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Original Article

RXFP2 as novel potential biomarker for abnormal differentiation induced by diethylstilbestrol in the gubernaculum of fetal mice

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Abstract: Environmental estrogens (EEs) have been correlated with abnormalities in the male urogenital system. However, the mechanism underlying the effect of these molecules remains unclear. In vitro and in vivo experiments were performed to examine the expression level and mechanism of relaxin family peptide receptor 2 (RXFP2) in the gubernaculum of fetal mice following diethylstilbestrol (DES) treatment. The in vivo results demonstrate that DES treatment increased the stillbirth rate gradually, decreased the gubernacular cone volume significantly, and disrupted the tissue structure, leading to incomplete testicular descent. In vitro experiments reveal that DES administration resulted in abnormal cellular morphology and structural disorder of gubernacular cells, which lost their original morphology in a dose-dependent manner. Moreover, DES-induced F-actin rearrangement and stress fiber formation in cultured cells. Protein quantitative analysis showed that the RXFP2 level in each experimental group was significantly lower than that of the normal group. In conclusion, DES affects the morphology and alters the gubernaculum structure, as well as the expression of RXFP2 protein. These data demonstrate that DES is toxic to gubernaculum in fetal mice, and that RXFP2 is associated with the abnormal gubernaculum morphology induced by DES. Taken together, these data suggest that RXFP2 may be a novel potential biomarker for abnormal differentiation of the gubernaculum.

Keywords: Diethylstilbestrol (DES), relaxin family peptide receptor 2 (RXFP2), insulin like factor 3 (INSL3), biomarker, gubernaculum, testicular descent

Introduction

Many epidemiological studies have proven that the reproductive capability of humans has gradually declined over the past few decades. Abnormalities in the development of the male genitourinary system have significantly increased, which may be closely related to exposure to environmental estrogens (EEs) during pregnancy [1-4]. The influence of EEs on human male reproductive health mainly manifests as congenital malformation, such as cryptorchidism, hypospadias, and disorders of sexual development (DSD), abnormal reproduction and sexual function, and urogenital system tumors in adults. These diseases are a part of what is known as testicular dysgenesis syndrome (TDS), which is caused by disrupted testicular development in utero [5-8]. As a core governing organ, the testicle influences or induces development of the entire male reproductive system. Therefore, studies to determine the factors that influence normal testicular descending development are particularly important.

Normal development of the testicles consists of two descent stages, transabdominal and ingui-

nal scrotal. In the transabdominal stage, the testicle descends to the groin, gubernacular cells rapidly proliferate, and the cranial suspensory ligament simultaneously degenerates. In the inguinal scrotal stage, the gubernaculum degenerates, and the testicle descends from the bottom of the abdominal cavity to the bottom of the scrotum [9-11]. In transabdominal descent, the gubernaculum typically undergoes proliferation, differentiation, migration, contraction and hormone metabolism [12]. Currently, the regulatory factor with widely accepted critical roles in this stage is insulin like factor 3 (INSL3), which has been shown to stimulate gubernaculum hyperplasia and control its development [13-16].

INSL3 plays its biological role by binding to a specific receptor called RXFP2 and forming the INSL3-RXFP2 ligand-receptor complex [17, 18]. In previous studies, EEs have been shown to inhibit INSL3 generation in Leydig cells in the testicles by varying degrees and further influencing testicular descent and testicle development, which may be indirectly realized by affecting the morphology and function of the gubernaculums [19]. However, the influence of EEs on gubernacular development remains unclear. There have been many reports describing the influence of EEs on INSL3 [14, 20], but the data regarding their direct effect on RXFP2 are still lacking.

To confirm whether EEs effects on the development of gubernaculum are directly related to RXFP2, a testicular descent mouse model and cultured gubernacular cells were established previously [7, 21]. In this study, we sought to investigate the mechanism of RXFP2 that is affected by estrogen during gubernaculum development. We exposed the mice and gubernacular cells to diethylstilbestrol (DES) to observe its influence on the morphology, structure, and RXFP2 of the gubernaculum tissues and gubernacular cells. Our data provide a new theoretical basis for the reproductive toxicity mechanism of EEs.

Materials and methods

Antibodies and reagents

Diethylstilbestrol (DES) and trypan blue were purchased from Sigma Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) with high sugar content and no phenolic red was purchased from Applichem Biotechnology (Darmstadt, Germany). Fetal bovine serum (FBS) without estrogen was purchased from BioInd (Haemek, Israel). Dimethyl sulfoxide (DMSO) was purchased from Sangon Biotech (Shanghai, China). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies (Tokyo, Japan). Goat anti-mouse RXFP2 polyclonal antibody was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Fluoresceinisothiocyanate (FITC)-conjugated rabbit anti-goat IgG was purchased from BOSTER Biological Technology (Wuhan, China).

Animals

Kunming mice aged 8 to 10 weeks were purchased and maintained at the Animal Research Laboratory in the Medical College of Shantou University. These mice were maintained under controlled conditions at a fixed temperature (23±1°C) and under a 12-h night/dark cycle, with free access to water and laboratory chow. All animals received excellent nutrition, were drug-free, and exhibited normal mental and neurological states. The Shantou University Medical College Animal Experimental Ethics Committee approved the use of Kunming mice for protocol purposes (NO. SUMC 2018-064). The procedures for care of the Kunming mice were in accordance with the guidelines for the treatment of experimental animals (Beijing Science Press (2016).

Grouping and treatment

Kunming mice were raised in cages with a female/male ratio of 2:1. Female mice were considered pregnant when a vaginal plug was found, at which point the gestational age was recorded as embryonic day 0 (ED 0). Pregnant mice were randomly assigned to one of six groups: normal, control, or an experimental group (DES at 0.10, 1.00, 10.00, and 100.00 μg/kg·d) (n = 10). Mice in the experimental groups were subcutaneously injected with DES during ED 9-ED 17, and those in the control group were subcutaneously injected with DMSO + physiological saline (1:2,000) of equal volume. Mice in the normal group were not medicated.

Calculating the gubernacular cone volume

Pregnant mice were euthanized by removing the cervical vertebra to immediately extract the

fetal mice. The lower abdomen of the fetal mice was fixed in 10% neutral formaldehyde to make sections. After hematoxylin and eosin (H&E) staining, microscopy was performed to visualize the gubernaculum and observe the morphological changes.

Sections of the specimens were made with the same thickness (d), except for the last one (d1), which was different from the others (d). A total of n sections and n+1 cross sections were obtained. The area of the cross section of the gubernacular cone under the microscope was measured (S), and the volume was calculated as: $V = \frac{d}{3}[S_0+S_{n-2}+2(S_2+S_4+...+S_{n-4}) + 4(S_1+S_3+...+S_{n-3})] + \frac{(d+d1)}{6}[2S_n+2S_{n-2} - \frac{d1}{d}S_{n-2} - \frac{d}{d1}S_n + \frac{(d+d1)^2}{dd1}S_{n-1}]$ [22].

