Serum 25OHD₃ of Obese Mice Is Affected by Liver Injury and Correlates with Testosterone Levels and Sperm Motility

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Keywords

Obesity · Vitamin D · Testosterone · Male reproduction

Abstract

Introduction: The concentration of 25-hydroxycholecalciferol (25OHD₃) in the serum of obese people is low and often accompanied by symptoms of low fertility. Therefore, vitamin D is recommended as a potential treatment option. However, after clinical trials, it was found that vitamin D cannot effectively increase the concentration of 25OHD₃ in the serum of obese people. How obesity causes low 25OHD₃ concentration and low fertility is unclear. **Methods:** We analyzed the physiological and pathological changes in obese mice induced by a high-fat diet (HFD) and the changes in mice after supplementing with 25OHD₃. **Results:** The concentration of 25OHD₃ in the serum of obese mice induced by HFD was significantly reduced, and these mice showed liver hypertrophy accompanied by abnormal liver injury, testicular hypertrophy, low testosterone levels, high leptin levels, and low sperm motility. The mRNA and protein expression of CYP2R1 of hydroxylated vitamin D₃ was significantly reduced; CYP11A1 and CYP11A2, which synthesize testosterone, were significantly reduced. After supplementing with

25OHD₃, there was an increase in serum 25OHD₃ concentration, testosterone level, and sperm motility, but it cannot improve the degree of obesity, CYP2R1 expression, and liver damage. **Conclusion:** Our research shows that there is a metabolic interference mediated by 25OHD₃ and testosterone between obesity and low sperm motility. The results of this study can provide a scientific basis for studying the mechanism of 25OHD₃ and hormone regulation and treating obese people with low sperm motility.

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Introduction

Obesity has become a global epidemic, and treatment is a considerable financial burden. The estimated annual medical cost of obesity in the USA was USD 147 billion in 2008; the medical cost for people who have obesity was USD 1,429, higher than that of normal weight [1]. Since 1999, the National Health and Nutrition Examination Survey has tracked the prevalence of obesity. From 1999–2000 through 2017–2018, the prevalence of obesity increased from 30.5% to 42.4%, and the prevalence of severe obesity increased from 4.7% to 9.2%. Be-

sides, obesity seems to occur easily in the adult population. The prevalence of obesity was 40.0% among young adults aged 20–39 years, 44.8% among middle-aged adults aged 40–59 years, and 42.8% among older adults aged 60 [2]. Obesity is not only associated with increased risk of diabetes, cardiovascular disease, cancer, and all-cause mortality but also with infertility [3, 4]. Several studies that occurred concurrently with the obesity epidemic showed that the quality of semen declined over time [5]. And, the increase in obesity rates parallels reports of poor incidence of sperm quality and male infertility [6, 7]. Male infertility rate accounts for 45–50% of infertile couples [8], and there is increasing evidence that male infertility is associated with obesity. Obesity may be responsible for this trend because it negatively affects reproductive injury through a variety of mechanisms, including hormonal disturbances, increased levels of inflammatory mediators and reactive oxygen species, and increased testicular calories. These accumulations may have a substantial harmful effect on spermatogenesis.

Vitamin D is a steroid hormone that is essential for maintaining calcium intake and bone metabolism [9]. Vitamin D deficiency (25OHD₃ below 20 ng/mL) is increasingly considered a public health problem [10, 11]. It is increasingly recognized that vitamin D itself is inactive. Vitamin D is converted to 25OHD₃ by cytochrome P450 (CYP) 2R1 and then converted to the active form of vitamin D by CYP27B1, 1,25(OH)2D₃ [12]. The role of vitamin D is mediated by binding the vitamin D receptor (VDR). Some recent studies have reported that the concentration of 25OHD₃ in obese people of different ages in many countries is significantly lower [13–15]; interestingly, the low concentration of 25OHD₃ or its derivatives showed a significant positive correlation with the quality of sperm [16, 17]. Several studies have shown that ingredients that mediate vitamin D activity, such as VDR, CYP24A1, CYP2R1, and CYP27B1, are highly expressed in the testis, epididymis, seminal vesicles, prostate, and sperm [18, 19]. It shows that vitamin D is important for sperm formation and sperm maturation. However, whether vitamin D deficiency can impair spermatogenesis and steroid production is controversial. Since obesity and 25OHD₃ deficiency are strongly correlated with male infertility, and the concentration of 25OHD₃ in obese people is found to be significantly lower, the purpose of this study is to use obese mouse models to study the correlation of 25OHD₃, physiological and pathological changes caused by obesity, and the motility of sperm.

