Mechanism of action of levocarnitine for the treatment of spermatogenic dysfunction in rats and spermatogonia

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Abstract

The mechanism of action of levocarnitine (LEV) in the treatment of spermatogenic dysfunction was unclear. We used GTW to construct a cell model (using spermatogenic GC-1 spg cells) and an animal model (using rats) of spermatogenic dysfunction. LEV and LY294002 (a PI3K pathway inhibitor) were then administered. By assessing proliferation, mitochondrial membrane potential, apoptosis, and sex hormone levels, the underlying mechanism was explored. We found that (I) by regulating PI3K/AKT pathway-related protein expression, LEV increased the percentages of GC-1 spg cells in the G0/G1 and G2/M phases, increased the mitochondrial membrane potential, and decreased apoptosis, thereby improving the sperm count and motility and (II) LEV significantly increased the T, FSH, and LH serum levels, thereby improving the spermatogenic function of rats.

1. Background

According to the World Health Organization, infertility is defined as the inability to conceive after 1 year of unprotected intercourse [1]. Worldwide, about 10–15% of couples have infertility [2]. The study of Agarwal et al. showed that male infertility rates were highest in Africa and Central/Eastern Europe, whereas corresponding rates for North America, Australia, and Central and Eastern Europe varied from 4.5–6%, 9%, and 8–12%, respectively [3]. While the most powerful negative predictive factor of fertility is increasing women's age at conception [4], other factors including lifestyle and environmental factors are believed to play an increasing role. Treatment measures include symptomatic treatments such as estrogen receptor modulators, sperm nutrition agents, and antibiotics, whose clinical efficacy remains to be proven [5-6]. Although assisted reproductive technology (ART) has been widely used [7], it has many limitations, as it leads to a low birth rate, is expensive, and involves genetic and ethical risks [8].

Levocarnitine (LEV), also called L-carnitine, is indispensable in the process of beta oxidation of fatty acids, which plays an important role in the production and transmission of energy in the body. LEV improves the percentage of forward-motion sperm and total sperm motility in patients with oligozoospermia (reduced sperm count) or asthenospermia (reduced sperm motility) in clinical settings [9]. We conducted this study to assess whether LEV acts through these proteins, along with proteins related to the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway.

Glucosides of *Tripterygium wilfordii* Hook F (GTW) are commonly used in the treatment of rheumatoid arthritis, but long-term use of GTW impairs reproductive function. In this study, GTW was used to establish cell and rat models of spermatogenic dysfunction [10]. After establishing the models, LEV and the PI3K pathway inhibitor LY294002 were administered. By assessing the expression of proteins and mRNA related to the PI3K/AKT signaling pathway protein and mRNA expression as well as assessing cell proliferation and apoptosis of spermatogonial GC-1 spg cells, the effect of LEV on spermatogenic function and the molecular mechanism were explored.

2. Methods

2.1. Ethical approval of study protocol

All animal procedures were performed in accordance with relevant guidelines and regulations of Good Animal Practice approved by the institutional and were approved by the Experimental Animal Welfare Ethics Committee of Dongzhimen Hospital, Beijing University of Chinese Medicine (NO.19-27) in Beijing, China.

2.2. Drugs and reagents

GTW tablets (batch no. 130801) were purchased from Anhui Xinlonghai Pharmaceutical Co., Ltd. (Suzhou, China). Before administration, 1,200 mg GTW tablets were dissolved in 300 ml deionized water and configured to a desired suspension. LEV (batch no. H19990372 10 ml: 1g; 1 g in each 10 mL) was purchased from Northeast Pharmaceutical Co., Ltd. (Shenyang, China). Before administration, 2 g L-carnitine was dissolved in 100 ml deionized water. LY294002 (S1105; a PI3K pathway inhibitor) was purchased from Selleck (Houston, TX, USA).

Dulbecco's Modified Eagle Medium (DMEM; high glucose; 06-1055-57-1ACS) and fetal bovine serum (FBS; 04-001-1ACS) were purchased from Biological Industries (Beit HaEmek, Israel), trypsin (25200056) was purchased from Invitrogen (Carlsbad, CA, USA), and 1% (v/v) penicillin–streptomycin solution (PB180120) was purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China). Annexin V-Fluorescein Isothiocyanate (FITC) Apoptosis Detection Kit I (556547) was purchased from Becton, Dickinson, and Company (Franklin Lakes, NJ, USA). Apoptotic Mitochondrial Membrane Potential Detection Kit (JC-1; KGA604) was purchased from Jiangsu Kaiji Biotechnology Co., Ltd. (Nanjing, China). Cell Counting Kit-8 (CCK8; CK04-1) was purchased from Dojindo Institute of Chemistry (Kyushu Island, Japan). Propidium Iodide/RNase Staining Buffer (550825) was purchased from Becton, Dickinson, and Company. SDS-PAGE Loading Buffer (5×) (GPP1820) and Total RNA Extraction Kit (DNase) (Cat# GPQ1801) were purchased from Beijing Genepool Biotechnology Co., Ltd. (Beijing, China). Acetone was purchased from Sinopharm Group Chemical Reagent Co. LTD (Shanghai, China). 812 embedding agent was purchased from Structure Probe, Inc. (West Chester, America). Antibodies against RET (AB134100), PI3K (AB191606), BAX (AB32503), and PCNA (AB18197), Actin (AB6276), GADPH (AB181602) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H+L) secondary antibodies (AB6789), were purchased from Abcam (Cambridge, UK). Antibodies against GFRa1 (SC-271546), BCL-2 (SC-492), and AR (SC-7305) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies against AKT (9272) and BAD (9292) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

2.3. Animals and cells

A total of 55 adult male specific-pathogen-free (SPF) Sprague–Dawley rats (15 for the preparation of NC, GTW-containing, and LEV-containing serum samples for the cell experiments and 40 for the animal experiments; 6 weeks old, 200±20 g) were purchased from Beijing Vitonliol Laboratory Animal

Technology Co., Ltd. (animal license no. SCXK (Beijing) 2016-0006). Before the experiment, the rats were kept in standard conditions and subjected to adaptive feeding for 7 days. Feeding and experiments were conducted in the SPF Animal Laboratory of Dongzhimen Hospital of Beijing University of Chinese Medicine.

