BPA Disturb TJ- Permiablity of Rat Sertoli Cells During Spermatogenesis in Vitro

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ABSTRACT Objective: To investigate the effects of BPA exposure on the testicular expression of Sertoli cell junctional proteins (SCJP) in male rats during spermatogenesis. Methods: Primary sertoli cells were isolated and cultured from Wistar rat for 4-5 days, and the tight junction-permeability barrier was established by dual-chamber culture model. The effect of BPA on tight junctions was measured by the method of transepithelial electrical resistance (TER) assay. With a series of concentration BPA (0,25,and 100μM) co-incubating the Sertoli cells in vitro for 24h, cell proliferative activity was assessed with MTT assay, and the expression of occludin, ZO-1, Cx43 were determined by Western blotting. Results: BPA perturbs the integrity of the TJ-permeablity in Sertoli cells in vitro, which was associated with the decline of selected proteins at the tight junction, and gap junction at the blood-testis barrier. The expression of Cx43 increased while the expression of occludin and ZO-1 reduced in the Sertoli cell following BPA treatment. Conclusion: The present study showed that occludin, ZO-1 and specifically Cx43 could be early targets for BPA, which may be one of the contributing factors leading to impairment in spermatogenesis.

Key words: Bisphenol A; Spermatogenesis; Tight junction; Sertoli cell junctional proteins

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Introduction

Bisphenol A is an endocrine disrupter which is associated with impaired spermatogenesis [1]. However the mechanistic basis of impaired spermatogenesis is unknown, whether BPA is a Sertoli cell toxicant has not yet been fully investigated. Sertoli cells were the unique somatocytes in seminiferous of testis. It provides mechanical and nutritional support and play a key role in spermatogenesis. Alterations in Sertoli cell function may lead to a germ cell loss and disruption of the seminiferous epithelium and in turn it will lead to impaired spermatogenesis. The zipper theory proposes that TJs at the basal domain of Sertoli cells break down to accommodate the passage of preleptotene and leptotene spermatocytes across the BTB, while new TJ fibrils are formed under the migrating releptotene and leptotene spermatocyes [2,3]. It is likely that germ cells are the major source of cytokines that regulate the timely opening and closing of the BTB. To date, three classes of integral membrane proteins have been found in the testis; They included occludins, claudins, and JAMs [4]. Although the structure and function of the Sertoli cell TJ that constitutes the BTB in the testis have been reviewed, the molecules and pathways that regulate TJ dynamics in the testis have remained largely obscure. This paper was to investigate the effects of BPA exposure on the testicular expression of Sertoli cell junctional proteins (SCJP) in male rats during spermatogenesis.

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1 Materials and methods

1.1 Animals and Antibodies and reagents

Male Wistar rats were purchased from Chinese food and Drug Administration (QingDao, ShanDong, China). Immunoblotting: rabbit anti-ZO-1, rabbit anti-OCLN, rabbit anti-Cx43, were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); BPA were obtained from Sigma-Aldrich Corp.

1.2 Experimental methods

1.2.1 Primary Sertoli cell cultures Sertoli cells were isolated from testes of 20-day-old Wistar rats as described earlier [4]. Cells were plated on Matrigel-coated dishes [Matrigel diluted 1:9 with F12/DMEM] and cultured in serum-free F12/DMEM (SFDM) supplemented with epidermal growth factor, insulin, transferrin, and bacitracin at 37℃ in a humidified atmosphere with 95% air and 5% CO_2. On day 2 (48 h after isolation), Sertoli cells were subjected to a hypotonic treatment (20 mM Tris, pH 7.4) for 5 min to remove residual germ cells. As such, the Sertoli cell purity in these cultures was greater than 98% with negligible contaminations of either germ cells (e.g., elongating and elongated spermatids). Sertoli cells were plated at 0.5×10^6 cells/cm^2 on Matrigel-coated 24-well dishes, treating with either vehicle control (0.1% DMSO), BPA at 25μM or 100μM on day 4-5 after the functional TJ-barrier was formed in cultured Sertoli cells, the effects of BPA on the steady-state levels of integral membrane proteins at the BTB were determined by Western blotting.

