Original

Di-n-butyl phthalate induced hypospadias relates to autophagy in genital tubercle via the PI3K/Akt/mTOR pathway

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Abstract: Objective: To explore the mechanisms of hypospadias induced by *in utero* exposure to din-butyl phthalate（DBP）. **Methods：** Timed-pregnant Sprague-Dawley rats were administered 750 mg/kg of DBP by gavage from GD (gestation days) 13 to GD 18, whereas control group received corn oil. Genital tubercles (GTs) and blood samples were collected from male fetuses on GD 19. The serum testosterone concentration, apoptosis activity, autophagosomes and their related proteins (light chain 3 (LC3-I, LC3-II)), and sequestosomes (SQSTM1/p62) in the GTs were then measured. Protein expression of protein kinase B (Akt), Beclin 1, phosphorylated Akt (p-Akt), p-S6, and phosphorylated mammalian target of rapamycin (p-mTOR) in the GTs were analyzed by Western blotting. **Results:** The incidence of hypospadias induced by DBP was 43.64% in male fetuses. The GT volume and GT volume/body weight of fetuses were significantly reduced in the hypospadias and the non-hypospadias groups. Apoptotic cell number was significantly decreased in the GTs of the hypospadias group, but unchanged in the non-hyposadias group. The ratio of LC3-II/LC3-I was higher in the GTs from DBP exposed fetuses compared to the control group. The ratio of LC3-II/LC3-I in the GTs was higher in the hypospadias group than in the non-hypospadias group. The number of autophagosomes was increased in the GTs of the hypospadias group. Protein expression of p-S6, p-mTOR, and p-Akt were significantly decreased in the GTs of hypospadiac rats. **Conclusions：** DBP-induced hypospadias might be associated with apoptosis and autophagy mediated by the PI3K/Akt/mTOR signaling pathway in the GT.
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1. Introduction

Hypospadias is a common genitourinary malformation with urethral opening on the ventral side of the penis. The prevalence of hypospadias is approximately 4 to 6 in 1,000 male children (Dolk et al. 2004; Paulozzi 1999; Paulozzi et al. 1997). The external genitalia originate from the genital tubercle (GT) by gathering mesenchymal cells at the border of the cloacal membrane. With development, paired genital swellings appear on both sides of the cloacal membrane, followed by urogenital folds along the flank of the cloacal membrane. Under the influence of androgens, urogenital folds are remodeled into a penis with the urethra and the genital swellings form the scrotum. Failure of fusion of the urogenital folds can result in hypospadias (Baskin 2004; Cunha and Baskin 2004).

The etiology of hypospadias is multifactorial, including genetic, endocrine, and environmental factors. Studies have shown that the risk of hypospadias is increased due to exposure to environmental endocrine disruptors (EEDs) (Canning 1999; Wang and Baskin 2008). Di-n-buty-phthalate (DBP), one of the EEDs, has been widely used in daily plastic products and found to adversely affect the male reproductive system, especially GT development (Ema et al. 1993; Ema et al. 2000; Fisher et al. 2003; Kim et al. 2004; Mylchreest et al. 2000; Phillips and Tanphaichitr 2008; Sharpe 2001; Wine et al. 1997). DBP has anti-androgenic effects (Mahood et al. 2005). However, it is not clear whether DBP-induced hypospadias is related to its anti-androgenic effects.

Apoptosis and autophagy are crucial processes involved in normal embryogenesis (Thorburn 2008). Apoptosis is essential for proper outgrowth of embryonic anlagen (Suzuki et al. 2003) and closure of the distal urethra (Morgan et al. 2003; van der Werff et al. 2000). Decreased apoptotic activity has been observed in the urethra in hypospadiac mice with a mutation in Hoxa13GFP gene (Morgan et al. 2003). In addition, apoptosis is closely related to autophagy, an important catabolic process that targets cytoplasmic components to lysosomes for degradation and recycling (Klionsky and Emr 2000). Autophagy serves an important cross talking point between type 1 and type 2 cell death pathways, and inhibition of autophagy can trigger apoptosis (Boya et al. 2005). Androgen deprivation can increase autophagy, while inhibition of autophagy by 3-methyladenine can promote apoptosis in androgen sensitive benign prostate epithelial cells (Li et al. 2014). However, the roles of autophagy and apoptosis in the pathogenesis of hypospadias induced by DBP are not clear.