The GC-1 spg cell line (no. CL-0600) derived from mouse testis tissue was purchased from Procell Life Science&Technology Co., Ltd. (Wuhan, China). The cells were observed under a microscope (to ensure they were healthy and growing as expected) and, after the cell confluency initially reached >80%, the cells were subcultured (using a 1:5 split ratio) for 48 h. At this point, the cell confluency again reached >80% and the cells were collected and cryopreserved in liquid nitrogen.

2.4. Preparation of NC, GTW-containing, and LEV-containing serum samples

After 1 week of adaptive feeding, 5 rats were randomly selected as the NC group. The remaining 10 rats were randomly divided into the GTW and LEV groups. Based on the human-equivalent dose method that uses the body surface area ratio (described in "*Pharmacological Experimental Methodology*"), [11] GTW and LEV were administered at doses that were equivalent to the clinical doses for adults. Briefly, once a day (at 10 am) for 7 days, 10 mL/kg GTW was administered to the GTW group, 0.2 g/kg LEV was administered to the LEV group, and 10 mL/kg deionized water was administered to the NC group [11]. GTW and LEV were diluted with water to allow administration of 10 mL/kg/d.

NC, GTW-containing, and LEV-containing serum samples were then obtained. Briefly, at 2 h after the last treatment administration, the rats were weighed and then anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and blood was collected from the abdominal aorta. After leaving the samples to stand for 2–3 h, they were centrifuged (1500g 25°C, 10 min). The serum was collected and inactivated in a water bath at 56°C for 30 min. Microporous membrane was used for filtration and sterilization.

2.5. In vitro experiments

2.5.1 Groups and treatments

GC-1 spg cells were randomly divided into four groups: the blank NC group, GTW group, GTW+LEV group, and LY group (treated with NC serum and the PI3K pathway inhibitor LY294002). The GC-1 spg cells were inoculated into 6-well plates (2×10^5 cells/well), with each well containing basic medium (2 ml DMEM with 10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin) [12]. After 24 h, the medium in the NC, GTW, GTW+LEV, and LY groups was replaced with 200 µL NC serum + 1.8 ml basic medium, 200 µL GTW-containing serum + 1.8 ml basic medium, 200 µL GTW-containing serum + 1.8 ml basic medium, and 200 µL NC serum + 1.8 ml basic medium + 50 µM LY294002, respectively. After another 24 h, the medium in

the NC, GTW, GTW+LEV, and LY groups was replaced with 200 μ L NC serum + 1.8 ml basic medium, 200 μ L NC serum + 1.8 ml basic medium, 200 μ L LEV-containing serum + 1.8 ml basic medium, and 200 μ L NC serum + 1.8 ml basic medium, respectively [13].

2.5.2 CCK8 was used to assess GC-1 spg cell proliferation

GC-1 spg cells were inoculated into 96-well plates (2000 cells/well). The cells were cultured in DMEM supplemented with 10% FBS, 100 IU/mL penicillin, and 100 g/mL streptomycin in an incubator at 37°C with 5% CO₂. After 24 h of culture, serum-free medium was replaced and the cells were cultured for a further 12 h. Thereafter, the cells in each group were treated with NC, GTW-containing, and/or LEV-containing serum (10%) for 24 or 48 h. Next, 10 μ L CCK8 solution was added to each well. The culture plate was placed in an incubator at 37°C with 5% CO₂ for 2 h. Finally, the optical density (OD) at 450 nm was determined using a microplate reader.

2.5.3 Flow cytometry

The GC-1 spg cells treated with NC, GTW-containing, and/or LEV-containing serum were subjected to trypsin digestion, centrifuged (1000 rpm, 4°C, 10 min), washed with cold PBS, and centrifuged again.

Regarding the apoptosis assessment, cells were collected and suspended in Binding Buffer (in the Annexin V-FITC Apoptosis Detection Kit I). Annexin V-FITC was added and mixed gently. Binding Buffer and Propidium Iodide were then added and the mixture was left at room temperature (25°C) for 25 min, and the fluorescence intensity was then detected by flow cytometry.

For the cell cycle assessment, cells were washed with PBS, mixed with 70% cold ethanol while vortexing, left at 4°C in the dark for 24 h, and then centrifuged (1000 rpm, 25°C, 10 min). The supernatant was removed and the cells were washed twice with PBS, resuspended in Propidium Iodide/RNase Staining Buffer, and incubated at room temperature (25°C) in the dark for 15 min before cell cycle assessment.

Lastly, to assess the mitochondrial membrane potential, cells were washed with PBS, resuspended in Working JC-1 Staining Solution, and then centrifuged (2000 rpm, 25°C, 5 min). The cells were washed twice with 1×Incubation Buffer (in the JC-1 kit) and then resuspended in 10×Incubation Buffer for assessment of the mitochondrial membrane potential.