Methods and Materials

Animal and Experimental Diets

A total of 80 Kunming mice about 2 weeks old were purchased from the Guangdong Experimental Animal Center. Upon arrival, all mice were fed normal-fat diet (NFD) and tap water freely and were housed in groups of 3 or 4 in an environmentally controlled room (19–26°C; relative humidity 40–70%; light and dark 12 h). All procedures have been approved by the Animal Care and Use Committee. After 1 week of acclimatization, mice with matching weights were divided into 4 groups and fed on one of the 4 diets. NFD group: NFD fed to 20 animals contained 20% calories from protein, 70% calories from carbohydrates, and 10% calories from fat (see online suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000518199); HFD group: 20 animals were fed 45% HFD (20% protein, 30% carbohydrate, and 50% fat); HFD+ group: 20 animals were fed 45% HFD (20% protein, 30% carbohydrate, 50% fat, and 20 ng/day 25OHD₃); and HFD++ group: 20 animals were fed 45% HFD (20% protein, 30% carbohydrate, 50% fat, and 40 ng/day 25OHD₃). According to the recommendations of the National Research Council, the control group's diet contained sufficient dietary calcium (5.5 g/kg diet) and vitamin D₃ (1,000 IU/kg diet). Mice were fed ad libitum throughout the study, and food intake and bodyweight were recorded twice a week. After 10 weeks, food was taken about 16 h before decapitation of 15 mice in each group. Trunk blood was quickly collected; liver, kidney, and testis were removed to weigh; and horsetail epididymis was collected for sperm analysis and then frozen in liquid N₂.

In the study, mice were monitored at least twice a day. Besides, we strictly enforced the humane endpoint rules to determine when the mice should be euthanized. When the mice reach our euthanasia standards, every effort was made to minimize suffering.

Serum and Plasma Analyses

Serum from treatment or control mice was collected and stored at –80°C until analysis. The radioimmunoassay (RIA) kit (DiaSorin Inc., Stillwater, MN, USA) was used to measure serum 25OHD₃ concentration. The detection limit of the 25OHD₃ RIA kit is 1.5–100 ng/mL. In this study, all values were within the detection limit.

Serum leptin levels were measured with the Mouse/Rat Leptin Quantikine ELISA Kit (R&D Systems). A competitive inhibition enzyme immunoassay was used to determine the levels of serum LH and FSH according to the manufacturer's protocol. All values fell within the detection limits.

Steroids were solubilized in PBS. Serum and testicular T concentrations were measured using ¹²⁵I-based T RIA kits according to the manufacturer's protocol. The T RIA kits' detection limit was from 10 to 800 pg/mL. In the present study, all values fell within the detection limits.

According to the manufacturer's instructions, serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST) were measured by using ALT (C009-2-1) and AST (C010-2-1) detection kits. AST and ALT levels were used to measure liver injury (Jiancheng, Nanjing, China).

Sperm Analysis

The cauda epididymides separated from mice of each group were cut in prewarmed (37°C) Tyrode's buffer (Sigma-Aldrich, USA) and then placed in a 5% carbon dioxide incubator. After 15-min incubation, sperm motility, progressive motility, and concentration were

Fig. 1. Changes in bodyweight and physical and chemical properties of obese mice induced by HFD. Bodyweight (a), food intake (b), weight gain (c), liver weight (d), liver injury (e), and testis weight (f). Data represent mean $\pm$ SEM derived from $n = 20$ for each group. *Significant differences ($p < 0.05$); **significant differences ($p < 0.01$, 2-way ANOVA with Holm-Sidak post hoc test). NFD, normal-fat diet; HFD, high-fat diet; HFD+, high-fat diet + 20 ng/day 25OHD₃; HFD++, high-fat diet + 40 ng/day 25OHD₃; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

analyzed by using a computer-assisted sperm motility analysis system (Integrated Semen Analysis System; Hview, Fuzhou, China). In brief, 5 μ L of semen was placed in a chamber and maintained at 37°C during the analysis. A minimum of 8 fields per sample were evaluated, and >200 individual trajectories were recorded.

