RESEARCH ARTICLE

# Lycopene restores spermatogenesis in cryptorchidism mice through Wnt signaling pathway

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**Lycopene (LYC) is a natural carotenoid with high concentrations of it being found abundantly in ripe tomatoes and other red fruits/vegetables such as papaya, pink grapefruits, and watermelons, which has been shown to positively impact male infertility. Wnt signaling pathway is more important owing to its function in production and maturation of sperm. The aim of this study was to verify the effect of LYC on restoring spermatogenic function in cryptorchidism mice and give deeper insight into the mechanisms of the relationship between Wnt signal pathway and the promotion of LYC. The cryptorchidism model of Kunming mouse was established, and LYC was given at the doses of 4, 10, and 20 μg/g/d continuously for 35 days. The changes of sperm count, body weight, and Hematoxylin-eosin (HE) staining sections of testicular tissue were analyzed. The expression levels of wnt1, wnt4, and DKK1 proteins in testicular tissue in each group were detected using Western blot. The results showed that the sperm counts in different LYC treatment groups were all significantly higher than that of cryptorchidism group, but still lower than the normal group. Testicular tissue section results indicated that spermatogenesis recovered in LYC-treated group, indicating that LYC could effectively restore spermatogenesis in cryptorchidism mice. In addition, expression levels of wnt1, wnt4, and DKK1 proteins in LYC-treated group were significantly up-regulated compared with those in cryptorchidism group. The result of this study suggested that LYC might regulate spermatogenesis in cryptorchid mice through up regulated the expression of wnt1, wnt4, and DKK1 proteins in the Wnt signaling pathway, which provided a theoretical basis for subsequent infertility treatment.**

**Keywords:** Lycopene; cryptorchidism; Wnt signal pathway; mechanism.

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## Introduction

Infertility is a major global health issue that affects about 14% of couples in the general population [1]. At present, about 8% men of childbearing age are troubled by infertility. Infertility caused by male factors directly or indirectly accounts for about 60% of the total infertility cases [2]. Spermatogenesis includes spermatogonia division and proliferation, spermatocyte meiosis, spermatocyte metamorphosis, differentiation, and the process of spermatogenic maturation [3-5]. The whole process is carefully regulated by many factors [5], including specific micro-environments, interactions between cells, regulation of external factors (hormones and cytokines), and internal factors (changes in chromatin structure and gene expression) [6-9]. However, external factors are ultimately through internal factors to complete the regulation of spermatogenesis. The in-depth study of the internal factors regulating

spermatogenesis, a gene level regulation, will enable people to understand the mechanism of spermatogenesis fundamentally.

Lycopene ($C_{40}H_{56}$, LYC) is a red-pigmented unsaturated linear carotenoid with a molecular weight of 536.85 Da, containing 11 conjugated and two non-conjugated double bonds [10, 11], which belongs to the group of natural carotenoids, founding in many fruits and vegetables, but predominantly in tomatoes and tomato-based products [10, 12]. Several studies showed that LYC preferentially accumulated in the testes, adrenal glands, livers, and prostate, while the concentration in the testes was ten times higher than that in other tissues [11, 13], which indicated that LYC was likely to play a major role in the process of spermatogenesis. Previous studies also showed some evidence that LYC could help to alleviate male infertility [11, 14, 15], while the other researchers demonstrated a possible role of oral LYC therapy in the improvement of semen parameters of infertility men [16-18]. The production and maturation of sperm involves abundant signaling pathways and regulatory mechanisms [19-21]. Among these pathways, Wnt signaling is more important owing to its extensive expression in male genital organs, and reproductive pathology is frequently observed during the Wnt pathway malfunctions. Wnt signaling is a highly conserved cell-to-cell communication mechanism, which is dependent and independent of β-catenin pathway with essential functions in development cell proliferation, tissue morphogenesis, tissue homeostasis, and diseases [22, 23]. Knocking out different genes in Wnt signaling pathway is normally accompanied by different kinds of manifestation of male infertility, which varies from complete absence of spermatozoa in the testes to congenital genital deformities. This phenomenon indicates that Wnt signaling may participate in various reproductive physiological activities including organogenetic development and spermatogenesis. According to recent study, incubating sperm with Wnt1 could significantly increase the percentage of cells with complete acrosome exocytosis, but the mechanisms were unclear [24]. Chassot *et al*. revealed that Wnt4 was involved in the mammalian testis determination pathway and required in early testis development [25]. In addition, Baetens *et al*. found that Sertoli cell differentiation was compromised in Wnt4 mutant testes. This defect occurred downstream of Sry, but upstream of Sox9 and Dhh [26]. Other studies indicated that Wnt4 was a balancing regulator that deeply participated in both male and female sex development [27, 28].