2.5.4 Scanning electron microscopy of GC-1 spg cell ultrastructure

After treatment with NC, GTW-containing, and/or LEV-containing serum, the GC-1 spg cells were subjected to trypsin digestion, centrifuged, fixed in 2.5% glutaraldehyde solution at room temperature (25°C) for 2 h, rinsed with 0.1 M PBS (pH 7.4), and postfixated with 1% osmic acid at 25°C for 2 h. The cells were then dehydrated, subjected to osmosis, (acetone: 812 embedding agent =1:1 2-4h, acetone: 812 embedding

agent =2:1 permeate overnight, pure 812 embedding agent 5-8h, pour the pure 812 embedding agent into the embedding plate, insert the sample into the embedding plate after 37°C oven overnight)and embedded(polymerization at 60°C oven for 48h). Ultrathin slices (60–80 nm thick) were prepared and stained with uranium acetate and lead citrate. Using a HT7700 transmission electron microscope (Hitachi, Tokyo, Japan), the cells and their contents (such as the plasma membrane, mitochondria, nucleus, and Golgi apparatus) were then evaluated.

2.6 In vivo experiments

2.6.1 Groups

After adaptive feeding for 1 week, 40 rats were weighed and randomly divided into four groups: blank normal control (NC) group (n=10), model (GTW) group (n=10), GTW+LEV group (n=10) and LY+LEV group (n=10).

2.6.2 Preparation of rat model of spermatogenic dysfunction using GTW

Rats in the GTW and GTW+LEV groups were gavaged with 10 ml/kg/d (1 ml contains 4 mg GTW; 40 times the amount used in humans) GTW for 4 weeks. Rats in the NC and LY+LEV groups were gavaged with 10 mL/kg/d deionized water for 4 weeks. After 4 weeks, 4 rats in each group were randomly selected and the right epididymis was removed for assessing sperm count and sperm motility in GTW-treated vs. NC rats. The remaining 6 rats in each of the four groups (GTW-treated [GTW and GTW+LEV] and non-GTW-treated [NC and LY+LEV]) were used in the subsequent experiments.

2.6.3 Treatment with LEV and LY294002

In weeks 5–8, 0.2 g/kg/d (6 times the amount in humans) LEV was gavaged to the rats in the GTW+LEV and LY+LEV groups, while 10 mL/kg/d deionized water was gavaged to the rats in the NC and GTW groups. Rats in the LY+LEV group were also given 0.3 mg/kg/week of the PI3K inhibitor LY294002 (using 0.154 mg/mL LY294002) for 1–8 weeks [6].

2.6.4 Preparation of blood and tissue samples

At the end of the treatment period, the rats were weighed and anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Blood samples were collected from the abdominal aorta, left to stand (2–3 h), and centrifuged. The levels of sex hormones, comprising estradiol (E2), follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL), and testosterone (T), in the serum were then assessed. Additionally, the testis and epididymal tissues were taken and weighed. The right epididymis was used to

assess the sperm motility and sperm count. The right testis was used for morphological observation, while the left testis was used to assess protein and mRNA expression.

2.6.5 Testis and epididymis organ coefficients

The weights of the testis and epididymis were measured using a precise electronic balance. The organ coefficients were calculated, as follows (as the ratio of testis or epididymis weight to body weight): organ coefficient = organ weight/body weight × 100%.

2.6.6 Testicular morphology

The testicular tissues were immersed in 4% paraformaldehyde for 4 h, transferred to 70% ethanol, and cut into 3-mm-thick fragments using a clean double-edged blade. Each sample was routinely dehydrated, paraffin embedded, sectioned, stained with hematoxylin and eosin (H&E), and examined under an optical microscope (BK-FL4; Chongqing Optec Instrument Co., Ltd., Chongqing, China) to assess the spermatogenic cells, supporting cells, seminiferous tubules, and other cells and structures.

2.6.7 Sperm count and motility assessment

After weighing, the right epididymis was placed in 2 ml normal saline (preheated to 33°C in a water bath at constant temperature for 15 min). The epididymis was then cut into pieces with scissors and incubated in the 33°C water bath. Gentle shaking allowed the sperm to fully swim out and mix with the normal saline. A stainless-steel sieve was used to filter the sperm mixture, the procedure was repeated, and the sperm was then centrifuged (4000 rpm, 25°C, 5 min). The sediment was obtained and 1 ml fixative was added to (and fully mixed with) the sedimented sperm. Thereafter, using a microscope, 10 X were randomly selected, the sperm cells in 10 X were counted, and the mean sperm count ($\times 10^6/\text{mL}$) was then calculated. Additionally, the sperm motility was assessed.

2.6.8 Sex hormone assays

The concentrations of E2, FSH, LH, PRL, and T in serum samples were determined by chemiluminescence assays using a Unicel Dxl 800 Immunoassay System (Beckman Coulter, Inc., Brea, CA, USA).