Quantitative Real-Time PCR

Total RNA from fresh the mouse liver, kidney, and testis was isolated using the TRIzol method according to the manufacturer's protocol (Invitrogen). Messenger RNA was isolated from total tissue using an Oligotex mRNA kit following the manufacturer's in-

structions, and then it was reverse transcribed to produce cDNA, which was used for rapid amplification of cDNA ends (RACE).

First-strand cDNA was made from 1 µg of total RNA from both groups of cells using Superscript III (Invitrogen). Aliquots of cDNA made from tissue RNA were used as the template for real-time and semiquantitative RT-PCR reactions with primers for CYP2R1, CYP27B1, CYP24A1, CYP11A1, and CYP17A1. Aliquots of cDNA made from the RNA of cultured cells were used as the template for semiquantitative RT-PCR reactions, which used the primers for CYP2R1, CYP27B1, CYP24A1, CYP11A1, and CYP17A1 to confirm recombinant gene expression. qPCR data were analyzed using the $2^{-\Delta\Delta C_t}$ method.

Target gene sequences were searched for on NCBI, and primers were designed. The target fragment amplified by real-time qPCR primers was <200 bp, and the primer fragments were synthesized by a company. The PCR solution was distributed in a 96-well PCR plate, and the analysis of each sample and gene was repeated 3 or more times so that there were at least 3 replicates for each data point. There is a detailed list of PCR information in online suppl. material (see online suppl. Table 2).

Western Blotting

The tissue samples of the liver, kidney, and testis were homogenized with a Polytron tissue homogenizer. The tissue homogenate was centrifuged at 12,000 g for 10 min to collect the supernatant. The BCA protein assay kit (Bio-Rad) was used to determine the protein concentration. Total protein (30 µg) was separated by the gradient (4–12%) SDS-PAGE gel (Novex) electrophoresis and then electrotransferred to a nitrocellulose membrane (Bio-Rad). After blocking, the membrane was probed with the primary antibodies of CYP2R1 (Sigma-Aldrich), CYP27B1 (Sigma), CYP24A1 (Abcam), CYP11A1 (Abcam), and CYP17A1 (Abcam) and their corresponding secondary antibodies (GE Healthcare). The bound antibody was detected using ECL Western Blot Detection Reagent (GE Healthcare) with Imager (Proteinsimple). The band signal was quantified using gel analysis software (UN-SCAN-IT gel, version 6.0). The membrane was stripped with stripping buffer (Thermo Scientific) and reprobed with mouse β-actin (Cell Signaling) primary antibody to adjust unequal loading and transfer.

Statistical Analyses

The data are expressed in the figures in the form of mean ± SEM expressed as a bar. All statistical analyses were performed using SAS (v9.4; SAS Institute, Cary, NC, USA). Repeated measures analysis of variance was used to compare weekly weight and cumulative food intake between diet groups that changed over time. One-way ANOVA was used to evaluate the statistical difference of other experimental measurement results between diet groups. A *p* value of <0.05 was considered significant.

Result

25OHD₃ Cannot Slow Down the Degree of Obesity and Abnormal Liver Injury in Mice Induced by HFD

Four groups of mice with different treatments were fed for 10 weeks, and NFD was used as a control group. Compared with NFD animals, the bodyweight and liver weight

of mice induced by HFD increased significantly, and the growth rate was accelerated. However, supplementation of 25OHD₃ did not reduce the degree of obesity in animals and even showed a higher growth rate at 4–7 weeks. However, the final bodyweight was not significantly different (Fig. 1a, c, d, f), and the feed intake of the 4 groups of mice did not change (Fig. 1b). Mice fed by HFD were found to have an abnormal liver injury (Fig. 1e). Supplementation with 25OHD₃ could not resist liver damage.