Using a hypospadias rat model induced by DBP, the present study investigated the roles of autophagy and apoptosis in the GT during embryogenesis. The rat model shares common features of human hypospadias, such as disruption of the urethral meatus, prevalence of distal penile deformities, and failure of epithelial fusion (Cunha et al. 2015). Some regulators of autophagy, such as mammalian target of rapamycin (mTOR), can inhibit autophagy *via* the phosphoinositide 3-kinase (PI3K) / protein kinase B (Akt) signaling pathway (Lum et al. 2005); whereas Beclin 1 can upregulate autophagy (He and Klionsky 2009). Therefore, we measured the expression of these regulators to explore the mechanism of autophagy in the GT.

2. Materials and Methods

2.1. Animals and treatment

Adult Sprague-Dawley rats (2 mo), purchased from the Soochow University Experimental Animal Center, were housed under a 12 h light-dark cycle at $22 \pm 4\,^{\circ}\mathrm{C}$ with 40-80% humidity. Animals were given fresh tap water and fed standard rat chow *ad libitum*.

After 2 weeks of acclimatization, 22 virgin female rats (body weights between 240-270 g) mated overnight with male rats at 1:1 ratio. If a vaginal plug was detected on the second morning after mating, we considered the rat to be at gestation day 0 (GD 0). The pregnant rats were then randomly divided into two groups. In the experimental group, pregnant rats (n = 12) were administered DBP (Cat. No. 524980, Sigma Chemical Co., St. Louis, MO, USA) by oral gavage at 750 mg/kg/day (4 ml/kg/d) in corn oil from GD 13 to GD 18. Control pregnant rats (n = 10) were given only corn oil (cat. No. C 8267, Sigma Chemical Co., St. Louis, MO, USA) in the same volume

as the experimental group. The exposure period and dosage of DBP were based on the established hypospadiac rat model (Jiang et al. 2007; Liu et al. 2012; Zhu et al. 2009).

All procedures and protocols were approved by the Institutional Animal Care Committee and followed the guidelines by the National Institutes of Health (NIH Publication No.85-23, 1996).

2.2. Rat fetuses

On GD 19, pregnant rats from the control and experimental groups were weighed and then anesthetized with sodium pentobarbital (100 mg/kg; Hengrui Medicine, Jiangsu, China) and sacrificed by exsanguination of the abdominal aorta. The fetuses were quickly removed from the uterus, weighed and counted, and the gonads and penises were carefully examined to identify male fetuses (Liu et al. 2012). After hypospadias was identified, the male fetuses in the experimental group were further divided into non-hypospadias and hypospadias groups. Blood samples were collected from male fetuses by decapitation for measurement of serum testosterone concentrations. Eight GTs each were randomly selected from the control, non-hypospadias, and hypospadias groups, and then stored at $-80\,^{\circ}\mathrm{C}$ until used for Western blotting. Twelve GTs (eight from the control and four from hypospadias fetuses) were randomly selected and fixed in 10% formalin for 12 h for histological examination. Eight GTs were randomly selected from the control and hypospadias groups, and fixed with 2.5% glutaraldehyde for at least 24 h for transmission electron microscopy examination. Four GTs each from the control, non-hypospadias, and hypospadias groups were randomly selected for TUNEL staining. The anogenital distance (AGD), diameter and length of GT were measured using a digital micrometer.

2.3. Histological Examination

The GT samples were fixed in 10% formalin for 12 h, and then transferred to 70% ethanol. After embedded in paraffin, the tissues were sectioned (6 μm) and processed routinely followed by hematoxylin and eosin (H & E) staining. The tissue sections were then examined with a Nikon L150 microscope (Nikon, Japan).

2.4. Western blotting

GT tissues were thawed and homogenized in lysis buffer. After incubation on ice for 30 min, the homogenate was centrifuged at 12,000 g for 30 min at $4\,^{\circ}\mathrm{C}$. Then the supernatant was collected and protein concentration was measured. Equal amount of protein (50 μg) from every group was boiled at $95\,^{\circ}\mathrm{C}$ for 5 min. After cooled on ice, protein was resolved through SDS-PAGE and then transferred to nitrocellulose membranes overnight at $4\,^{\circ}\mathrm{C}$. The membrane was then cut into strips based on protein size of interest. The strips were then blocked with 5%

Table 1. Effects of DBP on pregnancy outcomes

	Control	DBP
No. of pregnant rats	10	12
No. of fetuses per litter	12.20±1.69	9.58±2.19*
BW (g) of male pups	2.28±0.20	2.33±0.19
Total fetuses	62	55
Ratio of male/female pups	1.07±0.32	0.95±0.33
No. of hypospadiac pups (%)	0 (0)	24 (43.64)*