Scanning electron microscopy for observing the extent of testicular descent

The extent of testicular descent was observed under the scanning electron microscope and standardized. The distance between the inferior pole of the kidney to the bladder neck (a) was considered as 100 U, and that to the testicle center on the same side (b) and thus the extent of testicular descent was (b/a) × 100 U. A higher value indicated more complete testicular descent.

Immunohistochemical staining

The specimens were continuously sectioned at a thickness of 4 μm and were then dewaxed. After inactivation of endogenous enzymes and antigen repair by microwaving, primary antibody (goat anti-mouse RXFP2 polyclonal antibody, 1:100) was added, and the sections were incubated overnight. Then, the sections were washed three times with phosphate-buffered saline (PBS), and secondary antibody (horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG, 1:500) was added. The samples were incubated at 37°C for 20 min, developed with 3,3'-diaminobenzidine (DAB), dehydrated, cleared with xylene, sealed, and observed under the microscope. Detection of positive antibody signal was indicated by a pale brown or dark brown precipitate. PBS was used in place of the primary antibody as a negative control. Images were taken using a microscope and analyzed using Image Pro Plus (IPP) 6.0 software. Ten sections were selected from each group to record the optical density (OD) value of the positive substance.

Western blot analysis

The gubernaculum tissue was cut into pieces, lysed with radioimmunoprecipitation assay (RIPA) protein lysis buffer, completely ground on ice, and centrifuged at 12,000 × g for 30 min at 4°C to obtain a supernatant. The proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After 1 h, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane using wet transfer. The PVDF membrane was blocked with skim milk for 1 h before incubation with primary antibody (1:100, goat anti-mouse RXFP2 polyclonal antibody) at 4°C overnight. The membrane was washed three times with Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST), and incubated with secondary antibody (1:1,000, HRP-rabbit anti-goat IgG) at room temperature for 1-2 h. After the chemiluminescence reaction, the gel results were scanned and photographed with a gel imaging system, and the OD of the target band was analyzed using ImagePro software. The ratio of the measured protein gray value to the internal reference gray value indicated the relative protein level. These experiments were performed in triplicate, and the values were averaged.

Culture and grouping of gubernacular cells

The gubernaculum tissue was removed using a 3 × magnification surgical magnifying glass. The tissue was digested in Dulbecco's modified Eagle's medium (DMEM) containing Type I collagenase (1 mg/ml) at 37°C for 1 h, and then diluted in a 3- to 4-fold higher volume of medium without estrogen. The medium was decanted, filtered through a 25-μm filter, centrifuged at 1,000 rpm for 5 min (centrifugation radius 15 cm), and the supernatant was then discarded. The cell pellet was added to DMEM containing fetal bovine serum (FBS) but lacking estrogen (10% v/v), aerated to create a single-cell suspension, seeded into a 25-ml culture flask, and incubated in a 5% CO_2 incubator at 37°C with saturated humidity. Cells were randomly assigned to a normal, solvent control, or experimental group (four doses of DES at 0.01, 0.10, 1.00, and 10.00 μg/ml).

Gubernacular cell counting and determination of the survival rate

The cells in suspension at the time of subculture were counted. After using alcohol to wash the counting plate, a cover slip was placed on the plate. A small amount of uniform cell suspension was dropped into the gap between the plate and glass, and observed under a microscope to determine the quantity of cells that was uniformly dispersed. Cells in the four corners were counted, and those on the line were counted only when they were in the left upper corner, not in the right lower corner. The cell suspension concentration was calculated using the following equation: (number of cells/ml) = (total number of cells in four corners) × 10,000 × dilution fold.

Cell viability was detected using trypan blue, which stains only dead cells. A uniform cell suspension was thoroughly mixed with 0.2% trypan blue (9:1) and was maintained at room temperature for 2 to 3 min. Next, 10 μl of cell suspension was added to the gap between the counting plate and cover slip, and then observed under an inverted microscope. Cell viability the cells in the four corners of the counting plate was calculated as follows: cell viability (%) = [number of living cells/(number of living cells + number of dead cells)] × 100.

Immunofluorescence

Cells were subcultured for 1 to 2 days in a monolayer and exhibited good morphology. After discarding the culture medium, cells were added to medium containing DES at final concentrations of 0 (control group), 0.01, 0.10, 1.00, and 10.00 μg/ml (1 μl). The normal control group did not receive any DES. After culturing the cells for 48 h, the cover slip was washed lightly with PBS, and the cells were fixed with 4% paraformaldehyde for 15 min, washed three times with PBS, permeabilizaed in 0.2% Triton X-100 in PBS for 10 min, and blocked with 1% BSA. Then, primary antibody (goat anti-mouse RXFP2 polyclonal antibody, 1:100) was applied to the cover slip before storage in a wet box at 4°C overnight. The next day, the cover slip was washed three times with PBS, and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-goat IgG secondary antibody (1:500) was applied. F-actin was detected by incubation with 5 μg/ml FITC-phalloidin in PBS for 40 min. The cover slip was incubated at room temperature for 60 min, washed three times with PBS, sealed with glycerin, and visualized by a fluorescence microscopy.

Western blot analysis

Gubernacular cells from each group were lysed with RIPA buffer, and after centrifugation, protein was extracted from the supernatant. The proteins were separated by SDS-PAGE, and then transferred onto nitrocellulose membrane. The membrane was blocked with skim milk, rinsed, and then incubated with goat anti-mouse RXFP2 polyclonal antibody (1:100) at 4°C overnight, followed by incubation with the secondary antibody (HRP-rabbit anti-goat IgG 1:500) for 1-2 h at room temperature. After chemiluminescent reaction, the gel results were scanned and photographed with a gel imaging system, and the OD of the target band was analyzed using ImagePro software. The ratio of measured protein gray value to the internal reference gray value indicated relative protein level. These experiments were performed in triplicate, and the values were averaged.

Statistical analysis

All data are expressed as the mean ± standard deviation (SD). SPSS 20.0 software was used to analyze the data. The constituent ratio was determined by the chi-square test, and multiple comparisons between groups were analyzed by the homogeneity test of variances and one-way analysis of variance (ANOVA). Pairwise comparison was analyzed from two independent samples using the Student's t-test. $P < 0.05$ was considered as statistically significant.

Results

Dose-dependent relationship between stillbirth rate and DES exposure

We established a mouse model of DES exposure by subcutaneous injection of DES during pregnancy (**Figure 1A**). In the experimental groups, some pregnant mice exhibited vaginal bleeding with a high stillbirth rate. The stillbirth rates in the normal and control groups were 2.22% (2/90) and 2.15% (2/93), respectively.