25OHD₃ Can Restore the Concentration of 25OHD₃ in HFD-Induced Mice and Increase the Concentration of Testosterone and Leptin, as well as the mRNA and Protein Expression of Related Enzymes

The changes in the 4 groups of mice were further analyzed in terms of hormones. The levels of 25OHD₃ and testosterone in the serum of HFD-induced mice were significantly lower than those of the control group (Fig. 2a, b). Supplementing with 25OHD₃ restored the normal level of 25OHD₃. It may be due to the dosage of 25OHD₃ that mice of HFD+ and HFD++ groups had slightly higher concentration than the control group. At the same time, supplementing with 25OHD₃ improved the deficiency of testosterone, but it still cannot reach the level of the control group (Fig. 2b). The content of leptin in the HFD group was significantly higher than that in the control group (Fig. 2c). Supplementation with 25OHD₃ improved the excessive secretion of leptin, but it was still significantly higher than the control group. The results show that 25OHD₃ can alleviate hormone disorder caused by obesity in the body.

The mRNA and protein expressions of liver and kidney hydroxylase and testosterone synthase were detected (Fig. 3). HFD induced a significant downregulation of CYP2R1 mRNA and protein expression in mouse liver, and supplementation with 25OHD₃ did not affect. There was no significant difference in the expression of CYP27B1 in the kidney, and after supplementation with 25OHD₃, the expression of CYP24A1 in the kidney was significantly upregulated. This may be due to the excessive content of 25OHD₃ in the body, and the upregulation of CYP24A1 expression strengthens the degradation of 25OHD₃. HFD induced a significant decrease in the ex-

Fig. 2. HFD induces changes in mouse hormones, sperm quality, and related protein expression. Serum 25OHD₃ (a), testosterone (b), leptin (c), sperm motility (d), serum follicle-stimulating hormone (e), serum luteinizing hormone (f), and testosterone/LH ratio (g). HFD, high-fat diet; TM, total motility; PM, progressive motility.
(For figure see next page.)

Fig. 3. HFD induces changes in mouse hormone-related protein expression. Relative quantitative analysis of mRNA (**a**), image of the Western blot (**b**), and gray value (**c**). Data represent mean $\pm$ SEM derived from $n = 20$. HFD, high-fat diet.

pression of CYP11A1 and CYP17A1 in the testis of mice, and supplementation with 25OHD₃ could restore the normal expression level.

25OHD₃ Can Improve the Motility of Mouse Sperm Induced by HFD

The sperm motility of mice induced by HFD was significantly reduced (Fig. 2d), but there was no difference in concentration, which may provide a clue that the cause of low sperm motility may occur in the sperm maturation stage. Supplementing 25OHD₃ can improve sperm motility.

Discussion

By establishing a mouse obesity model, we found that the content of 25OHD₃ in the serum of obese mice was significantly reduced. This is not the first time this has been found in obese mice [20]. It is noteworthy that in several large-scale men's health surveys in recent years, it has also been found that the 25OHD₃ content in the serum of obese people is low [21–23]. Moreover, it does not seem to be restricted by age and exists in obese groups from adolescents to the elderly, and supplementing with vitamin D cannot effectively increase the concentration of 25OHD₃ [24]. There are 2 different explanations for the decrease of 25OHD₃ in serum. One is that the increase in body surface area after obesity leads to a decrease in the content of circulating 25OHD₃. The other is the decline of hydroxylation ability after obesity, so the concentration of 25OHD₃ is reduced. Both of these 2 explanations are reasonable, but based on the experimental results of this study, we prefer the latter. We have already described the activation pathway of vitamin D₃ in the body in the Introduction section. It can be converted into 25OHD₃ only after the liver undergoes the first hydroxylation by CYP2R1. In this study, the expression of CYP2R1 mRNA and protein in the liver of obese mice is significantly downregulated, which reduces the conversion efficiency of vitamin D₃, while the expression of CYP27B1 in the kidney is not affected, that is, the efficiency of 25OHD₃ conversion to the next product is not affected, which finally leads to a decrease in the content of 25OHD₃ in serum. Besides, the increase in serum ALT/AST indicates liver injury damage, and we speculate that this may be caused by fatty liver damage to liver injury that affects the expression of CYP2R1. There are many steroid hormone receptors in liver tissue, and there is increasing evidence that these receptors are involved in obesity mechanisms: the

increase in fat may lead to a decrease in the expression of vitamin D₃ receptors, which in turn reduces the utilization of vitamin D₃ [25, 26]. Although the results of this trial are not sufficient to prove that the decrease in 25OHD₃ concentration is simply due to the decrease in receptor expression, we believe that the results can explain to a certain extent that the decrease in 25OHD₃ concentration in obese people is associated with a decrease in liver injury and CYP2R1 expression.