*Significantly different from control, p<0.05.

non-fat dried milk in Tris-buffered saline containing 0.2% Tween-20 for 1 h at room temperature followed by incubation with different primary antibodies overnight at 4℃. The antibodies included Akt (Cell Signaling, #4691, 1: 1,000), phosphorylated AKT (p-Akt, #4060, Cell Signaling, 1: 2,000), sequestosome 1 (SQSTM1/p62) (#5114, Cell Signaling, 1: 2000), light chain 3 (LC3) (#12741, Cell Signaling, 1: 1,000), p-mTOR (#2971, Cell Signaling, 1: 1,000), p-S6 (sc-54279, Santa Cruz, 1: 300), Beclin 1 (sc-11427, Santa Cruz, 1: 500), and β-actin (a-1978, Sigma, 1: 1,000). After three washes, each for 10 min, with Tris-buffered saline containing 0.2% Tween-20, the membrane strips were incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (1: 4,000) for 2 h at room temperature. After three final washes blots were developed using enhanced chemiluminescence (ECL) reagents (Thermo, Illinois, USA). Protein bands were quantified using a UVP Bioimaging system EC3 apparatus (UVP, Upland, CA, USA).

2.5. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Frozen GT tissues were sectioned into 8 μm thick slides. TUNEL staining was then performed using One Step TUNEL Apoptosis Assay Kit (cat. No. C1089, Beyotime Institute, China) according to the manufacturer's instructions. Sections were randomly selected from control, non-hypospadias, and hypospadias groups. The number of apoptotic cells were counted in 3 high power fields randomly selected with at least 1,000 cells per field (n = 18, from 6 sections). Apoptosis index was expressed as the percentage of positive cells in total cell number.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected form 18 male fetuses per group. Serum from those fetuses was measured for testosterone concentrations by ELISA, as instructed by the Testosterone ELISA Kit (KGE010, R & D Systems, USA). Each sample was measured in triplicate.

2.7. Transmission electron microscopy

GTs at GD 19 were removed from fetuses and then fixed with 2.5% glutaraldehyde. After being dehydrated

in a series of ethanol concentrations, the specimens were embedded in Epon and then cut into ultrathin sections with an ultramicrotome (LEICA, Germany). The sections were treated with 2% uranyl acetate and lead citrate, and then examined for autophagosomes and autolysosomes under a Tecnai G 2 transmission electron microscope (FEI, USA). The autophagosomes and autolysosomes were identified based on characteristic double- or multiple-membranes vacuoles wrapping cellular constituents. At least 400 cells from each specimen were counted in different randomly selected fields.

2.8. Statistical analysis

Data were analyzed using GraphPad Prism, version 5.0 (GraphPad Software, San Diego CA). Significant differences between the two groups were tested by the independent-samples t-test. All results were presented as mean ± standard deviation (SD). A $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of DBP on pregnancy outcomes