Although previous studies have shown that LYC plays a major role in the process of spermatogenesis, the exact mechanism whether LYC could restore cryptorchidism mice reproductive, and its specific protective mechanisms remains a mystery. The objectives of this study were to verify whether LYC could restore spermatogenic function in cryptorchidism mice and give a deeper insight into mechanisms of the relationship between Wnt signal pathway and the promotion of LYC. Understanding the physiology and pathology of male reproductive function is great significance for treating male infertility.

## Materials and methods

### Experimental animals

Forty (40) 15-day-old male Kunming mice were purchased from the Experimental Animal Center of Zhengzhou University (Zhengzhou, Henan, China). The mice were kept in a dry and clean environment with adequate food and water supply, and room temperature at 25℃, 12 h dark/light cycle. The experiments started after 1 week of adaptive feeding. All procedures of this research were approved by the Animal Experimental Committee of Huanghuai University (Zhumadian, Henan, China).

### Cryptorchidism animal model construction

A total of 32 mice underwent cryptorchidism operation as described in the previous study [29]. Briefly, after ether anesthesia, the mouse was placed in supine position with the limbs fixed.

The abdomen was disinfected with alcohol, and a 1 cm incision was made at the median line of the abdomen. Both testicles were pulled from the scrotum into the abdominal cavity. The traction straps of both testes were cut short, and the epididymal fat pad was fixed on the abdominal wall. One week recovery was taken after the operation before LYC administration treatment.

**Lycopene administration treatment**

After cryptorchidism operation, the mice were randomly divided into four groups including cryptorchid, cryptorchid + 4 μg/g/d LYC treatment, cryptorchid + 10 μg/g/d LYC treatment, and cryptorchid + 20 μg/g/d LYC treatment with 8 mice in each group. LYC obtained from Solarbio, Beijing, China was dissolved in olive oil and fed the animals with gavage. The dosage was determined according to the weight of the mouse. 8 mice were included in normal control group with the treatment of same amount of olive oil. All the animals were treated with continuous gavage for 35 days.

**Sperm counting**

The mice were sacrificed by cervical dislocation after the experiment. The epididymis was removed from the upper side of the genitalia and put into a tube containing 1 mL of 37℃ normal saline. Spermatozoa in the suspension were then counted using ML-608JZ-II sperm counter (MaiLang, Nanning, Guangxi, China) and analyzed as described previously [29].

**Preparation of testicular tissue sections and Hematoxylin-eosin (HE) staining**

After removing the adipose tissue from one testicle, the tissue was fixed in Bouin's solution (Servicebio, Wuhan, Hubei, China) for 12 h. The sections were dehydrated by different gradients of alcohol as 75% alcohol for 4 h, 85% alcohol for 2 h, 90% alcohol for 2 h, 95% alcohol for 1 h, then absolute ethyl alcohol for 30 min before transparent by xylene I for 5 – 10 min and xylene II for 5-10 min. The sections were immersed in melted paraffin for 1 h and then embedded using RM2016 microtome (Leica Instrument, Shanghai, China) with a thickness of 5 μm before HE staining (Servicebio, Wuhan, Hubei, China).