1.2.2 SDS-PAGE and western blot analysis Rats (n=3 for each time point) at different ages were sacrificed, and testes were removed frozen in liquid nitrogen immediately, and then they were stored at -80℃. Testis lysates were prepared in the NP-40 lysis buffer [50mM Tris, pH 8.0 containing 0.15M NaCl, 10% glycerol

(v/v), 1% NP-40 (v/v), 2mMEGTA], supplemented with protease inhibitor cocktail(~10μl/ml) and phosphatase inhibitor cocktails 1 and 2 (~20μl/ml) (Sigma-Aldrich) as described[5]. 100μg of protein from each sample was resolved by SDS-PAGE with 12% total acrylamide concentration. Immunoblotting was performed as described[6].

1.2.3 Assessment of the Sertoli cell BTB integrity in vitro Sertoli cells cultured in vitro on Matrigel-coated dual-chamber are known to assemble the TJ-permeability barrier that mimics the BTB in vivo physiologically and ultrastructurally[7,8],which has been used extensively by investigators in the field to study the BTB dynamics. To assess the establishment of the Sertoli cell TJ-barrier, TER across the sertoli cell epithelium was monitored daily with a series of concentration BPA (0,25,and 100 μM). BPA was removed by rinsing the Sertoli cells with fresh F12/DMEM (2 times, 5min each) to detected the reversibility of BPA-induced TJ-barrier disruption. TER= (Determination of value-Blank background value) /Effective area of film growth (0.6cm^2).

1.2.4 Cell viability assay MTT was used as an indicator of cell viability as determined by the transformation of tetrazolium salt MTT to an insoluble formazan salt. Sertoli cells were cultured at 0.5× 10^6 cm^2 on Matrigel-coated 96-well plate and treated with different concentrations of BPA for 24h. MTT (0.5 mg/ml) was added to each well for 2 h at 35℃. The supernatants were removed and the formazan crystals were solubilized overnight with a solution made of isopropanol containing HCl 0.1N and 10% Triton X-100. Formazan was quantified at 570 nm using a microplate reader Softmax Pro spectrophotometer.

1.2.5 Statistical analysis Statistical analyses were performed by two-way analysis of variance (ANOVA) using the repeated measures model followed by Dunnett's test to compare changes between treatment groups and their corresponding controls using the GBSTAT statistical analysis software package.

2 Results and analysis

2.1 Analysis of occludin, ZO−1, and Cx43 expressions in Sertoli cells

Immunoblot analysis to assess any alteration in the steady-state levels of proteins found at different junction types in the seminiferous epithelium following BPA treatment. The steady-state levels of various junction proteins in testis samples from two BPA treatment groups, 25μM, 100μM concentrations were assessed by immunoblotting. The levels of integral membrane proteins of the tight junction and gap junction were shown. We can see statistically significant, decline in the protein level of TJ protein occludin and ZO-1 was detected. Significant increase in the protein level of GJ protein also could be detected. (a) $P < 0.05$ versus control; (b) $P < 0.05$ versus control and 25μM BPA.