2.7 Western blotting

In the in vitro experiments, the GC-1 spg cells were treated (with NC, GTW-containing, and/or LEV-containing serum) and then cultured in a 6-well plate (2×10^5 cells/well). After 48 h, the cells were washed three times with precooled PBS, cell lysis buffer was added, and the mixture was centrifuged (12000 rpm, 25°C, 10 min). The protein concentration of the total proteins from the cells was determined using a

bicinchoninic acid (BCA) protein assay kit and, for the in vivo experiments, the protein concentration in testicular tissue samples was determined using the same method. After adjusting the protein concentration with Loading Buffer (5×) and boiling the proteins for 10 min, the cell and tissue samples were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane. Next, the membrane was washed with PBS, incubated with primary antibody at 4°C overnight(12h), rinsed, incubated with secondary antibody(25°C 50min) , and rinsed again. An enhanced chemiluminescence (ECL) substrate was then added. Quantity One version 4.6.2 software (Bio-Rad, Hercules, CA, USA) was used to conduct densitometric analysis of the protein bands, with the target protein expression normalized by dividing the intensity of the target protein by the intensity of the control (GADPH and Actin). The in vivo experiments only involved assessing the protein expression of a subgroup of the proteins (GFRa1, PI3K, AKT, BCL-2).

2.8 RT-qPCR

A SLAN®-96S Real-Time PCR System (Shanghai Hongshi, Shanghai, China) was used for RT-qPCR. Total RNA was extracted from the GC-1 spg cells and testicular tissue homogenates using Total RNA Extraction Kit (DNase $\times$), the RNA purity and concentration were determined, and cDNA was prepared by reverse transcription. Primer sequences are shown in **Tables1**. The samples were added to a 96-well plate for RT-qPCR. The number of cycles (Ct value) required for the fluorescent signal to reach the set threshold in each reaction was recorded. Relative gene expression was then determined using the $2^{-\Delta\Delta C_t}$ method, with normalization of the target gene to the reference gene (GAPDH and Actin). The in vivo experiments only involved assessing the mRNA expression of a subgroup of mRNAs (GFRa1, PI3K, AKT, BCL-2).

2.9 Statistical analysis

Continuous data are expressed as mean $\pm$ SEM. The unpaired *t*-test was used to compare two groups, and analysis of variance (ANOVA) was used to compare multiple groups. All statistical data were inputted by a specially assigned person and analyzed in SPSS 21.0. $P < 0.05$ was considered statistically significant.

3. Results

3.1 GTW was used to construct a GC-1 spg cell model of spermatogenic dysfunction

3.1.1 GTW inhibited GC-1 spg cell proliferation and promoted apoptosis

After 24 and 48 h of culture with GTW-containing serum in the GTW group, Cell Counting Kit-8 (CCK8) assays showed that the optical density (OD) was significantly lower in the GTW group than the blank normal control (NC) group ($P<0.01$) (Fig. 1A). Additionally, the apoptosis rate (based on flow cytometry) was significantly higher in the GTW group than the NC group ($P<0.01$) (Fig. 1B). The results indicated that GTW-containing serum inhibited spermatogenic GC-1 spg cell proliferation and promoted apoptosis.

3.1.2 GTW regulated the cell cycle of GC-1 spg cells

Compared to the NC group, the percentages of GC-1 spg cells in the G0/G1 and G2/M phases in the GTW group were significantly decreased, and the percentage in the S phase was significantly increased ($P<0.01$) (Fig. 1C). This indicated that GTW-containing serum inhibited the proliferation of GC-1 spg cells by regulating the cell cycle.

3.1.3 GTW reduced the mitochondrial membrane potential of GC-1 spg cells

The Apoptotic Mitochondrial Membrane Potential Detection Kit (JC-1) combined with flow cytometry showed that the proportion of GC-1 spg cells exhibiting mitochondrial membrane depolarization significantly increased in the GTW group compared to the NC group ($P<0.01$) (Fig. 1D).

3.1.4 GTW damaged the GC-1 spg cell ultrastructure

Scanning electron microscopy showed that the plasma membrane of the GC-1 spg cells in the NC group was intact, there were pseudopodia and projections on the membrane, and the organelles were abundant and slightly swollen. The nucleus was round, and the nuclear membrane was clear. There were many mitochondria; most of them were slightly swollen, with intact membranes, disintegrate weakened local matrix, and a cristae structure was completion. The rough endoplasmic reticulum was abundant, some of the structure was slightly dilated, and ribosome attachment was observed on the surface. There were also many autolysosomes.

Compared to the NC group, the cell ultrastructure in the GTW group was significantly changed, with partially uplifted plasma membrane edges and ruptured plasma membranes, fewer pseudopodia and projections on the membrane, and reduced local electron density in the cell matrix. The nucleus was irregularly shaped, with local depressions, dissolved matrix and a ruptured or missing mitochondria cristae. The rough endoplasmic reticulum was considerably expanded, with local degranulation and accumulation of flocculent material. There were fewer autolysosomes. There was also hypertrophy of the Golgi apparatus, and an obvious expansion of the Golgi. Lastly, there was a considerable increase in lipid droplets, which were aggregated (Fig. 2).

3.2 LEV improved the GTW-induced spermatogenic dysfunction of GC-1 spg cells

3.2.1 Serum cytotoxicity evaluation of LEV-containing serum samples

To ensure that the LEV-containing serum samples did not exhibit cytotoxicity toward GC-1 spg cells, normal GC-1 spg cells were used to preliminarily evaluate the cytotoxicity of LEV-containing serum at various concentrations compared to NC serum before establishing the cell model (Fig. 3A). The results showed that the LEV-containing serum samples showed no cytotoxic [difference](#) [p>0.05] at any of the concentrations tested, and the experiments could be designed reasonably within the range of the tested concentrations.