**Western immunoblot analysis**

Testes were removed and lysed with RIPA buffer (Sangon Biotech, Shanghai, China). The samples were centrifuged at 12,000 rpm for 30 min at 4℃. Supernatants were collected, and 5× loading buffer was added. Total protein concentrations were determined using the BCA kits (Sangon Biotech, Shanghai, China) following manufacturer's instruction. The samples were analyzed by SDS-PAGE gel electrophoresis and transferred to the nitrocellulose membranes. Wnt1, Wnt4, and DKK1 proteins were detected using the rabbit polyclonal antibodies (Proteintech, Wuhan, China) with the enhanced chemiluminescent reagent (ThermoScientific, Swedesboro, NJ, USA). Images were acquired using the ChemiDoc MP System (Bio-Rad, Hercules, CA, USA). The integral optical density of each band was measured using Image-J software (National Institutes of Health, Bethesda, MD, USA).

**Statistical analysis**

Prism8 (GraphPad, Boston, MA, USA) was employed for statistical analysis of this study. The student t-test was used for results comparison between the experimental groups. The *P* value less than 0.05 was defined as the significant difference.

## Results

**Sperm count**

The results showed that mice in the cryptorchid group had no sperm appeared. However, compared with the cryptorchidism group, the sperm counts in the cryptorchidism + LYC administration groups were significantly increased ($P < 0.01$), but still lower than that in the control group ($P < 0.01$) (Figure 1). The results indicated that LYC could effectively restore spermatogenesis in cryptorchidism mice.