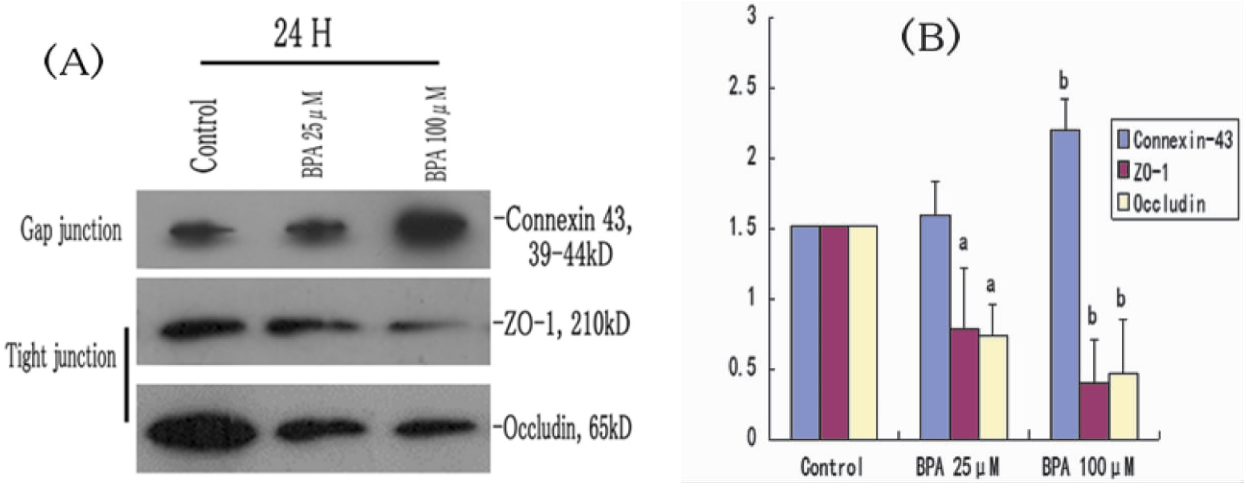

Fig.1 Influence of treatment of BPA on the SCJP：A：The levels of integral membrane proteins of the tight junction and gap junction were shown. We can see statistically significant, decline in the protein level of TJ protein occluding and ZO-1 was detected. Significant increase in the protein level of GJ protein (Cx43) also could be detected. (a) $P < 0.05$ versus control ; (b) $P < 0.05$ versus control and 25μM BPA.

2.2 BPA perturbed the Sertoli cell TJ−permeability barrier function via the redistribution of proteins at the cell−cell interface

When Sertoli cells were cultured in vitro, a functional TJ-permeability barrier was established by day 4. This was manifested by the ability of the cell epithelium to resist the passage of a short pulse of electrical current as quantified by the transepithelial electrical resistance (TER) (Fig.2). This in vitro system has been widely used by investigators in the field to study the BTB dynamics. While BPA at 25μM had no apparent effect on the Sertoli cell TJ-barrier function ($P > 0.05$), BPA at 100μM was shown to perturb the TJbarrier obviously ($P < 0.05$).

2.3 BPA inhibited the cell survival of Sertoli cells

The cytotoxicity of BPA on Sertoli cells cultured at 0.5×10^6 cells/cm^2 was assessed. BPA was added to cell cultures on day 5 at the desired concentrations. The MTT assay showed that BPA inhibited the proliferation activity of Sertoli cells significantly. Sertoli cell survival decreased obviously, and less than 66.43% when the

concentration was greater than 102μM, the results showed remarkable significance (P<0.05). Cell Survival dropped to 11.56% when the concentration was 103μM (P<0.01). However, it indicated that BPA was non-cytotoxic at 25μM and 100μM.(Tab.1)