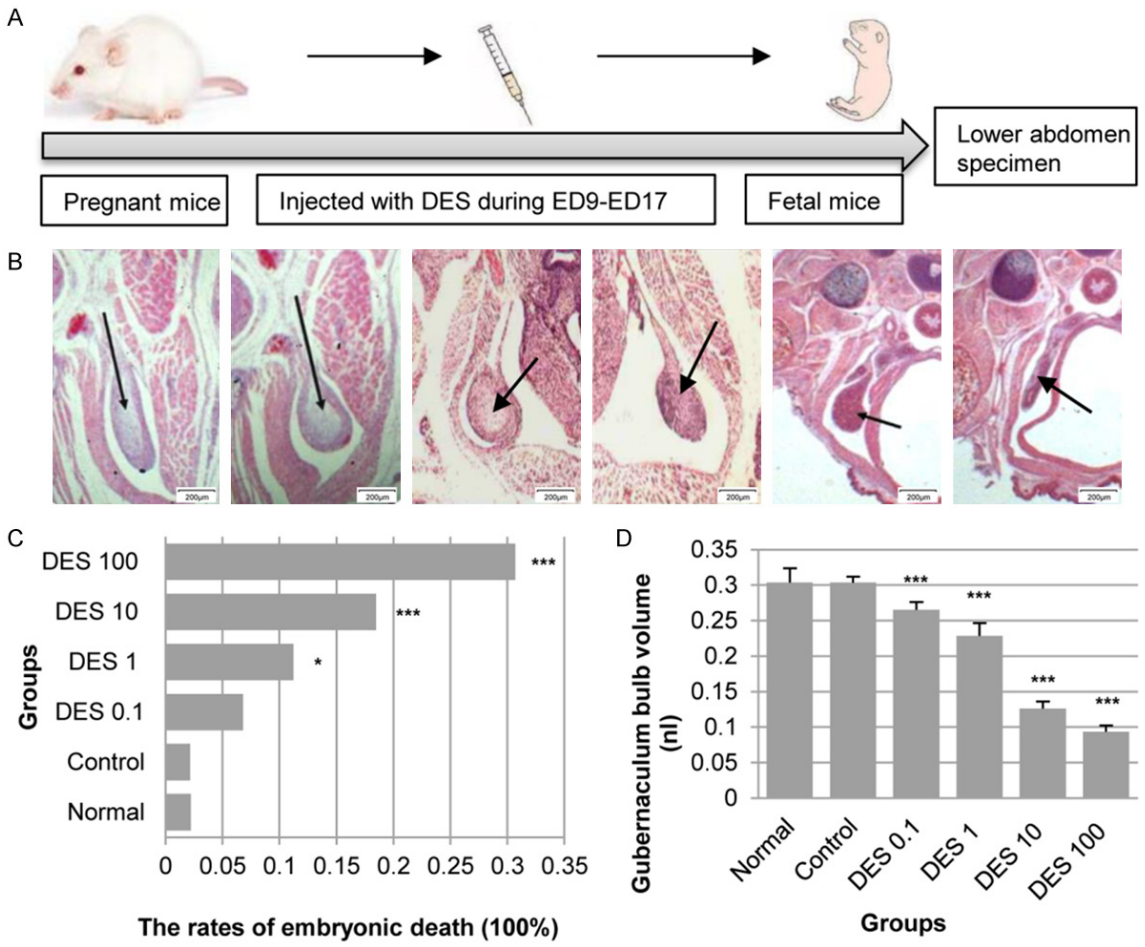

Figure 1. Influence of DES on the stillbirth rate and gubernaculum development. A. Establishment of animal models. Pregnant mice were subcutaneously injected with DES during ED9-ED17. The lower abdomen of fetal mice was fixed in formaldehyde to make sections. B. Changes in morphological structure of the gubernaculum in each group. From left to right: the normal group, solvent control group, and experimental groups (DES 0.1, 1.0, 10, 100 μg/kg·d). As the DES dose increased, the gubernaculum became more poorly developed and the morphology became more irregular. No obvious boundary was observed between mesenchymal tissues and muscle-derived cells, and the tissue structure was relatively disordered with the gubernacular cone volume decreasing gradually. C. Comparison of the stillbirth rate. The stillbirth rate of fetal mice increased as the DES dose increased. D. The gubernacular cone volume of each group compared with the normal group. The volume continued to decrease significantly with increasing DES dose. *P < 0.05 vs. the normal group, ***P < 0.001 vs. the normal group.

However, the stillbirth rate of animals exposed to 0.1, 1.0, 10, and 100 μg/kg·d DES was 6.82% (6/88), 11.24% (10/89), 18.48% (17/92), and 30.68% (27/88), respectively, demonstrating an increase in stillbirth rate with increasing DES exposure. No statistical difference was found between the control mice and those treated with 0.1 μg/kg·d DES compared with the normal group (chi-square test, P > 0.05). However, the other experimental groups (DES at 1.0, 10, and 100 μg/kg·d) displayed a dose-dependent effect compared with the normal group (chi-square test, P < 0.05 or P < 0.001) (**Figure 1C**).

Disruption of the gubernaculum and gubernacular cone volume morphology with DES exposure

H&E staining illustrates the normal and control groups have well-developed gubernaculum, and that the gubernacular cone appears round with mesenchymal tissues and sparse cells in the center and muscle-derived cells in a dense pattern in the periphery. The boundaries between the mesenchymal tissue and muscle-derived cells were clear. In the experimental groups, the gubernaculum was not well-developed and exhibited irregular morphology,