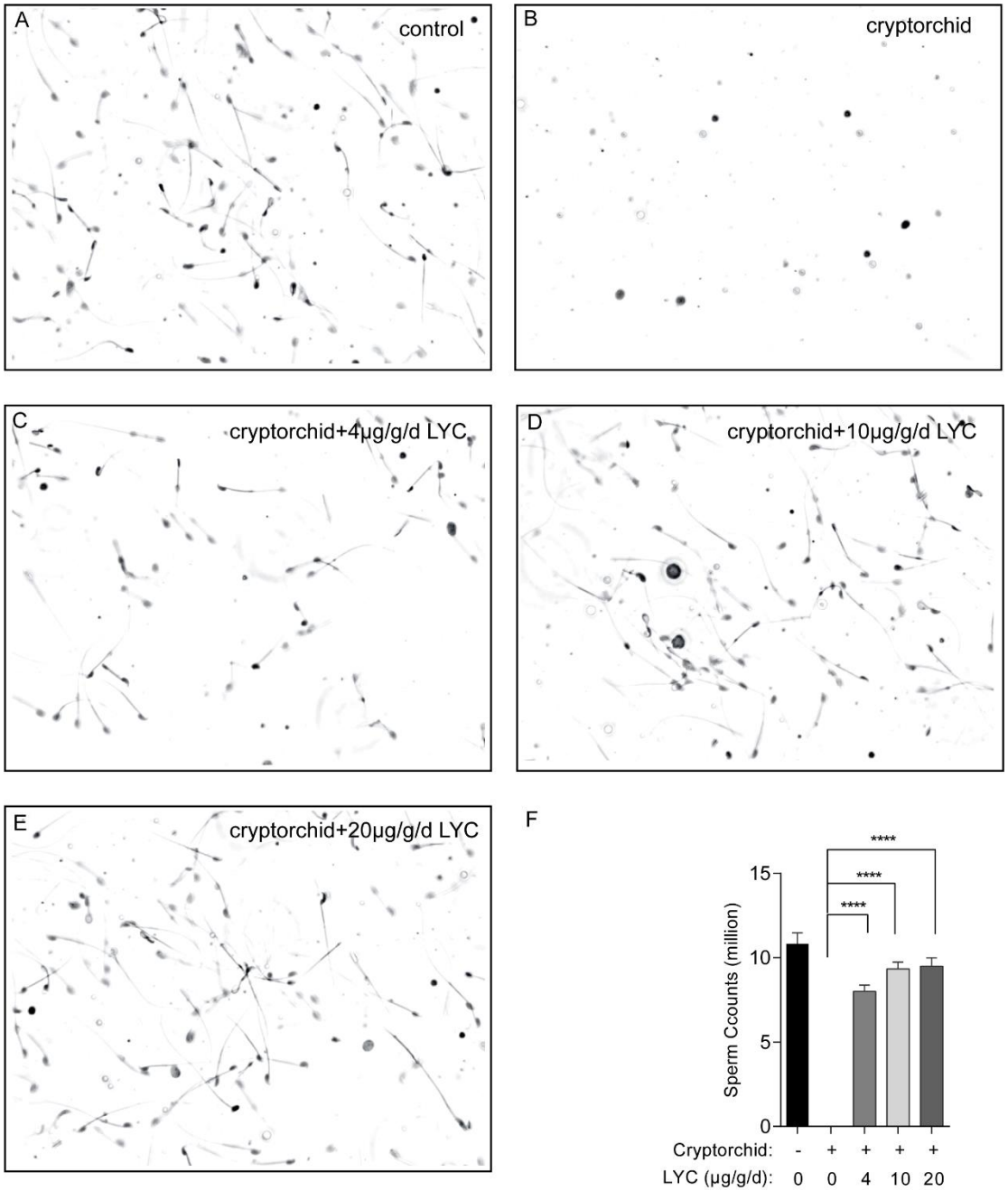


**Figure 1.** Sperm counts in different experimental groups. **A.** normal control. **B.** cryptorchidism. **C.** cryptorchidism + 4 μg/g/d LYC. **D.** cryptorchidism + 10 μg/g/d LYC. **E.** cryptorchidism + 20 μg/g/d LYC. F. quantification of A-E. Date was presented as the mean + SEM from 8 randomly chosen figures. **** $P < 0.0001$.

**Weight gain and testicular tissue weight in mice**

Compared with the control group, the animals' weekly body weights of the experimental groups were not significantly different at the same level and showed a continuous increasing trend (Figure 2A). However, the analysis of testicular tissue weight showed that the testicular weight of the cryptorchidism group was significantly lower than that of the control group and the LYC administration groups ($P < 0.01$) (Figure 2B).

**HE staining**

HE staining of testicular tissue showed that spermatogenic tubules were arranged neatly in

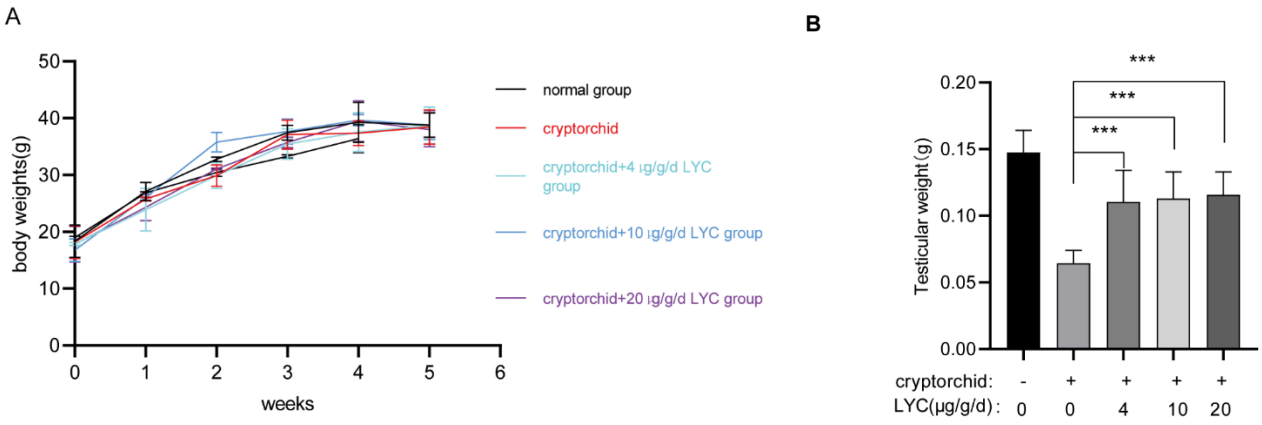


**Figure 2.** The body weight and testicular weight of mice in different groups. **A.** the weights of each group of mice weighed weekly. **B.** testicular weights of mice in different groups weighed on 35th day. ***$P < 0.001$.