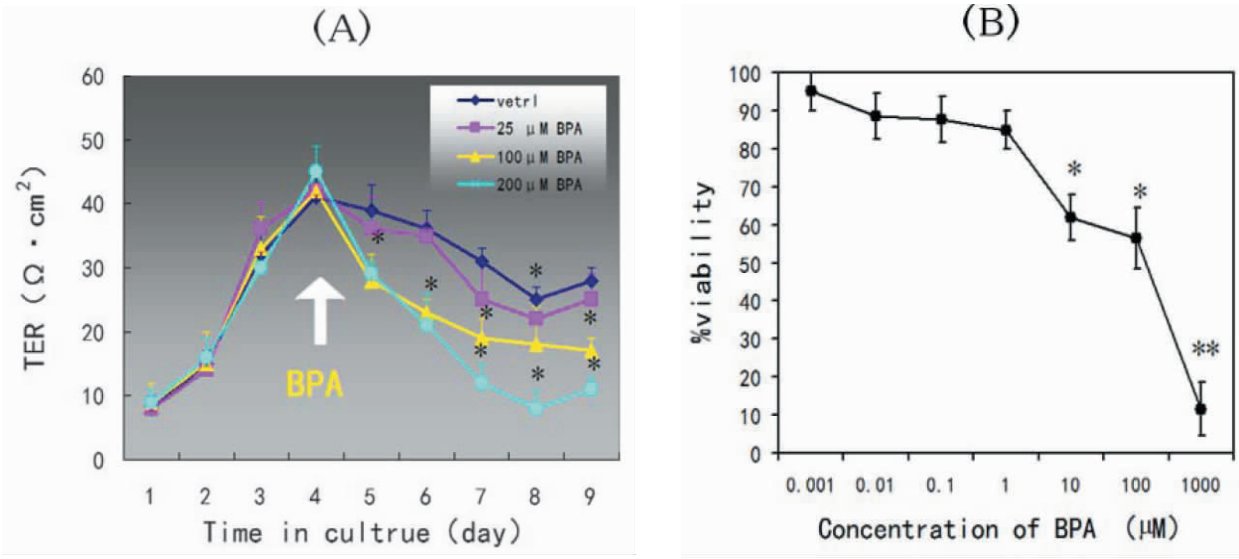

Fig.2 A study to assess the effect of BPA on the tight junction-permeability barrier function and proliferative activity of Sertoli cells in vitro: A: Sertoli cells were plated at 0.5×10^6 cells/cm^2 on Matrigel-coated dual-chamber and the TJ-barrier assembly was monitored by TER measurement. On day 4, cells were treated with 0.1% DMSO (vehicle control, VeCtrl), BPA . For the BPA treatment groups, cells were washed 24 h thereafter replenished with F12/DMEM. BPA at 25μM had no apparent effect on the Sertoli cell TJ-barrier function(P>0.05), but BPA at 100μM was shown to perturb the TJ-barrier obviously (P<0.05). B: The MTT assay showed that BPA inhibited the proliferation activity of Sertoli cells significantly. Cell survival dropped to 11.56% when the concentration of BPA rised to 10^3μM (P<0.01).

Table 1 BPA inhibited the cell survival of Sertoli cells

Concentration (μM)	OD	Cell Survival (%)
Blank	0.986± 0.025	100.00
10^{-3}	0.937± 0.030	95.08
10^{-2}	0.874± 0.031	88.64
10^{-1}	0.865± 0.026	87.72
10^{0}	0.837± 0.023	84.93
10^{1}	0.611± 0.035	62.00*
10^{2}	0.556± 0.023	56.43*
10^{3}	0.114± 0.038	11.56**

Note: *Compare with Blank group P<0.05 ;**Compare with Blank group P<0.01

3 Discussions

BPA is an estrogenic environmental contaminant, free BPA in the systemic circulation acts as an endocrine disruptor. BPA affects the male reproductive system as evidenced by decreases in daily sperm production in mice[9], and the decline in plasma free testosterone levels and the appearance of multinucleated giant cells in mouse seminiferous tubles,and the atrophy of rat seminiferous tubules[10].

Toyama and Yuasa [7] show that neonatal exposure with BPA affects spermatogenesis between the Sertoli cell and spermatids when the treated animals reached puberty. At stage VIII of the epithelial cycle during spermatogenesis, the migration of the early spermatocytes towards the adluminal compartment is highly coordinated by the Sertoli cell junctional complexes. BTB is a highly dynamic structure as it undergoes extensive restructuring at stages VIII-IX of the epithelial cycle to facilitate the transit of primary leptotene spermatocytes[8].However, the BTB undergoes rapid restructuring to facilitate preleptotene/leptotene spermatocyte migration. The migration of preleptotene and leptotene spermatocytes across the BTB at stage VIII of the seminiferous epithelial cycle in the adult rat testis involves extensive restructuring of the TJ and AJ. The promoting effects of testosterone on the BTB integrity [11,12] is mediated by an increase in protein recycling of internalized integr-

al membrane at he BTB site[6]. Cytokines, such as interferon-γ , TGF-βs, and TNF-α, were shown to perturb junctions by lowering the steady-state TJ and AJ protein levels at the intestinal barrier[13-15] and the BTB[16-20].Therefore,the Sertoli cell TJ-permeability barrier function, could be mediated by their differential effects on protein endocytosis, recycling,and/or transcytosis at the BTB[6]. These specialized junctional proteins are essential for the development and maintenance of spermatogenesis and that alterations of Sertoli-Sertoli cell interactions may lead to sterility in the male[21].