On GD 19, a total of 55 fetuses were recovered from pregnant female rats treated with DBP and 62 fetuses from the controls. The ratio of male/female fetuses in DBP treated pregnant rats was similar to the control rats (Table 1). However, the number of fetuses per pregnant rat was significantly less in the DBP treatment group compared to the control group ($p < 0.05$, Table 1).
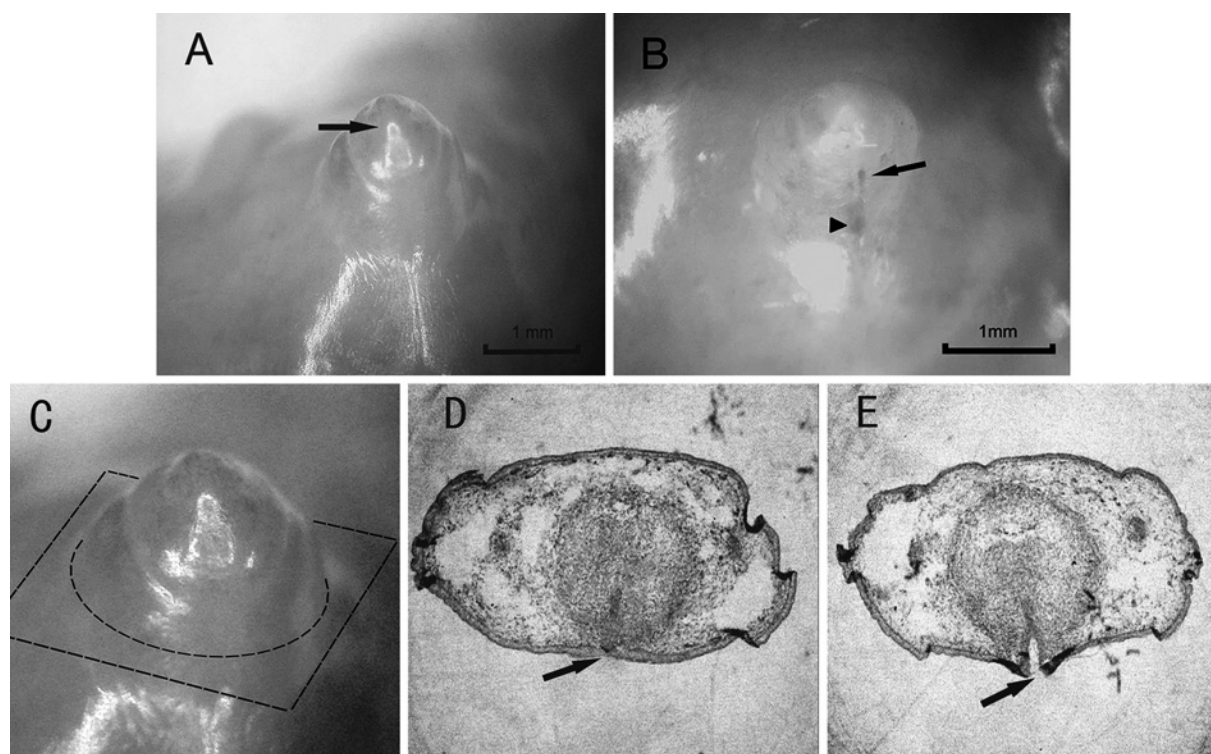
Gross and histological examinations showed urethral grooves in the GTs in the experimental group, which was in contrast to fully closed GTs in the control group (Fig. 1). Hypospadias was observed in 43.64% male fetuses exposed to DBP *in utero*, whereas no fetuses presented with hypospadias in the control group ($p < 0.05$, Table 1). No other morphological abnormality was observed in either male or female fetuses in either group.

3.2. Effects of DBP on male fetuses

The AGD, the GT volume, the AGD/body weight and the GT volume/body weight in the hypospadias and non-hypospadias groups were significantly decreased compared to those in the control group ($p < 0.05$, Table 2); however, no significant change was observed in these variables between hypospadias and non-hypospadias groups ($p > 0.05$, Table 2).

3.3. Serum testosterone levels were reduced in male fetuses exposed to DBP

Serum testosterone levels were measured in 18 fetuses in each group. There was an approximate 75% and 67% reduction in serum testosterone levels in the hypospadias (3.94 ± 1.57 nmol/m*l*) and non-hypospadias (5.89 ± 2.08 nmol/m*l*) groups compared to the control group ($15.74 \pm$

Fig. 1. Representative images and histological examination of genital tubercles (GTs) from male rat fetuses at GD 19. (A) Normal GT with the urethral orifice at the tip of the penis (arrow). (B) GT with hypospadias. The urethral opening is on the ventral surface of the penis (arrow). A visible cleft is on the ventral prepuce (arrowhead). (C) GT under a dissection microscope. The circle and square represent the cross-sections of (D) a normal GT with closed urethra (arrow), and (E) a hypospadiac GT with open urethral groove (arrow), respectively. Magnification: 25×.

Table 2. Effects of DBP on male fetus genitalia

| | Control | DBP | |
		Non-hypospadias	Hypospadias
Total fetuses	62	31	24
AGD (mm)	2.10±0.15	1.66±0.14*	1.64±0.17*
AGD/BW of male pups	0.93±0.08	0.72±0.08*	0.71±0.09*
GT volume/BW	2.98±0.25	2.21±0.37*	2.11±0.45*
GT volume (mm³)	1.30±0.63	0.96±0.16*	0.92±0.24*

*Significantly different from control, $p<0.05$.

3.56 nmol/m*l*) ($p < 0.001$). The serum testosterone level in the hypospadias group was significantly lower than that in the non-hypospadias group ($p < 0.01$).