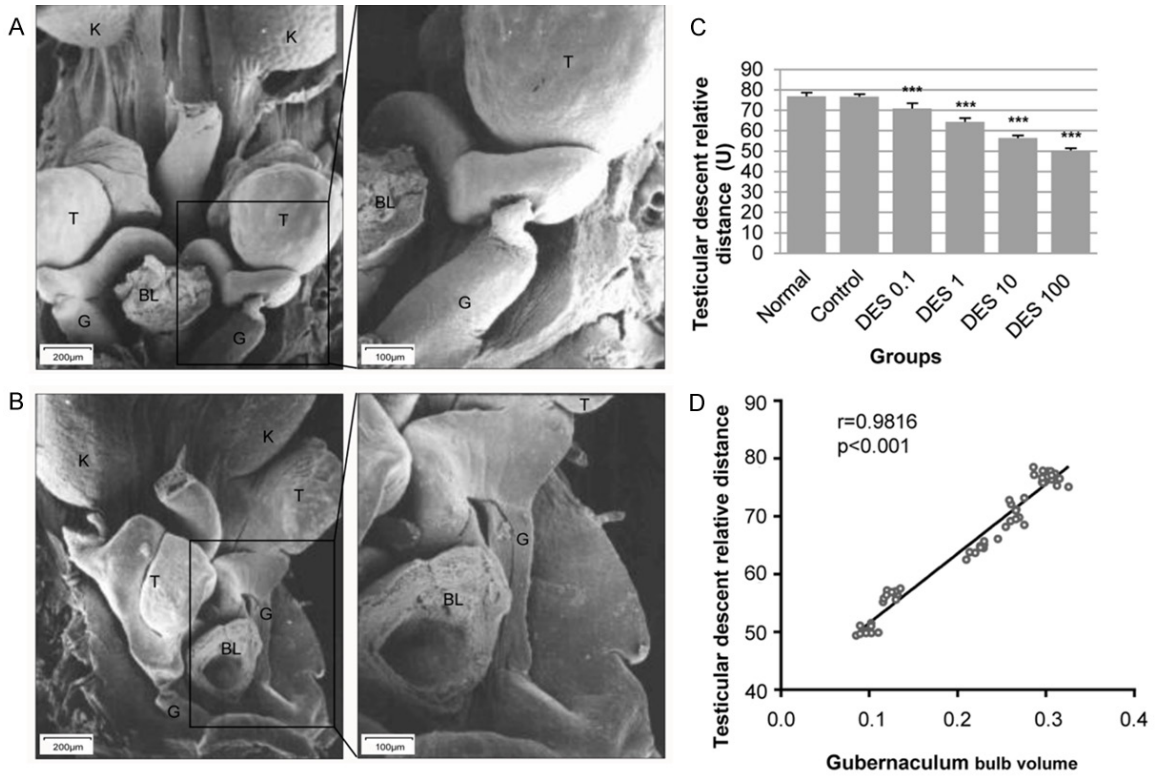

Figure 2. Examination of the extent of testicular descent by scanning electron microscopy. A. The testicle and gubernaculum were well-developed in the normal group. The testicles descended to the neck of the bladder, which was relatively far from the inferior pole of the kidney. B. The testicle and gubernaculum were poorly developed in the DES-treated groups. Testicular descent was short, ending far from the neck of the bladder and near the inferior pole of the kidney. C. The relative distance of testicular descent in each group compared with the normal group. The distance of testicular descent in the DES-treated groups was shorter than those in the normal or control groups. D. The gubernacular cone volume had a positive correlation with the relative distance of testicular descent. ***P < 0.001 vs. the normal group.

namely diffuse boundaries and disordered tissue structure (**Figure 1B**). Moreover, the gubernaculum bands in the normal and control groups were short, while the gubernacular cones were well-developed and large with a cylindrical shape. The gubernaculum bands in the experimental groups were long and poorly developed, possessing a small volume. With increasing DES dose, the volume decreased significantly compared with the normal group (one-way ANOVA, P < 0.001) (**Figure 1B** and **1D**).

Decrease in testicular descent in fetal mice exposed to DES

We also examined the extent of testicular descent by scanning electron microscopy. Animals in the normal and control groups possessed testicles that had descended to the neck of the bladder, which was relatively far from the inferior pole of the kidney. The testicle and gubernaculum were well-developed in these mice (**Figure 2A**). In contrast, the testicle and gubernaculum were poorly developed in the DES experimental groups, with testicular descent far removed from the neck of the bladder and near to the inferior pole of the kidney (**Figure 2B**). The testicular descent distances in the experimental groups were shorter than those in the normal and control groups (one-way ANOVA, P < 0.001) (**Figure 2C**). Correlation analysis between the gubernacular cone volume and relative distance of testicular descent indicated that the length became shorter as the volume of the gubernacular cone shrank (r = 0.9816, P < 0.001) (**Figure 2D**).

Dose-dependent decrease in RXFP2 protein level in gubernacular tissue based on DES exposure

The immunohistochemistry results demonstrate that RXFP2-positive cells were pale

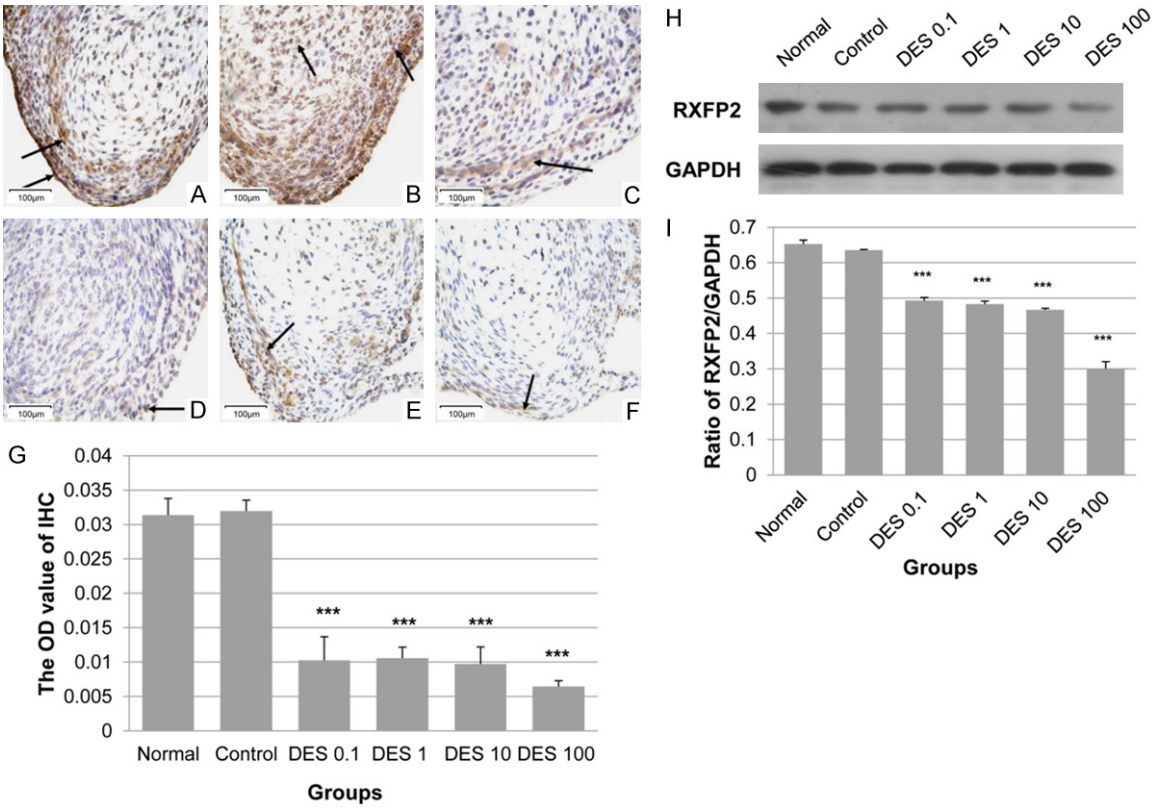

Figure 3. Examination of RXFP2 expression in gubernacular tissue by immunohistochemistry and western blot. RXFP2-positive cells exhibit a pale brown or dark brown signal, and were found in muscle-derived cells and the mesenchymal tissues in the gubernaculum. No statistical difference was found between the (A) normal group and (B) control group. Positive staining was relatively weak (C-F) in each experimental group compared with the (A) normal group. (G) Comparison of RXFP2 expression in the gubernaculum tissue between groups indicates that the reduction in RXFP2 expression induced by DES is dose-dependent. Positive optical density represents the relative quantity of RXFP2 expression. (H, I) Quantitative analysis of RXFP2 protein in the fetal mouse gubernacular tissue indicates that there was no significant difference between RXFP2 expression in the normal and the control groups. However, RXFP2 expression in the experimental groups was decreased significantly. Higher DES dose led to lower expression of RXFP2. ***P < 0.001 vs. the normal group.