In the Introduction section, we have described the recently reported study of low content of 25OHD₃ in infertile people. Since the test data have not been made public, we cannot know whether the patient was obese. Obese mice in this study also showed symptoms of low sperm motility. Besides, we found that the level of testosterone measured in the testes of obese mice was almost 6 times lower than that in normal mice. The physiological requirements for elevated levels in the testis are not fully understood. However, normal sperm production requires levels >70 nmol/L [27]. The testosterone content of obese mice is far from enough to maintain spermatogenesis and maturity. The mRNA and protein expression of CYP11A1 and CYP17A1, which synthesize testosterone, is significantly reduced, which may be one of the direct reasons for the low efficiency of testosterone synthesis. Besides, the level of leptin in obese mice was significantly increased. It has been reported that in obese individuals, the level of leptin increases for a long time, leading to "leptin resistance" [28]. This resistance is concentrated in the middle of the hypothalamus. In these men who resist, leptin cannot stimulate the HPG axis, resulting in lower testosterone levels [29, 30]. The effect of male obesity on the androgen axis is well known; however, the effects of abnormal levels of obese hormones on sperm parameters are complex and may be multifactorial. Although it is believed that the disordered change of the androgen axis in spermatogonial stem cells plays a role, the exact cause of poor sperm by which factor is still elusive. In a randomized clinical trial, vitamin D deficiency was found to be related to semen quality and sex steroid levels in infertile men. In >1,400 subjects, men with low 25OHD₃ concentrations had significantly lower sperm motility and testosterone levels than normal men [31]. This shows to a certain extent that the reduction of 25OHD₃ is related to the level of testosterone. And, it has been reported that 25OHD₃ can increase testosterone levels [32], while vitamin D₃ supplementation does not affect testosterone levels. Further experiments are needed to prove how they influence each other.

Based on the results of this research, we speculate that due to the reduction of CYP2R1 expression, it is impossible to synthesize enough 25OHD₃, which interferes with the progress of other biochemical reactions, which implies that the target of 25OHD₃ is related to the regulation of reproductive hormones. Also, we are supported by some literature studies. A series of clinical observational studies on humans have shown that orchiectomy or testicular injury harms the circulating levels of 25-hydroxyvitamin D₃ [33, 34].

What we need to emphasize is that the supplementation of 25OHD₃ in this study cannot slow down the degree of obesity in mice, and even the growth rate will increase. Studies have reported that mice that knock out the VDR can resist obesity [35], and a large number of reports show that 25OHD₃ can improve the growth rate of animals [36]. We suggest that people with obesity and infertility can properly supplement 25OHD₃, but it cannot resist obesity and improve liver injury.

Conclusion

By constructing an obese mouse model, this study found that obesity can lead to decreased 25OHD₃ concentration and insufficient sperm motility in mice. Supplementation with 25OHD₃ can increase serum 25OHD₃ concentration and increase sperm motility, which may be regulated by 25OHD₃ and reproductive hormones. The results of this study can provide a scientific basis for studying the mechanism of 25OHD₃ and hormone regulation and treating obese people with low sperm motility.