3.4. Exposure to DBP in utero decreased apoptotic activity in the GT

Compared to the control, the number of TUNEL-positive cells was significantly decreased in the GTs of the hypospadias group ($p < 0.001$, Fig. 2); however, there was no significant change in the non-hypospadias group ($p > 0.05$, Fig. 2C).

3.5. Exposure to DBP in utero promoted autophagy in the GT

Autolysosomes were observed in the GTs from both groups under electron microscopy (Fig. 3; arrowheads). In contrast to the autolysosomes, the autophagosomes (double-membrane vesicles) were only detected in the GTs from male fetuses with hypospadias (Fig. 3B, D, and E; arrows). Consistently, proteins forming the autophagosomes (LC3I and LC3II) were significantly increased in the hypospadias and non-hypospadias groups ($p < 0.001$, Fig. 4A). Compared to non-hypospadias, the
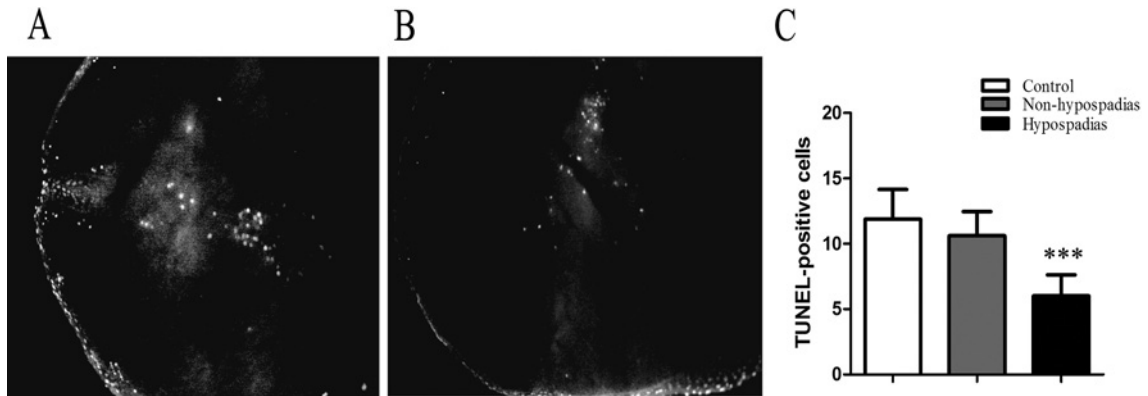
Fig. 2. Effects of DBP on apoptosis in the GTs from male fetuses at GD 19. Apoptotic cells (red dots, 20×) in the GT in the control (A) and hypospadiac (B) rats detected by TUNEL. (C) Quantitative analysis of the number of apoptotic cells in the GT. Three high power fields per section were randomly selected and at least 1,000 cells per field were counted on coded sections in a blind manner (n=18, from 6 sections). ***, p<0.001.
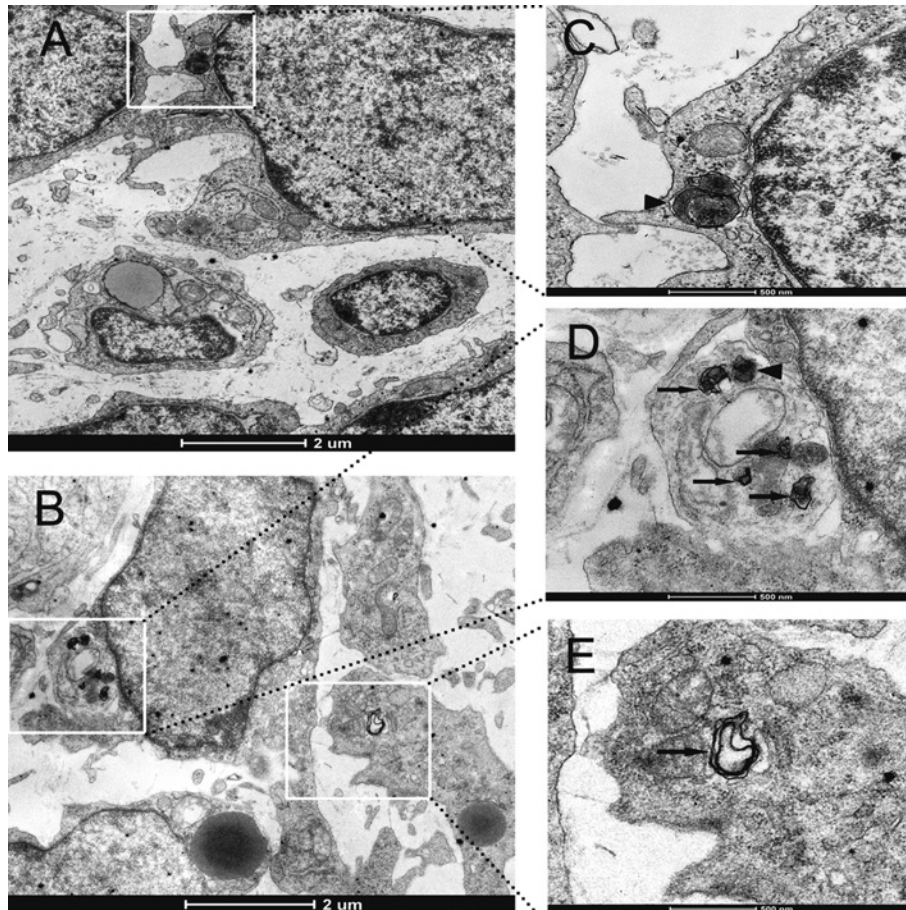