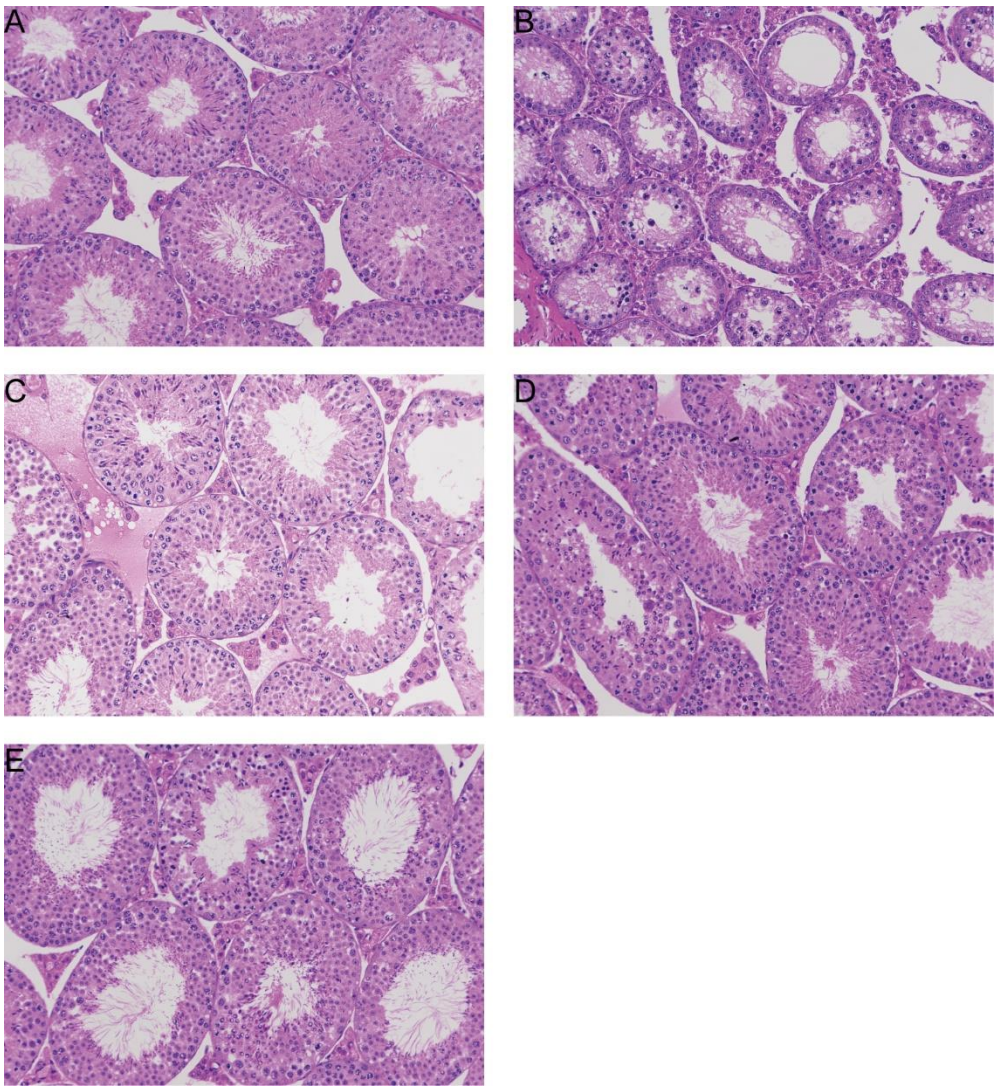


**Figure 3.** Representative images of HE staining sections of testes from mice in different groups. **A.** control. **B.** cryptorchidism. **C.** cryptorchidism + 4 μg/g/d LYC. **D.** cryptorchidism + 10 μg/g/d LYC. **E.** cryptorchidism + 20 μg/g/d LYC. (200 x).

control group, spermatogenic cells at all levels were clear and tightly structured, and spermatogenesis was visible in the tubules. In the cryptorchidism group, the distance between spermatogenic tubules widened, the thickness of the tube wall was uneven, the arrangement of spermatogenic cells was disordered, the number of spermatogenic cells was small, and a large blank area could be seen in the official cavity. Compared with the cryptorchidism group, the spacing between spermatogenic tubules in the 4, 10, and 20 μg/g/d LYC treated groups were reduced, the spermatogenic cells were arranged relatively neatly, and the development level of spermatogenic cells was observed (Figure 3).

