao Ma, Pengcheng Ma and Yiqing Wang. 2021. Research Progress on the Influence of Obesity on male reproduction. *Journal of Chongqing Medical University*, 46(04):439-443. DOI:10.13406/j.cnki.cyx.002772.
3. Huihui Li, Jing Wang, Yun Zhang, Hongwei Sui and Munong Xu. 2017. Determination of Fatty Acids in Pork and Beef Soup and Broth Foam. *Food Technology*, 42(10):128-132.DOI:10.13684/j.cnki.spkj.2017.10.026.
4. Jun Tao and Yan Li. 2022. Research progress of testosterone and hyperlipidemia, energy metabolism disorder and obesity. *Journal of Microcirculation*, 32(01):76-79+86.
5. Can Xu, Duo Gong, Changhui Liu and Chaoke Tang. 2022. Recent advances in the study of high density lipoprotein function. *Advances in Physiological Science*, 53(05):363-367.
6. Erhui Wang, Shi Guo, Yanbei Huo, Jingyan Xi and Haiyang Liang.2018. Research Progress of nutritional obesity on male reproductive function. *Journal of Xinxiang Medical College*,35(10):940-942. DOI:10.7683/xyxyxb.2018.10.024.
7. Joseph Bagi Suleiman, Victor UdoNna, ZaidaZakaria, Zaidatul AkmalOthman, Ainul Bahiyah AbuBakar and MahaneemMohamed. 2020.Obesity-induced testicular oxidative stress, inflammation and apoptosis: Protective and therapeutic effects of orlistat. *Reproductive Toxicology*, 96:113-122. <https://doi.org/10.1016/j.reprotox.2020.05.009>.
8. Chaohui Lv, Yiming Mu, Baoan Wang, Hua Zhen, Juming Lu, Jiangyuan Li and Changyu Pan. 2002. Free saturated fatty acids induced apoptosis of rat testicular Leydig cells. *Chinese Journal of Endocrinology and Metabolism*, (06):63-65. DOI:10.3760/j.issn:1000-6699.2002.06.024.
9. Anitta Kinga Sárvári, Elvira Laila Van Hauwaert, Lasse Kruse Markussen, Ellen Gammelmark 1, Ann-Britt Marcher, Morten Frenedo Ebbesen, Ronni Nielsen, Jonathan Richard Brewer, Jesper Grud Skat Madsen and Susanne Mandrup. 2021. Plasticity of Epididymal Adipose Tissue in Response to Diet-Induced Obesity at Single-Nucleus Resolution. *Cell Metabolism*, 33(2): 437-453e5. <https://doi.org/10.1016/j.cmet.2020.12.004>.
10. AK Sárvári, Hauwaert E , Markussen L K , et al. 2020. Plasticity of Epididymal Adipose Tissue in Response to Diet-Induced Obesity at Single-Nucleus Resolution[J]. *Cell Metabolism*, 33(2). <https://doi.org/10.1016/j.cmet.2020.12.004>.