Fig. 3. Electron microscopic images of autophagy in GTs from male fetuses at GD 19. A, C: control group; B, D, E: hypospadias group; Arrowheads indicate autolysosomes and arrows indicate autophagosomes.

LC3II/LC3I ratio was significantly increased in the hypospadias group (p < 0.001, Fig. 4A). Expression of polyubiquitin-binding protein SQSTM1/p62 in the GT was significantly decreased in the hypospadias and non-hypospadias groups compared to the control group (p < 0.001, Fig. 4B). In the hypospadias group, the expression
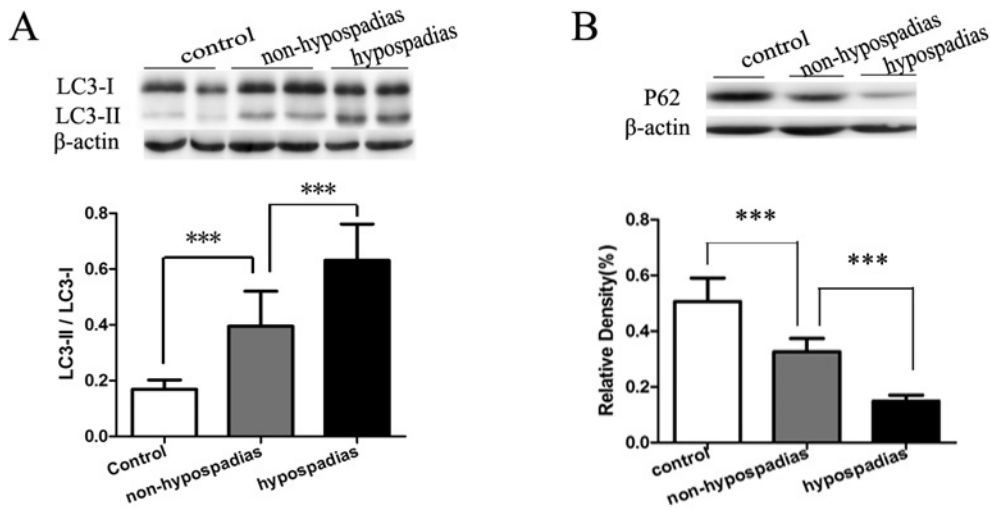
Fig. 4. The effects of DBP on LC3 and p62 protein levels in GTs from hypospadiac male fetuses (n=8). ***, p<0.001.
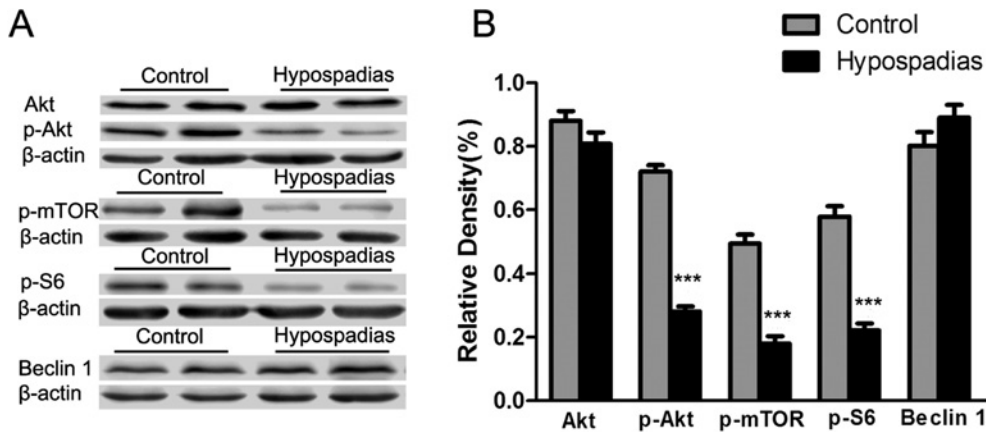
Fig. 5. The effect of DBP on protein levels of the autophagy pathway in GTs from hypospadiac male fetuses (n=8). ***, comparison between the hypospadias group and the control group, p<0.001.