References

- 1 Finkelstein EA, Trogdon JG, Cohen JW, Dietz W. Annual medical spending attributable to obesity: payer- and service-specific estimates. *Health Aff.* 2009;28(5):w822–31.
- 2 Hales CM, Carroll MD, Fryar CD, Ogden CL. Prevalence of obesity and severe obesity among adults: United States, 2017–2018. *NCHS Data Brief.* 2020;(360):1–8. Hyattsville, MD: National Center for Health Statistics.
- 3 Flegal KM, Kit BK, Orpana H, Graubard BI. Association of all-cause mortality with overweight and obesity using standard body mass index categories: a systematic review and meta-analysis. *JAMA.* 2013;309(1):71–82.
- 4 Guh DP, Zhang W, Bansback N, Amarsi Z, Birmingham CL, Anis AH. The incidence of co-morbidities related to obesity and overweight: a systematic review and meta-analysis. *BMC Public Health.* 2009;9:88.
- 5 Rolland M, Le Moal J, Wagner V, Royère D, De Mouzon J. Decline in semen concentration and morphology in a sample of 26,609 men close to general population between 1989 and 2005 in France. *Hum Reprod.* 2013;28(2):462–70.
- 6 Katib A. Mechanisms linking obesity to male infertility. *Cent European J Urol.* 2015;68(1):79–85.
- 7 Phillips KP, Tanphaichitr N. Mechanisms of obesity-induced male infertility. *Expert Rev Endocrinol Metab.* 2010;5(2):229–51.
- 8 Lamb DJ, Lipshultz LI. Male infertility: recent advances and a look towards the future. *Curr Opin Urol.* 2000;10(4):359–62.
- 9 Wagner CL, Greer FR. Prevention of rickets and vitamin D deficiency in infants, children, and adolescents. *Pediatrics.* 2008;122(5):1142–52.
- 10 Schoenmakers I, Pettifor JM, Peña-Rosas JP, Lamberg-Allardt C, Shaw N, Jones KS, et al. Prevention and consequences of vitamin D deficiency in pregnant and lactating women and children: a symposium to prioritise vitamin D on the global agenda. *J Steroid Biochem Mol Biol.* 2016;164:156–60.
- 11 Hilger J, Friedel A, Herr R, Rausch T, Roos F, Wahl DA, et al. A systematic review of vitamin D status in populations worldwide. *Br J Nutr.* 2014;111(1):23–45.
- 12 Christakos S, Dhawan P, Verstuyf A, Verlinden L, Carmeliet G. Vitamin D: metabolism, molecular mechanism of action, and pleiotropic effects. *Physiol Rev.* 2016;96(1):365–408.
- 13 Filgueiras MS, Rocha NP, Novaes JF, Bressan J. Vitamin D status, oxidative stress, and inflammation in children and adolescents: a systematic review. *Crit Rev Food Sci Nutr.* 2020;60(4):660–9.

Statement of Ethics

All procedures have been approved by the Animal Care and Use Committee. This trial was also approved by the Ethics Committee of Zhao Qing University (APV-00514) and the Scientific Research Foundation of Shaoguan University for the Introduction of Talents.

Conflict of Interest Statement

The authors report no conflicts of interest in this work.

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Author Contributions

F.Y. and W.K. conceived and designed the experiments. F.Y. and W.K. performed the experiments. F.Y. and C.Y. analyzed the data and generated the figures. F.Y. and W.K. wrote the manuscript. All authors gave final approval for the submitted version.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online suppl. material files. Further enquiries can be directed to the corresponding author.