brown or dark brown and expressed in muscle-derived cells, while the mesenchymal tissues in the gubernaculum consisted mainly of muscle-derived cells (**Figure 3A-F**). No statistical difference was found between the normal and the control groups (two independent samples, Student's t-test, P > 0.05). Moreover, positive staining was relatively weak in each experimental group compared with the normal group, with a higher DES dose producing in more significant reduction (one-way ANOVA, P < 0.001) (**Figure 3G**). Quantitative analysis of RXFP2 protein level in fetal mouse gubernacular tissue indicated that there was no significant difference in RXFP2 expression between the normal and the control groups (two independent samples, Student's t-test, P > 0.05). However, the experimental groups exhibited a significant decrease in RXFP2 protein that was dependent on DES dosage. (one-way ANOVA, P < 0.001) (**Figure 3H** and **3I**).

DES alters the morphology and decreases the survival rate of gubernacular cells

Most gubernacular cells in the normal and control groups were fibroblast-like while a few had an epithelioid cell phenotype. The cell body displayed either a fusiform shape or an irregular triangular shape, with a protruding cytoplasm. After subculture, the fibroblast-like cells grew with high homology and a 90% survival rate (**Figure 4A**, **4B** and **4H**). Growth of the experimental group cells was significantly inhibited as the DES dose increased, growing slowly with a smaller protuberance but a larger intercellular

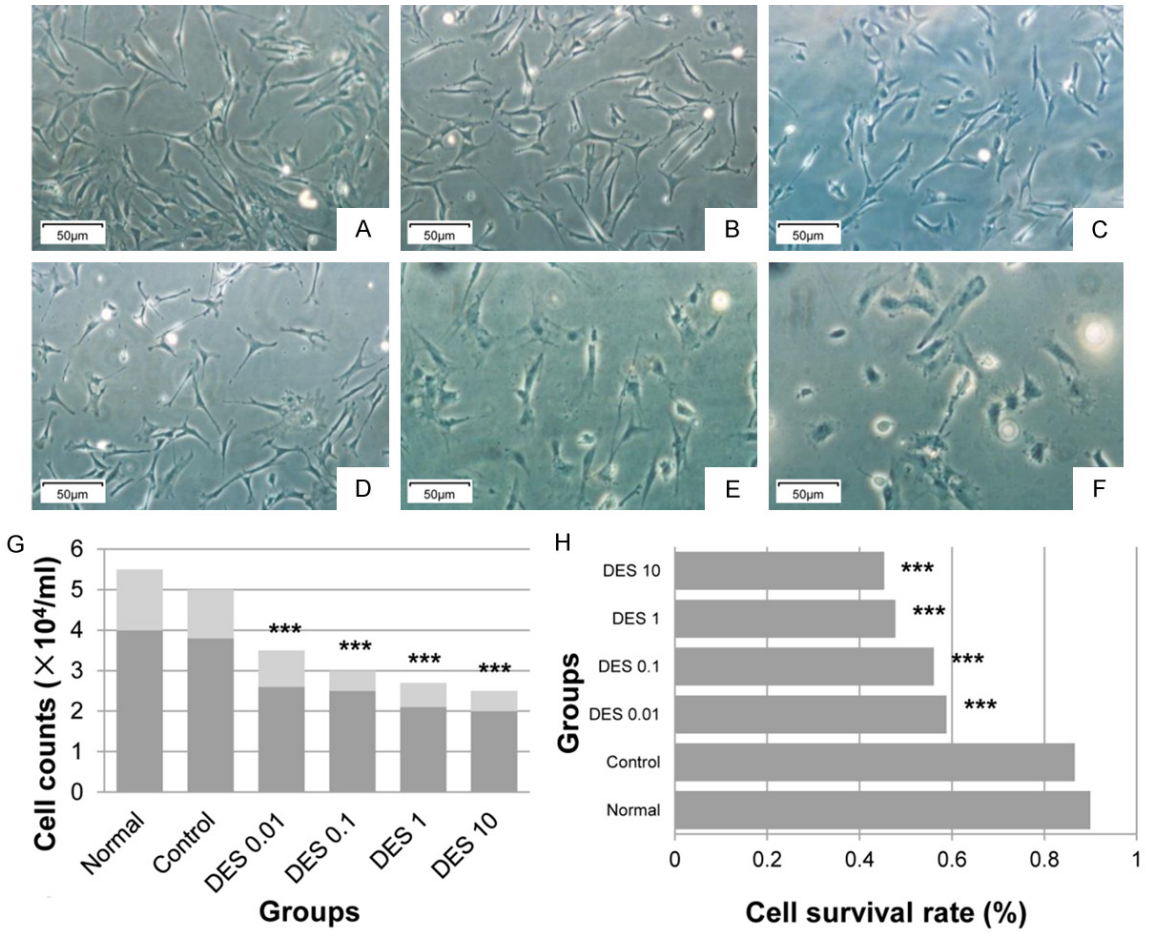

Figure 4. Influence of DES on gubernacular cell morphology, growth, and survival rate. Most of the gubernacular cells in the (A) normal group and the (B) control group were fibroblast-like cells while only a few were epithelioid cells. The cell body exhibited a fusiform or irregular triangular shape with a protruding of the cytoplasm. (C-F) Cell growth in the experimental groups was significantly inhibited as the DES dose increased. Cells grew slowly, with less protuberance, and an enlarged intercellular gap junction. (F) A higher DES dose led to a loss of morphology of fibroblasts, with decreased cytoplasm, generation of cells with an irregular elliptical shape. (G) Comparison of cell count in each group. Dark gray color represents low cell counts, and light gray color represents high cell counts. (H) The homology in the normal group was high, and the survival rate was 90%. However, the rate in the DES groups was reduced. ***P < 0.001 vs. the normal group.

gap junction (**Figure 4C-F**). A higher DES dose led to loss of fibroblast morphology and decreased cytoplasm, as well as exhibition of an irregular elliptical shape (**Figure 4F**). The number of cells and survival rate were also decreased in the experimental group (one-way ANOVA and chi-square test, P < 0.001) (**Figure 4G** and **4H**). Taken together, these data demonstrate that DES affects the morphology and survival rate of gubernacular cells.