3.2.2 LEV inhibited GC-1 spg cell apoptosis

The apoptosis rate was significantly increased in the GTW group and the LY group (treated with the PI3K pathway inhibitor LY294002) compared to the NC group ($P<0.01$). Compared to the GTW group, the apoptosis rate in the GTW+LEV group was significantly reduced ($P<0.01$) (Fig. 3B). The results showed that LEV inhibited GTW-induced apoptosis of GC-1 cells.

3.2.3 LEV increased the percentages of GC-1 spg cells in the G0/G1 and G2/M phases

Compared to the NC group, the percentages of GC-1 spg cells in the G0/G1 and G2/M phases in the GTW and LY groups were significantly reduced and the percentage in the S phase was significantly increased ($P<0.01$). Compared to the GTW group, GTW+LEV significantly increased the percentages of GC-1 spg cells in the G0/G1 and G2/M phases and significantly decreased the percentage in the S phase ($P<0.01$) (Fig. 3C). The results showed that LEV promoted the proliferation of GC-1 spg cells by regulating the cell cycle.

3.2.4 LEV increased the mitochondrial membrane potential of GC-1 spg cells

Compared to the NC group, the proportion of GC-1 spg cells exhibiting mitochondrial membrane depolarization was significantly increased in the GTW and LY groups ($P<0.01$). Compared to the GTW group, the proportion in the GTW+LEV group was significantly decreased ($P<0.05$) (Fig. 3D). The results showed that LEV ameliorated the GTW-induced decrease in mitochondrial membrane potential in GC-1 spg cells.

3.2.5 LEV repaired the GTW-induced ultrastructure damage of GC-1 spg cells

Scanning electron microscopy showed that, in the GTW and LY groups (in contrast to in the NC group), the plasma membranes of the GC-1 spg cells were largely ruptured, the organelles were free, there were fewer pseudopodia and projections on the membrane, vacuoles were increased, and the whole cell matrix had disintegrated. The nucleus was irregularly shaped, with local depressions, multiple nucleoli, and a clear nuclear membrane. There were many mitochondria, and most of them were severely swollen, with fractured or missing cristae. The rough endoplasmic reticulum was considerably expanded and degranulated. There were many autolysosomes. There was also hypertrophy of the Golgi apparatus, and obvious expansion of the Golgi. There were few lipid droplets. Compared to in the GTW group, the ultrastructure of cells in the GTW+LEV group was considerably changed, with the morphology of each type of organelle being somewhat recovered, but not to the extent in the NC group (Fig. 4).

3.3 LEV improved the spermatogenic function of GC-1 spg cells by regulating PI3K/AKT pathway-related protein and mRNA expression

3.3.1 PI3K/AKT pathway-related protein expression detected by western blotting

Compared to the NC group, the protein expression of GFRa1 and RET (upstream regulators of the PI3K/AKT pathway), PI3K and AKT, and BCL-2 (an anti-apoptotic factor) in GC-1 spg cells in the GTW group was significantly decreased ($P<0.01$), while the protein expression of BAD and BAX (pro-apoptotic factors) was significantly increased ($P<0.01$). Compared to the NC group, the GFRa1 and RET protein expression in the LY group was not significantly different (i.e., LY294002 did not affect the upstream regulators), while the PI3K, AKT, and BCL-2 protein expression was significantly decreased and the BAD and BAX protein expression was significantly increased ($P<0.01$). The GFRa1, RET, PI3K, AKT, and BCL-2 protein expression was significantly increased in the GTW+LEV group compared to the GTW group ($P<0.01$), while the BAD and BAX protein expression was significantly decreased ($P<0.01$) (Fig. 5A, Fig.6). The results indicated that LEV downregulated BAD and BAX protein expression by upregulating GFRa1, RET, PI3K, AKT, and BCL-2 expression, thus improving the GTW-induced spermatogenic dysfunction of GC-1 spg cells.

3.3.2 PI3K/AKT pathway-related mRNA expression detected by real-time quantitative polymerase chain reaction (RT-qPCR)

Compared to the NC group, GFRa1, RET, PI3K, AKT, and BCL-2 mRNA expression in GC-1 spg cells in the GTW group was significantly decreased ($P<0.01$), while the BAD and BAX mRNA expression was significantly increased ($P<0.01$). Compared to the NC group, GFRa1 and RET mRNA expression in GC-1 spg cells in the LY group was not significantly different, while the PI3K, AKT, and BCL-2 mRNA expression was significantly decreased and the BAD and BAX mRNA expression was significantly increased ($P<0.01$). The GFRa1, RET, PI3K, AKT, and BCL-2 mRNA expression was significantly increased in the GTW+LEV group compared to the GTW group ($P<0.01$ or $P<0.05$) (Fig. 5B). The results indicated that LEV downregulated BAD and BAX mRNA expression by upregulating GFRa1, RET, PI3K, AKT, and BCL-2 mRNA expression, thus improving the GTW-induced spermatogenic dysfunction of GC-1 spg cells.

3.4 LEV improved GTW-induced spermatogenesis dysfunction in rats

3.4.1 Body, testis, and epididymis weights

Before the experiment, there was no difference in the weight of the rats in each group, and the weight of the rats in each group increased gradually as the experiment went on. After the experiment, the body, testis, and epididymis weights were significantly lower in the GTW group than the NC group ($P<0.01$). Compared to the GTW group, these weights were significantly increased in the GTW+LEV group ($P<0.01$ or $P<0.05$). Compared to the GTW+LEV group, these weights were significantly decreased in the LY+LEV group ($P<0.01$ or $P<0.05$) (Fig. 7A–C).