of polyubiquitin-binding protein SQSTM1/p62 was significantly lower than that in the non-hypospadias group (p < 0.001, Fig. 4B).

3.6. Expression of autophagy proteins in the GT

The autophagy pathway is regulated by a variety of proteins. One of the stimulating proteins of the pathway, Beclin 1, showed similar expression levels in the GTs between normal fetuses and fetuses with hypospadias (Fig. 5). Some direct or indirect inhibitory proteins of the autophagy pathway (such as p-Akt, p-mTOR, and p-S6) were significantly decreased in the GTs from fetuses with hypospadias compared to GTs from normal male fetuses. There was no significant difference in Akt expression between the two groups (Fig. 5).

4. Discussion

In the present study, we generated hypospadiac male rats by timed exposure to DBP during pregnancy to investigate the mechanisms involved in DBP-induced hypospadias. Our results demonstrate that DBP significantly decreased the serum testosterone concentration in non-hypospadiac and hypospadiac male fetuses. Apoptotic cell numbers in the GTs were significantly decreased in fetuses at GD 19 in the hypospadias group; however, there was no change in the non-hypospadias group. Autophagy in the GTs was increased in fetuses at GD 19 with or without hypospadias after DBP exposure. In addition, the levels of autophagy in the hypospadias group were higher compared to the non-hypospadias group. DBP is a known environmental male reproductive toxicant, which

can cause hypospadias, cryptorchidism and anorectal malformations (Ema et al. 1993; Ema et al. 2000; Fisher et al. 2003; Kim et al. 2004; Mylchreest et al. 2000; Phillips and Tanphaichitr 2008; Sharpe 2001; Wine et al. 1997; Zhu et al. 2016). To the best of our knowledge, our findings are the first to provide evidence that apoptosis and autophagy play major roles in DBP-induced hypospadias.

Hypospadias takes place during early embryogenesis if urethral folds fail to fuse in the midline to make the urethral groove and form the tubular penile urethra (Yamada et al. 2003). The established hypospadiac male rat model in the present study indicates that there is increased susceptibility to DBP-induced hypospadias between GD 13 to GD 18 during pregnancy. The toxic effects of DBP to the external genitalia were found only in male fetuses, including decreased ratio of AGD/body weight and GT volume/body weight. Such sex-specific toxicity may be due to anti-androgen effects of DBP. DBP exposure can induce testicular dysgenesis, delay testicular development, and affect testosterone secretion in rat embryos (Li et al. 2015). It is known that male genitalia development is androgen-dependent and disruption of the androgen signaling pathway leads to feminization of male external genitalia, such as hypospadias (Mylchreest et al. 2000; Steinhardt 2004; Wang and Baskin 2008). Another study showed a potential correlation between low testosterone levels and the occurrence of hypospadias in male rat offspring after prenatal exposure to DBP (Jiang et al. 2016). In the present study, serum testosterone concentration in DBP-exposed male fetuses with or without hypospadias was significantly decreased. In addition, testosterone levels were lower in the hypospadiac fetuses compared to the non-hypospadiac male fetuses. These findings suggest that low levels of testosterone may be one of the important factors contributing to hypospadias induced by DBP.

In addition to testosterone, male genitalia development is dependent on other regulatory factors. Studies have shown that apoptosis is a prerequisite for proper embryonic development of male GT and anterior urethra (Suzuki et al. 2003; van der Werff et al. 2000). In the Hoxa 13 GFP-mutant hypospadias mouse model, loss of apoptosis was found in the urethra, consequently resulting in closure defects in the distal urethra (Morgan et al. 2003). In our hypospadias rat model, TUNEL-positive cells were significantly decreased in the GTs from male fetuses with hypospadias at GD 19. Interestingly, we found no changes between the non-hypospadias and control groups. Taken together, our findings suggest that decreased apoptosis in the GT plays an important role in DBP-induced hypospadias in rats.