DES impairs the actin cytoskeleton of gubernacular cells

FITC-phalloidin against F-actin was used to examine whether the actin cytoskeleton differed between untreated and DES-treated gubernacular cells. We found that the normal and control groups exhibited short processes and a diffusive filamentous staining with FITC-phalloidin, mainly around the cell periphery (**Figure 5A**). However, the stress fibers of DES-exposed cells were distributed throughout the cytoplasm (**Figure 5B**), suggesting that DES impairs the actin cytoskeleton of gubernacular cells in vitro.

Subcellular localization of RXFP2 and influence of DES on RXFP2 expression in gubernacular cells

The results from our immunofluorescence experiments indicated that RXFP2 was expressed on the cell membrane with relatively

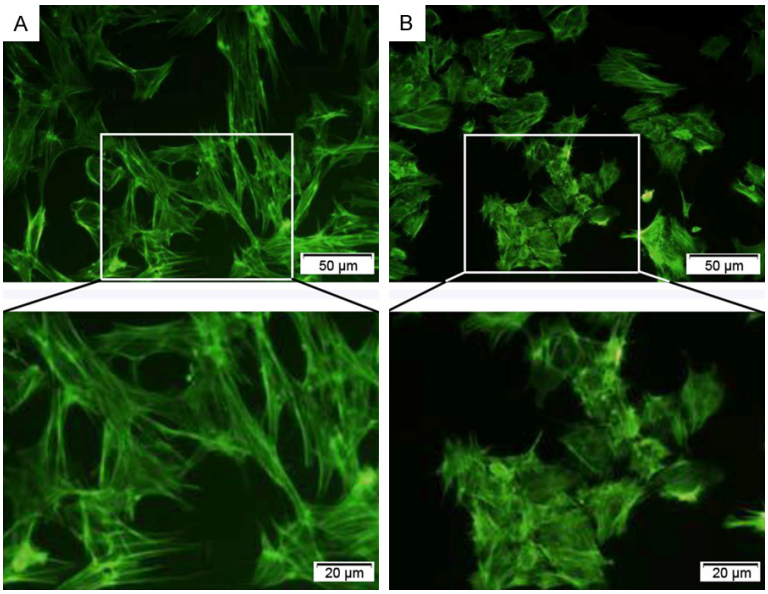

Figure 5. DES impairs the F-actin cytoskeleton of gubernacular cells. A. The normal and control groups exhibited short processes and a diffuse filamentous staining pattern with FITC-phalloidin that was localized mainly around the cell periphery. B. Cells exposed to DES exhibited stress fibers distributed throughout the cytoplasm, not just around the cell periphery. DES impairs the actin cytoskeleton of gubernacular cells.

high intensity (**Figure 6A-C**). Western blot analysis demonstrated that there was no significant difference in RXFP2 expression in gubernacular cells between the normal and control groups (two independent samples, Student's t-test, $P > 0.05$); however, the level of RXFP2 protein was significantly decreased in the experimental groups (one-way ANOVA, $P < 0.001$) (**Figure 6D** and **6E**).

Discussion

With social development and increasing industrialization, environmental contamination and disruption of the ecological balance when conquering and transforming nature have severe effects on human health, especially reproductive health [23-26]. Environmental estrogen, also called exogenous estrogens (EEs), is found widely in the environment and possesses estrogen-like activity that can damage the endocrine balance, leading to a series of undesired clinical outcomes [27-29]. Studies in both animals and epidemiology have shown that EEs can cause multiple abnormalities and deformities in the urogenital system [30-32]. Laboratories of the National Institute of Environmental Health Sciences use DES as a reference estrogen in the studies focused on the effects of EEs [15, 33-35]. Thus, in the current study, DES was used as an intervention method to investigate the reproductive toxicity of EEs.

Our results indicated that, on day 18 of pregnancy in mice, the gubernaculums in the normal and control groups were well-developed, and that testicles descended to a site near the neck of the bladder, which is relatively far from the kidneys. After administration of DES, the stillbirth rate gradually increased, the gubernacular cone volume significantly decreased, and tissue structure became disordered. The boundary between the mesenchymal tissues and myogenous cells was diffuse, the gubernaculum band was thin and long, and testicular descent was incomplete, with the distance to the kidney still very short on day 18 of pregnancy. A higher dose of DES led to more obvious abnormalities, suggesting a dose-dependent effect. These data suggest that DES exerted a toxic effect on the gubernaculum and could affect the growth and development of mesenchymal tissues and myogenous cells, further influencing gubernaculum development and testicular descent. All of the results indicated that DES significantly inhibited the development of the gubernaculum.

The first stage of testicular transabdominal descent in mice takes place in the fetus, where proliferation, differentiation, migration, shrinking, and hormone metabolism of the gubernaculums occurs [9, 11]. Gubernacular cell contractility and morphological changes coordinate with each other [9]. After DES administration, a smaller gubernacular cone volume and less complete testicular descent occurred with a significant positive correlation. These data suggest that the morphology of the gubernaculum was influenced by DES, which further affected contractility, leading to incomplete testicular descent.