3.4.2 Sperm count and motility

The sperm count was significantly lower in the GTW group than the NC group ($P<0.01$), but significantly higher in the GTW+LEV and LY+LEV group than in the GTW group ($P<0.01$) (Fig. 7D). The percentage of forward-motion + non-forward-motion sperm was also significantly lower in the GTW group than the NC group ($P<0.01$), but significantly higher in the GTW+LEV and LY+LEV groups than the GTW group ($P<0.01$) (Fig. 7E).

3.4.3 Serum sex hormone levels

The levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone (T) were significantly lower in the GTW group than the NC group ($P<0.01$), but significantly higher in the GTW+LEV and LY+LEV groups than the GTW group ($P<0.01$) (Fig. 7F–J). Compared to the NC group, prolactin (PRL), and estradiol (E2) levels in the GTW group were not significantly different ($P>0.05$) (Fig. 7H,I).

3.4.4 Testicular morphology

Optical microscopy showed that the spermatozoa in the testicular tissues of the NC rats were present in a normal, organized arrangement. The structure of each layer was complete and orderly. There were many normal spermatogenic and sperm cells at all levels in the lumen. In the GTW rats, the structure of the seminiferous tubules was highly disordered. Narrowing of the space between the seminiferous tubules, partially degenerated epithelia, numerous vacuoles, few mature sperm in the lumen, abnormal sperm morphology, a large increase in the percentage of sperm cells among the cells in the lumen, and missing spermatogenic cells at various levels in many of the small tubules. The testicular morphology was better in the GTW+LEV and LY+LEV groups than the GTW group, with larger lumens and considerably higher sperm counts. In addition, the structures of some seminiferous tubules was somewhat recovered, but not to the standard in the NC group (Fig. 8).

3.5 LEV improved the spermatogenic function of rats by regulating PI3K/AKT pathway-related protein and mRNA expression

3.5.1 PI3K/AKT pathway-related protein expression detected by western blotting

Compared to the NC group, the GFRa1, PI3K, AKT, and BCL-2 protein expression in the GTW group was significantly decreased ($P<0.01$). These protein levels were significantly increased in the GTW+LEV and LY+LEV groups compared to the GTW group ($P<0.01$). There were no significant differences between the GTW+LEV and LY+LEV groups (Fig. 9A, Fig. 10).

3.5.2 PI3K/AKT pathway-related mRNA expression detected by RT-qPCR

Compared to the NC group, the GFRa1, PI3K, AKT, and BCL-2 mRNA expression was significantly decreased in the GTW group ($P<0.01$). These mRNA expression levels were significantly increased in the GTW+LEV and LY+LEV groups compared to the GTW group ($P<0.01$). There were no significant differences between the GTW+LEV and LY+LEV groups (Fig. 9B). These results indicated that LEV improved the spermatogenic function of rats with GTW-induced spermatogenic dysfunction by upregulating the GFRa1, PI3K, AKT, and BCL-2 proteins and mRNA expression.

4. Discussion

In the in vivo experiments of rats with GTW-induced spermatogenic dysfunction, LEV significantly improved the sperm count, motility, and testicular pathology and increased the serum FSH, LH, and T levels. In vitro, LEV inhibited GC-1 spg cell apoptosis and increased the cell proliferation. In addition, LEV

increased the GFRa1, RET, PI3K, AKT, BCL-2 protein and mRNA expression and decreased the BAD and BAX protein and mRNA expression. Thus, the mechanism of action of LEV is closely related to the PI3K/AKT signaling pathway and the expression of the above related proteins.

In recent years, GTW has been widely used for the establishment of rat models of spermatogenic dysfunction [14]. Our previous study found that GTW administered via the stomach(40mg/kg/d) for 4 weeks can be used to establish a rat model of spermatogenic dysfunction [11]. The in vitro experiments showed that GTW-containing serum decreased the proportions of GC-1 spg cells in the G0/G1 and G2/M phases and increased the proportion that exhibited mitochondrial membrane depolarization, thus promoting apoptosis and decreasing cell proliferation, which, in turn, affected the cell morphology and function. The in vivo experiments confirmed that GTW disrupted the testicular morphology and function, decreasing the serum sex hormone levels. Our results indicate that we successfully established GTW-induced cell and rat models of spermatogenic dysfunction [10].

LEV has been shown to improve sperm count and motility [15,16], but the mechanism was previously unclear. The PI3K/AKT signaling pathway, by inhibiting BAX and promoting BCL-2, decreases the opening of mitochondrial permeability transition pores (mPTPs) and the release of small pro-apoptotic molecules into the cytoplasm, thereby decreasing apoptosis. We found that GTW decreased the PI3K, AKT, and BCL-2 expression, and increased the BAD and BAX expression, while affecting spermatogonial function by decreasing cell proliferation and mitochondrial function and increasing apoptosis. Thus, the abnormal expression of proteins related to the PI3K/AKT pathway resulted in dysfunction, in line with previous studies[11]. In contrast, LEV (in the GTW+LEV group) increased the PI3K, AKT, and BCL-2 protein expression and decreased the BAD and BAX protein expression. This decreased the proportion of cells exhibiting mitochondrial membrane depolarization, increased the proportions of cells in the G0/G1 and G2/M phases, and decreased apoptosis compared to the levels in the GTW group. LEV may improve mitochondrial function, the cell cycle, and cell proliferation by regulating the expression of related mRNA and proteins in the PI3K/AKT signaling pathway. It has been reported that mitochondria regulate the cell cycle and cell proliferation [17]. Additionally, it is known that sperm motility mainly depends on the movement of the sperm flagellum, and the energy for this is mainly provided by mitochondrial oxidative phosphorylation or glycolysis [18]. Oxidative phosphorylation leads to the production of mitochondrial reactive oxygen species (mtROS) in the sperm [19]. Excessive mtROS followed by oxidative damage to mitochondrial structures and function alters the mitochondrial membrane potential in the sperm, resulting in decreased sperm motility and, ultimately, male infertility [19]. It has been reported that LEV, which acts as an energy reserve, enters sperm cells via the plasma membrane when they are in the epididymal cavity [20]. As the sperm cells move from the head to the tail of the epididymis, LEV alters the composition and structure of their plasma membranes, which ensures that the membrane fluidity is maintained, which is important for sperm function[21].