In androgen-sensitive cells, such as prostate epithelial cells, the androgen signaling pathway is closely associated with autophagy, which affects cell survival (Boutin et al. 2013; Li et al. 2014). Autophagy is the major intracellular degradation and recycling system, which plays an important role in the turnover of unnecessary or dysfunctional proteins and organelles. These cellular constituents are sequestered in double- or multiple-membranes vacuoles called autophagosomes, which subsequently fuse with lysosomes and are degraded by hydrolases (Cuervo 2004; Galluzzi et al. 2008). Prostate cancer cells (HRPCa cells) (Boutin et al. 2013) and benign prostate epithelial cells (Li et al. 2014) show significantly elevated autophagy after androgen deprivation. The present study found significantly decreased levels of testosterone in male rats after DBP treatment. Therefore, we subsequently investigated how autophagy in the GT changed in response to lower levels of testosterone. We measured expression of two autophagy-related proteins, LC 3 and SQSTM1/p62 in male GTs. Autophagy marker LC3 is the mammalian equivalent of yeast Atg 8. Cleavage of LC3 immediately after its synthesis produces a cytosolic LC3-I form subsequently converted into the PE-conjugated form, LC3-II, during autophagy (Mizushima et al. 2010). The presence of LC3 in autophagosomes and the conversion of LC3-I to LC3-II are well-characterized markers of autophagy (Kabeya et al. 2000). P62 is an ubiquitin binding protein, which binds autophagosomal membrane protein LC3/Atg8 and subsequently aggregates to the autophagosome. Lysosomal degradation of autophagosomes leads to a decrease in p62 levels during autophagy. In the present study, the expression of LC3-II and the ratio of LC3-II/LC3-I were significantly increased both in the non-hypospadias and hypospadias groups accompanied by decreased p62 expression. Our findings suggest that autophagy was increased in the GT in male rats exposed to DBP in utero. We further confirmed the findings with transmission electron microscopy, which showed an increased number of autophagosomes and autophagolysosomes in the GTs of fetuses with hypospadias. In addition, we found that LC3-II and the ratio of LC3-II/LC3-I in the hypospadias group were significantly increased compared to levels in the non-hypospadias group. Thus, the present study provides a new clue that exposure to DBP in utero can affect autophagy in the GT, which may play a role in the pathogenesis of hypospadias.

A complex cross talk exists between autophagy and apoptosis. Exogenous and endogenous stresses, such as oxidative stress, hypoxia, nutrient deficiency, pathogen infection, or anticancer drug treatments, can markedly enhance autophagy activity in cells (Mizushima et al. 2008; Rubinsztein 2006). Apoptosis can be triggered by inhibiting macroautophagy (Boya et al. 2005). DBP induced autophagy plays a cytoprotective role against apoptosis in male germ cells (Zhang et al. 2016). In the present study, we found increased autophagy and decreased apoptosis in the GTs of hypospadiac male fetuses. Hence, it is possible that autophagy also plays an important role in resisting normal apoptosis, which may contribute to DBP-induced hypospadias in rats.

Autophagy is a complicated process in which at least two signaling pathways are involved in regulation - Beclin 1 and the PI3K/AKT/mTOR signaling pathways. Activation of Beclin 1 or inhibition of the PI3K/AKT/mTOR pathway can increase autophagy (He and Klionsky 2009). mTOR, the central player in autophagy regulation, is a serine/threonine kinase that inhibits autophagy in the presence of growth factors and abundant nutrients. The PI 3K/Akt signaling molecules link receptor tyrosine kinases to activate mTOR and consequently repress autophagy (Lum et al. 2005). In the present study, protein expression of p-Akt, p-mTOR, and p-S6 were significantly decreased in the GT in hypospadiac male rats. Because Beclin 1 protein level was not significantly different between the DBP treatment group and the control group, our findings suggest that exposure to DBP *in utero* increased autophagy *via* inhibiting the PI3K/Akt/mTOR pathway rather than *via* activating the Beclin 1 pathway.

In conclusion, the present study demonstrated that autophagy was increased in GTs from fetuses with and without hypospadias after exposure to DBP *in utero*. Further evidence indicated that decreased apoptosis and/or upregulated PI3K/AKT/mTOR signaling in the GT might play critical roles in the pathogenesis of hypospadias related to DBP exposure.

Conflicts of interest: No confliction of interest.

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