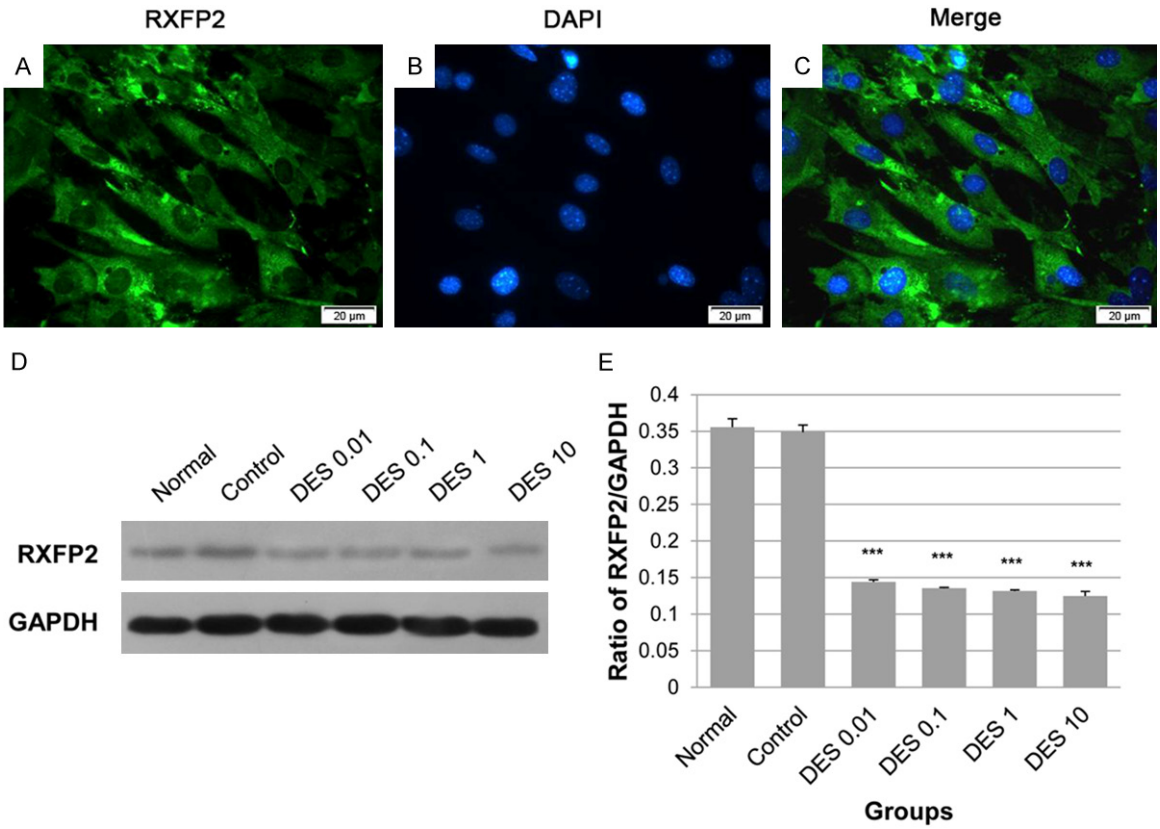

Figure 6. Influence of DES on RXFP2 expression in gubernacular cells. A-C. Immunofluorescence experiments demonstrate that RXFP2 was expressed on the cell membrane with relatively high intensity. D, E. Quantitative analysis of RXFP2 protein in mouse gubernacular cells indicated that there was no significant difference between RXFP2 expression in the normal and the control groups; however, RXFP2 expression was significantly decreased in the DES-treated groups. ***P < 0.001 vs. the normal group.

To exclude interference from other endocrine factors, gubernacular cells were cultured in vitro and examined for their response to DES treatment. We found that DES administration resulted in abnormal cellular morphology and structure in gubernacular cells. With increasing DES dose, the cells lost their original morphology in a dose-dependent manner. Moreover, DES induced F-actin rearrangement and stress fiber formation in the cultured cells. With increased numbers of stress fibers, cells sustain a sub-stress state over the long term, decreasing the contraction and reaction capacity of the cultured cells significantly. These results suggest that gubernacular cells were affected by the significant toxicity of DES, which inhibited the growth and development of gubernacular cells. These data are consistent with the abnormalities observed in gubernaculum morphology and structure caused by DES at the tissue level.

The development of descending testicles is very complicated and occurs in two stages that involving multiple factors that influence or regulate the process [36-38]. Studies have established that INSL3 plays a critical role in the first stage. INSL3 and RXFP2 bind together to form a receptor-ligand complex INSL3-RXFP2 that mediates the biological effects [17, 18]. Although in vivo toxicity studies during the embryonic period and in vitro gubernacular cell culture studies have demonstrated the effect of DES on gubernaculum development, the underlying mechanism remains unclear [39].

In this study, we found that RXFP2 was highly expressed in the gubernaculum and was mainly distributed on the cell membrane. Interestingly, both in vitro and in vivo experiments indicated that RXFP2 protein was reduced in response to DES treatment in a dose-dependent manner. As a specific receptor of the critical regulatory

factor INSL3 during the first stage of testicular descent, reduction of RXFP2 expression decreases complex formation with INSL3, thereby blocking testicles from descending through the abdomen [17, 18, 40, 41]. Collectively, these data suggest that the effect of DES on the morphological structure of the gubernaculum occurs via RXFP2, suggesting that RXFP2 may be involved in mediating the effect of DES on gubernaculum development.

Conclusions

In conclusion, in vitro and in vivo studies demonstrate that DES causes abnormal morphology in the gubernaculum, as well as reduces the expression of RXFP2 in this tissue. These results indicate that DES imparts reproductive toxicity on the gubernaculum in fetal mice, and that RXFP2 is associated with the morphological abnormalities induced by DES. Thus, our data suggest that RXFP2 is a novel potential biomarker for abnormal differentiation of the gubernaculum, which may reflect its primary role in INSL3-mediated signaling.

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Disclosure of conflict of interest

None.

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References

[1] Wong EW and Cheng CY. Impacts of environmental toxicants on male reproductive dysfunction. Trends Pharmacol Sci 2011; 32: 290-299.

[2] Bajkin I, Bjelica A, Icin T, Dobric V, Zavisic BK and Stojanoska MM. Effects of phthalic acid esters on fetal health. Med Pregl 2014; 67: 172-175.

[3] Sidorkiewicz I, Zareba K, Wolczynski S and Czerniecki J. Endocrine-disrupting chemicals-Mechanisms of action on male reproductive system. Toxicol Ind Health 2017; 33: 601-609.

[4] Stenz L, Escoffier J, Rahban R, Nef S and Paoloni-Giacobino A. Testicular dysgenesis syndrome and long-lasting epigenetic silencing of mouse sperm genes involved in the reproductive system after prenatal exposure to DEHP. PLoS One 2017; 12: e0170441.

[5] Mathur PP and D'Cruz SC. The effect of environmental contaminants on testicular function. Asian J Androl 2011; 13: 585-591.

[6] Barthold JS, Reinhardt S and Thorup J. Genetic, maternal, and environmental risk factors for cryptorchidism: an update. Eur J Pediatr Surg 2016; 26: 399-408.

[7] Duan S, Jiang X, Zhang X, Xie L, Sun Z, Ma S and Li J. Diethylstilbestrol regulates the expression of LGR8 in mouse gubernaculum testis cells. Med Sci Monit 2016; 22: 416-421.

[8] Xing JS and Bai ZM. Is testicular dysgenesis syndrome a genetic, endocrine, or environmental disease, or an unexplained reproductive disorder? Life Sci 2018; 194: 120-129.

[9] Nation TR, Buraundi S, Farmer PJ, Balic A, Newgreen D, Southwell BR and Hutson JM. Development of the gubernaculum during testicular descent in the rat. Anat Rec (Hoboken) 2011; 294: 1249-1260.

[10] Hutson JM, Vikraman J, Li R and Thorup J. Undescended testis: what paediatricians need to know. J Paediatr Child Health 2017; 53: 1101-1104.

[11] Hutson JM, Li R, Southwell BR, Newgreen D and Cousinery M. Regulation of testicular descent. Pediatr Surg Int 2015; 31: 317-325.

[12] Favorito LA, Costa SF, Julio-Junior HR and Sampaio FJ. The importance of the gubernaculum in testicular migration during the human fetal period. Int Braz J Urol 2014; 40: 722-729.