GFRa1 and RET are upstream regulators of the PI3K/AKT pathway and play key roles in spermatogonial cell division, sperm cell proliferation, and differentiation [22]. We found that LEV significantly increased the GFRa1 and RET expression, which was also positively associated with the PI3K and AKT expression.

However, compared to the NC group, GFRa1 and RET expression did not change significantly after LY treatment, as LY targets PI3K and its downstream proteins.

Spermatogenesis and maturation are not only regulated locally by the interaction between spermatogenesis-related genes and supporting cells in the testes, but also by neuroendocrine regulation. Sex hormones are closely related to male reproductive function, with the hypothalamus–pituitary–testicular reproductive axis playing an important role in spermatogenesis [23]. We found that the serum T, FSH, and LH levels in the GTW rats were decreased, which would affect spermatogenic function. The epididymis is the site of sperm maturation. The level of T in the epididymis fluid influences the physiological function of the epididymis. Low T level decreases sperm production and maturation, ultimately affecting the sperm quality [24]. FSH can promote T secretion in the testes and LH can promote spermatogenesis [25]. After LEV treatment, serum T, FSH and LH levels were significantly increased. To further explore the underlying mechanism, we investigated the expression of several proteins closely related to sex hormone levels.

In conclusion, we found that GTW induced spermatogenic dysfunction (based on in vitro and in vivo experiments), and LEV ameliorated the GTW-induced spermatogenic dysfunction. LEV may operate via the PI3K/AKT signaling pathway, with increases in GFRa1, RET, PI3K, AKT, and BCL-2 protein expression and decreases in BAD and BAX protein expression, so that the percentages of GC-1 spg cells in the G0/G1 and G2/M phases increase, mitochondrial membrane potential is improved, apoptosis decreases, and the sperm count and motility improve. This study explored the potential mechanism of LEV in the treatment of oligospermia and provides an experimental basis for the application of LEV in the treatment of male infertility.

Declarations

Ethical Approval and Consent to participate

All animal procedures were performed in accordance with relevant guidelines and regulations of Good Animal Practice approved by the institutional and were approved by the Experimental Animal Welfare Ethics Committee of Dongzhimen Hospital, Beijing University of Chinese Medicine (NO.19-27) in Beijing, China.

Consent for publication

All the authors grant consent of the publication.

Availability of supporting data

All the supporting data are availability.

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Author contributions

H.L., B.W., J.W., and F.M. designed the study. J.W., and B.B. carried out the experiments. F.M., S.D. and J.W. analyzed the data. F.M., J.W., and B.B. wrote and edited the manuscript. H.D. and J.F. helped in the execution of the research. All authors approved the final version of the manuscript.

Competing interests

The authors declare that there are no competing interests.

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Table

Table 1. Primer sequences used for real-time quantitative polymerase chain reaction (RT-qPCR)

mer name			Primer sequences (5' to 3')	Product size (bp)
PDH	In vivo experiment	Forward primer	TGGAGTCTACTGGCGTCTT	138
		Reverse primer	TGTCATATTTCTCGTGGTTCA	
	In vitro experiment	Forward primer	AGGTCGGTGTGAACGGATTTG	123
		Reverse primer	TGTAGACCATGTAGTTGAGGTCA	
Ra1	In vivo experiment	Forward primer	TTGGCAATGGCTCAGATGTGA	75
		Reverse primer	CAGTGGTAGTGGTGGCAGTG	
	In vitro experiment	Forward primer	GAGTCAAGGTCTGTCAGCAACT	172
		Reverse primer	TCAGGCAATCTTCCAGGTCATT	
K	In vivo experiment	Forward primer	CTGGTAGACGATGACGAGGATT	162
		Reverse primer	CACTACAGAGCAGGCATAGCA	
	In vitro experiment	Forward primer	AATGATGCTTGGCTCTGGAATG	186
		Reverse primer	TGCTGCTTGATGGTGTGGAA	
T	In vivo experiment	Forward primer	GCTTCTACGGTGCGGAGATTGTGT	125
		Reverse primer	GTCCGTTATCTTGATGTGCCCCGTC	
	In vitro experiment	Forward primer	ATGAACGACGTAGCCATTGTG	116
		Reverse primer	TTGTAGCCAATAAAGGTGCCAT	
L-2	In vivo experiment	Forward primer	GGGCTACGAGTGGGATACTGGAG	101
		Reverse primer	CGGGCGTTTCGGTTGCTCT	
	In vitro experiment	Forward primer	ATGCCTTTGTGGAACATATATGGC	120
		Reverse primer	GGTATGCACCCAGAGTGATGC	
T	In vitro experiment	Forward primer	GCAGCCTTCCAGCATCAACT	274
		Reverse primer	GCCACCACATTCCTCACACT	
D	In vitro experiment	Forward primer	GGCAGCCACCAACAGTCATCAT	105
		Reverse primer	TCCTCCTCCATCCCTTCATCCT	
X	In vitro experiment	Forward primer	CAGGGTTTCATCCAGGATCGAGCAGG	185
		Reverse primer	CGGGGGGAGTCCGTGTCCACGTCAG	
		Reverse primer	GCATCCCTACCGACACCAC	