Recent data indicate that tight junction is a platform for signal transduction and that disruption of this platform may affect Sertoli cell function[22].To date, the integral membrane proteins that are known to constitute the BTB between adjacent Sertoli cells in rodent testes include :TJ proteins (e.g. Occluding, JAM-A, and claudins); basal ES proteins (e.g. N-cadherin, nectin-2); and gap junction proteins (e.g.connexin43). In vitro study was also performed to explore the direct effects of BPA on Sertoli cells. BPA was reported to significantly reduce the steady-state protein levels of occludin, N-cadherin and connexin43 in the SerW3 Sertoli cell line[23], implicating its disruptive effects on the BTB in vitro. This was in agreement with in vitro observations that known testicular toxicants such as gossypol or $CdCl_2$ induce inter-Sertoli gap and tight junction disruption[24]. The disruptive effects of BPA on the Sertoli cell BTB function appear to be mediated via changes in the kinetics of integral junction protein.

In conclusion, the results of the present study clearly show that intercellular junction proteins including occludin, ZO-1, N-cadherin and specifically Cx43 are primary and sensitive targets for cytotoxic agents in Sertoli cells. Indeed estrogen receptor beta (ERβ) is localized on the Sertoli cells[25] and BPA is known to interact with ERβ[26].Thus it is likely that BPA may modulate activity of ERβ in Sertoli cells leading to altered expression of SCJPs, thereby affecting the integrity of Sertoli cell-germ cell complex. Thus SCJP may serve as one of the potential markers to study estrogen like compound induced effects on testes.

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双酚 A 干扰大鼠生精过程时 TJ 渗透性屏障的体外研究

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摘要 **目的**:研究体外大鼠睾丸支持细胞紧密连接蛋白(SCJP)在类雌激素 - 双酚 A(BPA)干扰下的损伤机制。**方法**:对 Wistar 大鼠睾丸支持细胞(Sertoli 细胞)离体原代培养 4-5d,通过双室培养模型建立体外紧密连接(TJ)渗透性屏障,并测量其跨上皮电阻值(TER)反应紧密连接结构的形成及 BPA 对紧密连接的损害程度。设溶剂(DMSO)做阴性对照,以终浓度为 25μM、100μM 的 BPA 作用于支持细胞 24h,MTT 法测不同浓度 BPA 作用的 Sertoli 细胞增殖活性。Western bloting 观察 occludin、ZO-1、Cx43 表达的变化。**结果**:成功分离并培养 Wistar 大鼠睾丸支持细胞,并建立良好的体外 TJ 屏障模型。双室培养支持细胞上皮 TER 值在培养的 d4 达到顶峰,然后在 d4-9 维持相对较稳定的状态,d4 以 200μM,100μM,25μM BPA 染毒,分别于染毒后 24,48,72,96 和 120h 测 TER:与 DMSO 溶剂对照组相比,200μM,100μM 的 BPA 组 TER 值明显下降($P<0.05$),而 25μM 的 BPA 组在染毒后 TER 值无明显变化($P>0.05$)。MTT 结果显示:经不同浓度 BPA 作用 24h 后,Sertoli 细胞的吸光度(OD 值)随着染毒剂量的增加而逐渐降低。10^{2}、10^{3}μM 浓度组与溶剂对照组有显著性差异($P<0.05$),而 10^{-2}、10^{-1}、10^{0}、10^{1}μM 组和溶剂对照组无显著性差异($P>0.05$)。Western blot 结果显示:occludin、ZO-1、Cx43 在各剂量组均有表达,与溶剂对照组相比,occludin、ZO-1 表达均分别随作用剂量的增加而降低:25μM 组、100μM 组与溶剂对照组相比,差异均存在显著性($P<0.05$);100μM 组与 25μM 组相比,差异亦存在显著性($P<0.05$)。Cx43 的表达却随染毒剂量的增加而增加,与溶剂对照组相比,25μM 组表达无明显增加($P>0.05$),而 100μM 组则明显增加($P<0.05$);与 25μM 组相比,100μM 组表达明显增加($P<0.05$)。**结论**:双酚 A 可通过损伤支持细胞连接蛋白正常表达,破坏了 TJ 屏障渗透性,从而影响正常的精子形成过程。

关键词 双酚 A 精子形成 紧密连接 支持细胞连接蛋白

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