[13] Huang Z, Rivas B and Agoulnik AI. Insulin-like 3 signaling is important for testicular descent

but dispensable for spermatogenesis and germ cell survival in adult mice. Biol Reprod 2012; 87: 143.
[14] Bay K and Anand-Ivell R. Human testicular insulin-like factor 3 and endocrine disrupters. Vitam Horm 2014; 94: 327-348.
[15] Zhang X, Ke S, Chen KH, Li JH, Ma L and Jiang XW. Diethylstilbestrol affects the expression of GPER in the gubernaculum testis. Int J Clin Exp Pathol 2015; 8: 7217-7222.
[16] Duan S, Zhang X, Jiang X, Xie L, Sun Z, Ma S and Li J. An insight into insulin-like factor 3 regulate its receptor RXFP2 in mouse gubernaculum testis cells. Int J Clin Exp Pathol 2015; 8: 14806-14811.
[17] Arrighi S, Bosi G, Groppetti D, Aralla M and Cremonesi F. An insight into testis and gubernaculum dynamics of INSL3-RXFP2 signalling during testicular descent in the dog. Reprod Fertil Dev 2010; 22: 751-760.
[18] Sagata D, Minagawa I, Kohriki H, Pitia AM, Uera N, Katakura Y, Sukigara H, Terada K, Shibata M, Park EY, Hasegawa Y, Sasada H and Kohsaka T. The insulin-like factor 3 (INSL3)-receptor (RXFP2) network functions as a germ cell survival/anti-apoptotic factor in boar testes. Endocrinology 2015; 156: 1523-1539.
[19] Shono T, Shima Y, Kondo T and Suita S. In utero exposure to mono-n-butyl phthalate impairs insulin-like factor 3 gene expression and the transabdominal phase of testicular descent in fetal rats. J Pediatr Surg 2005; 40: 1861-1864.
[20] Anand-Ivell R and Ivell R. Insulin-like factor 3 as a monitor of endocrine disruption. Reproduction 2014; 147: R87-95.
[21] Jiang XW, Li JH, Huang TH and Deng WD. Effect of prenatal exposure to diethylstilbestrol on gubernacular development in fetal male mice. Asian J Androl 2004; 6: 325-329.
[22] Naiqing Z. The volume of the object is calculated by the cross section value and thickness of the slice. acta academiae medicinae shanghai 1988; 15: 314.
[23] Nordkap L, Joensen UN, Blomberg Jensen M and Jorgensen N. Regional differences and temporal trends in male reproductive health disorders: semen quality may be a sensitive marker of environmental exposures. Mol Cell Endocrinol 2012; 355: 221-230.
[24] Guerrero-Bosagna C and Skinner MK. Environmentally induced epigenetic transgenerational inheritance of male infertility. Curr Opin Genet Dev 2014; 26: 79-88.
[25] Cao-Lei L, Elgbeili G, Massart R, Laplante DP, Szyf M and King S. Pregnant women's cognitive appraisal of a natural disaster affects DNA methylation in their children 13 years later: project ice storm. Transl Psychiatry 2015; 5: e515.
[26] Heindel JJ, Balbus J, Birnbaum L, Brune-Drisse MN, Grandjean P, Gray K, Landrigan PJ, Sly PD, Suk W, Cory Slechta D, Thompson C and Hanson M. Developmental origins of health and disease: integrating environmental influences. Endocrinology 2015; 156: 3416-3421.
[27] Manfo FP, Nantia EA and Mathur PP. Effect of environmental contaminants on mammalian testis. Curr Mol Pharmacol 2014; 7: 119-135.
[28] Skakkebaek NE, Rajpert-De Meyts E, Buck Louis GM, Toppari J, Andersson AM, Eisenberg ML, Jensen TK, Jorgensen N, Swan SH, Sapra KJ, Ziebe S, Priskorn L and Juul A. Male reproductive disorders and fertility trends: influences of environment and genetic susceptibility. Physiol Rev 2016; 96: 55-97.
[29] Adeel M, Song X, Wang Y, Francis D and Yang Y. Environmental impact of estrogens on human, animal and plant life: a critical review. Environ Int 2017; 99: 107-119.
[30] Titus-Ernstoff L, Troisi R, Hatch EE, Palmer JR, Hyer M, Kaufman R, Adam E, Noller K and Hoover RN. Birth defects in the sons and daughters of women who were exposed in utero to diethylstilbestrol (DES). Int J Androl 2010; 33: 377-384.
[31] Ivell R, Heng K, Nicholson H and Anand-Ivell R. Brief maternal exposure of rats to the xenobiotics dibutyl phthalate or diethylstilbestrol alters adult-type Leydig cell development in male offspring. Asian J Androl 2013; 15: 261-268.
[32] Lombo M, Getino-Alvarez L, Depince A, Labbe C and Herraez MP. Embryonic exposure to bisphenol a impairs primordial germ cell migration without jeopardizing male breeding capacity. Biomolecules 2019; 9: 307.
[33] Harris RM and Waring RH. Diethylstilboestrol--a long-term legacy. Maturitas 2012; 72: 108-112.
[34] McLachlan JA. Environmental signaling: from environmental estrogens to endocrine-disrupting chemicals and beyond. Andrology 2016; 4: 684-694.
[35] Escher J and Robotti S. Pregnancy drugs, fetal germline epigenome, and risks for next-generation pathology: a call to action. Environ Mol Mutagen 2019; 60: 445-454.
[36] Hutson JM, Southwell BR, Li R, Lie G, Ismail K, Harisis G and Chen N. The regulation of testicular descent and the effects of cryptorchidism. Endocr Rev 2013; 34: 725-752.
[37] Acer T and Hicsonmez A. The separation of the epididymis and the abnormal attachment of the gubernaculum cause undescendence in the rat testes. Pediatr Surg Int 2014; 30: 1155-1161.
[38] Hannan MA, Kawate N, Kubo Y, Pathirana IN, Bullesbach EE, Hatoya S, Inaba T, Takahashi M and Tamada H. Expression analyses of insulin-like peptide 3, RXFP2, LH receptor, and 3beta-

hydroxysteroid dehydrogenase in testes of normal and cryptorchid dogs. Theriogenology 2015; 84: 1176-1184.
[39] Zhang X, Ping HY, Li JH, Duan SX and Jiang XW. Diethylstilbestrol regulates mouse gubernaculum testis cell proliferation via PLC-Ca2+ -CREB pathway. Cell Biochem Funct 2018; 36: 13-17.
[40] Donizetti A, Fiengo M, Del Gaudio R, Iazzetti G, Pariante P, Minucci S and Aniello F. Expression pattern of zebrafish rxfp2 homologue genes during embryonic development. J Exp Zool B Mol Dev Evol 2015; 324: 605-613.
[41] Ayers K, Kumar R, Robevska G, Bruell S, Bell K, Malik MA, Bathgate RA and Sinclair A. Familial bilateral cryptorchidism is caused by recessive variants in RXFP2. J Med Genet 2019; 56: 727-733.