Figures

Figure 1

Construction of the model of spermatogenic dysfunction based on spermatogenic GC-1 spg cells (A) CCK8 detection of optical density (OD) after 24 and 48 h of culture with GTW-containing serum; (B) apoptosis rate detected by flow cytometry; (C) percentages of spermatogenic GC-1 spg cells in the G0/G1, G2/M, and S phases; (D) proportion of cells exhibiting mitochondrial membrane depolarization. Values are mean $\pm$ SEM. Student's t-test was used. GTW group was compared to the NC group. **P<0.01

NC (×1.2K)

GTW (×1.2K)

NC (×5.0K)

GTW (×5.0K)

Figure 2

Ultrastructure of GC-1 spg cells. Protuberances (PS), nuclei (N), nucleoli (Nu), mitochondria (M), rough endoplasmic reticulum (RER), autophagolysosome (ASS), Golgi apparatus (Go), Lipid droplet (LD).

Figure 3

LEV improved the GTW-induced spermatogenic dysfunction of spermatogenic GC-1 spg cells. (A) Cytotoxicity evaluation of LEV-containing serum samples; (B) apoptosis rate detected by flow cytometry; (C) percentages of GC-1 spg cells in the G0/G1, G2/M, and S phases; (D) proportion of cells exhibiting mitochondrial membrane depolarization. Values are mean $\pm$ SEM. Student's t-test was used. **, ## $P < 0.01$. * vs. NC group; # vs. GTW group.

Figure 4

Ultrastructure of GC-1 spg cells. Protuberances (PS), nuclei (N), nucleoli (Nu), mitochondria (M), rough endoplasmic reticulum (RER), autophagolysosome (ASS), Golgi apparatus (Go); Lipid droplet (LD).

Figure 5

LEV improved the spermatogenic function of GC-1 spg cells by regulating PI3K/AKT pathway-related protein and mRNA expression and CYP19A1, CYP17A1, AR, and SRD5A2 protein and mRNA expression. PI3K/AKT pathway-related (A) protein expression detected by western blotting and (B) mRNA expression detected by RT-qPCR. Student's t-test was used. **, ## $P < 0.01$; *, # $P < 0.05$. * vs. NC group; # vs. GTW group; & LY+LEV group vs. GTW+LEV group.

Figure 6

Electrophoretogram of western blotting(PI3K/AKT pathway-related protein).

Figure 7

LEV improved GTW-induced spermatogenesis dysfunction in male Sprague-Dawley rats. (A) Body weight, (B) Epididymis weight; (C) Testis weight; (D) Sperm count; (E) Percentage of forward-motion (PR) + non-forward-motion (NP) sperm; (F) FSH levels; (G) LH levels; (H) E2 levels; (I) PRL levels; (J) T levels. Values are mean $\pm$ SEM (n=6 rats per group). Student's t-test was used. **, ## and && P<0.01; *, # and & P<0.05. * vs. NC group; # vs. GTW group; & LY+LEV group vs. GTW+LEV group.

Figure 8

Testicular morphology. Testicular tissues of rats were stained with hematoxylin and eosin (H&E) to observe the pathologic changes using scanning electron microscopy ($n=6$ rats per group). (A) In the NC group: (i) the seminiferous tubules are closely arranged, and the boundary between the basal membrane and the stroma is clear and even (arrow 1) and (ii) all levels of spermatogenic and sperm cells are arranged in order (arrows 2–5). (B) In the GTW group: (i) the tubules are sparsely arranged, there is interstitial edema, the tubule epithelium is separated from the stroma, and the tubules have cavity-like structures (arrow 6); (ii) there is degeneration, nuclear shrinkage, and necrosis of spermatogenic and sperm cells in the tubules (arrows 7 and 9); (iii) the tubules are damaged to different degrees, and their number and morphology are significantly changed (arrows 8 and 10); and (iv) some spermatogenic cells at all levels have disappeared, and there is proliferation of interstitial cells (arrow 9). (C, D) In the GTW+LEV and LY+LEV groups: (i) some of the tubules are closely arranged, and the boundary between the basal membrane and the stroma is clear and even (arrows 11 and 16); (ii) some of the tubules have returned to normal (arrows 13, 14, 18, and 19); and (iii) all levels of spermatogenic cells are regularly aligned (arrows 12, 15, 17, and 20).

Figure 9

LEV improved the spermatogenic function of rats with spermatogenic dysfunction by regulating PI3K/AKT pathway-related protein and mRNA expression. PI3K/AKT pathway-related (A) protein expression detected by western blotting and (B) mRNA expression detected by RT-qPCR. Values are mean $\pm$ SEM (n=6 rats per group). Student's t-test was used. **, ## P<0.01. * vs. NC group; # vs. GTW group.

Figure 10

Electrophoretogram of western blotting(PI3K/AKT pathway-related protein)