**Detection of Wnt1, Wnt4, and DKK1 protein expression in the testicular tissue**

The expression levels of Wnt1 and Wnt4 proteins in the cryptorchidism group decreased significantly compared with that in the control group. However, the expression levels of Wnt1 and Wnt4 proteins in LYC-treated group were significantly higher than that in cryptorchidism group, which were basically the same as that in control group (Figure 4). In addition, the expression level of DKK1 protein in cryptorchidism group was significantly lower than that in control group, while the expression levels of DKK1 protein in LYC-treated groups were significantly higher than that in cryptorchidism group. These results indicated that LYC might up-regulate the expression of Wnt1, Wnt4, and DKK1 proteins and promote spermatogenesis in cryptorchidism mice.

## Discussion

Male reproductive activities include spermatogenesis, sperm maturation, launch and sperm capacitation and other physiological processes, and sperm in female reproductive system and fertilization [6]. It is a complex physiological process controlled by neuroendocrine and now generally believes that male infertility is usually the result of various diseases or environmental effects such as immunity, varicocele and reproductive system infection, and a disease or factor that affects multiple physiological processes of male reproduction at the same time [30, 31]. Testicular dysfunction is the most common cause of male infertility. Our previous findings suggested that cryptorchidism might cause spermatogenesis to stop at the primary spermatocyte stage [29, 32]. In this study, the cryptorchidism model was used to explore the protective effect of LYC on reproductive damage in cryptorchidism mice. LYC, as a naturally occurring carotenoid, has a strong antioxidant capacity, which can reduce the oxidative damage of tissue cells and may inhibit the inflammation and oxidative stress induced by polysaccharides, thus protecting reproductive ability [11, 33-35]. In recent years, many researchers have paid attention to the role of LYC on male reproduction, but the underling mechanism is still unclear.

In the present study, it was observed that the different concentrations of LYC could significantly restore the sperm counts, which was consistent with the previous reports [36, 37]. Although the sperm counts in the LYC treated groups were still lower than that in the control group, there were no significant differences in sperm morphology between the control group and LYC treated groups. Histological analysis indicated that germ cell development was arrested at the stage of primary spermatocyte in the cryptorchidism group. However, sperms were seen when treated cryptorchidism animals with 4, 10, and 20 μg/g/d LYC, which indicated that LYC might decrease the rate of apoptosis and stimulate germ cells differentiation. The weight of testes in the cryptorchidism group were smaller than those of control groups, mainly because of the position of testes in cryptorchidism mice that the testicular tissue was in the abdominal cavity, where the temperature was relatively high [29, 38].

To clarify the relationship between the role of LYC and Wnt signaling in male reproductive physiology and pathology, protein expression levels in the testes in different groups were