- 14 Zakharova I, Klimov L, Kuryaninova V, Nikitina I, Malyavskaya S, Dolbnya S, et al. Vitamin D insufficiency in overweight and obese children and adolescents. *Front Endocrinol*. 2019;10:103.
- 15 Sousa-Santos AR, Afonso C, Santos A, Borges N, Moreira P, Padrão P, et al. The association between 25(OH)D levels, frailty status and obesity indices in older adults. *PLoS One*. 2018;13(8):e198650.
- 16 Abbasihormozi S, Kouhkan A, Alizadeh AR, Shahverdi AH, Nasr-Esfahani MH, Sadighi Gilani MA, et al. Association of vitamin D status with semen quality and reproductive hormones in Iranian subfertile men. *Andrology*. 2017;5(1):113–8.
- 17 Zhu CL, Xu QF, Li SX, Wei YC, Zhu GC, Yang C, et al. Investigation of serum vitamin D levels in Chinese infertile men. *Andrologia*. 2016;48(10):1261–6.
- 18 Blomberg JM, Jørgensen A, Nielsen JE, Bjerum PJ, Skalkam M, Petersen JH, et al. Expression of the vitamin D metabolizing enzyme CYP24A1 at the annulus of human spermatozoa may serve as a novel marker of semen quality. *Int J Androl*. 2012;35(4):499–510.
- 19 Cheng JB, Levine MA, Bell NH, Mangelsdorf DJ, Russell DW. Genetic evidence that the human CYP2R1 enzyme is a key vitamin D 25-hydroxylase. *Proc Natl Acad Sci U S A*. 2004;101(20):7711–5.
- 20 Roizen JD, Long C, Casella A, O’Lear L, Caplan I, Lai M, et al. Obesity decreases hepatic 25-hydroxylase activity causing low serum 25-hydroxyvitamin D. *J Bone Miner Res*. 2019;34(6):1068–73.
- 21 Almesri N, Das NS, Ali ME, Gumaa K, Giha HA. Gender-dependent association of vitamin D deficiency with obesity and hypercholesterolemia (LDLC) in Adults. *Endocr Metab Immune Disord Drug Targets*. 2020;20(3):425–36.
- 22 Olmos JM, Hernández JL, Pariente E, Martínez J, González-Macías J. Serum 25-hydroxyvitamin D in obese Spanish adults: the Camargo Cohort Study. *Obes Surg*. 2018;28(12):3862–71.
- 23 Walsh JS, Bowles S, Evans AL. Vitamin D in obesity. *Curr Opin Endocrinol Diabetes Obes*. 2017;24(6):389–94.
- 24 Shah S, Wilson DM, Bachrach LK. Large doses of vitamin D fail to increase 25-hydroxyvitamin D levels or to alter cardiovascular risk factors in obese adolescents: a Pilot Study. *J Adolesc Health*. 2015;57(1):19–23.
- 25 Hammes SR, Levin ER. Minireview: recent advances in extranuclear steroid receptor actions. *Endocrinology*. 2011;152(12):4489–95.
- 26 Zhang XK, Su Y, Chen L, Chen F, Liu J, Zhou H. Regulation of the nongenomic actions of retinoid X receptor- α by targeting the coregulator-binding sites. *Acta Pharmacol Sin*. 2015;36(1):102–12.
- 27 Zirkin BR, Santulli R, Awoniyi CA, Ewing LL. Maintenance of advanced spermatogenic cells in the adult rat testis: quantitative relationship to testosterone concentration within the testis. *Endocrinology*. 1989;124(6):3043–9.
- 28 Considine RV, Considine EL, Williams CJ, Nyce MR, Magosin SA, Bauer TL, et al. Evidence against either a premature stop codon or the absence of obese gene mRNA in human obesity. *J Clin Invest*. 1995;95(6):2986–8.
- 29 Münzberg H, Myers MG. Molecular and anatomical determinants of central leptin resistance. *Nat Neurosci*. 2005;8(5):566–70.
- 30 Farooqi IS, O’Rahilly S. Leptin: a pivotal regulator of human energy homeostasis. *Am J Clin Nutr*. 2009;89(3):980S–4S.
- 31 Blomberg Jensen M, Gerner Lawaetz J, Andersson A-M, Petersen JH, Nordkap L, Bang AK, et al. Vitamin D deficiency and low ionized calcium are linked with semen quality and sex steroid levels in infertile men. *Hum Reprod*. 2016;31(8):1875–85.
- 32 Pilz S, Frisch S, Koertke H, Kuhn J, Dreier J, Obermayer-Pietsch B, et al. Effect of vitamin D supplementation on testosterone levels in men. *Horm Metab Res*. 2011;43(3):223–5.
- 33 Foresta C, Selice R, De Toni L, Di Mambro A, Carraro U, Plebani M, et al. Altered bone status in unilateral testicular cancer survivors: role of CYP2R1 and its luteinizing hormone-dependency. *J Endocrinol Invest*. 2013;36(6):379–84.
- 34 Foresta C, Selice R, De Toni L, Di Mambro A, Carraro U, Plebani M, et al. Altered bone status in unilateral testicular cancer survivors: role of CYP2R1 and its luteinizing hormone-dependency. *J Endocrinol Invest*. 2013;36(6):379–84.
- 35 Jahn D, Dorbath D, Schilling AK, Gildein L, Meier C, Vuille-Dit-Bille RN, et al. Intestinal vitamin D receptor modulates lipid metabolism, adipose tissue inflammation and liver steatosis in obese mice. *Biochim Biophys Acta Mol Basis Dis*. 2019;1865(6):1567–78.
- 36 Wang K, Chen Y, Zhang D, Wang R, Zhao Z, Feng M, et al. Effects of 25-hydroxycholecalciferol supplementation in maternal diets on reproductive performance and the expression of genes that regulate lactation in sows. *Anim Sci J*. 2020;91(1):e13391.