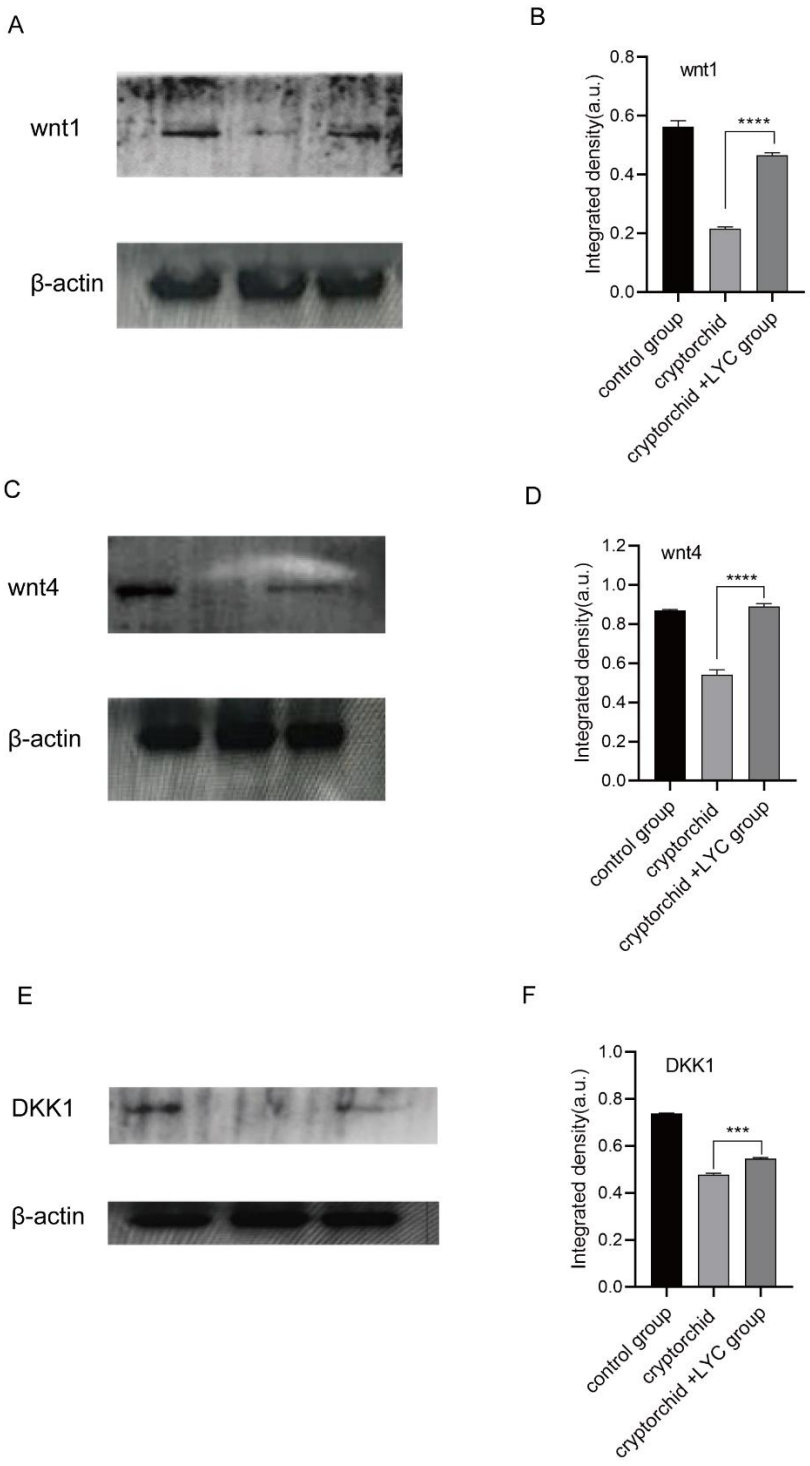


**Figure 4.** Western blot analysis of Wnt-signal related protein levels in different groups. **A, C, E:** Wnt1, Wnt4, DKK1 protein expression levels in testes. **B, D, F:** the quantification of the results in A, C, E. ***$P < 0.001$, ****$P < 0.0001$.

detected. Wnt1 was reported as the leading member of the Wnt protein family, which was an indispensable element for embryonic neurogenesis [39]. The early study had established the conditional Wnt1-/- mice and found no gross abnormality of spermatogenesis

[40]. In addition, incubating sperm with Wnt1 could significantly increase the percentage of cells with complete acrosome exocytosis [24]. Wnt4 is also an indispensable element for the formation of male genital structures. The researchers have discovered that Sertoli cell differentiation was impaired and testicular blood vessels underdeveloped in Wnt4-/- mice, which led to the decrease in testis size and sperm count [41]. DKK1, as a member of the DKKs family, is a well-known negative regulator of Wnt signaling. The results of expression analysis showed that Dkk1 in testis mainly existed in spermatogonia and Leydig cells [42]. In this study, the expression levels of wnt1, wnt4, and DKK1 proteins were up-regulated in the LYC treated groups compared to the cryptorchidism groups, which indicated that Wnt signaling pathway might play a key role in the reproduction of male infertility.

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