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Evaluation of ezrin and fascin 1 in the PFOS treated Sertoli cell culture: An in vitro study

PFOS ile tedavi edilen Sertoli hücre kültüründe ezrin ve fascin 1'in araştırılması: İn vitro bir çalışma

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ABSTRACT

Aim: Depending on the findings resulting from the knock-downing of ezrin and of fascin 1 in vivo, we aim to show the defects or disruption of the blood-testis barrier (BTB) structure and F-actin bundling after Perfluorooctanesulfonate (PFOS) treatment in primary Sertoli cell culture.

Study Design: Primary Sertoli cell isolation was occurred with control and PFOS-treated (20M) groups. Sertoli cells were prepared for both experiments as 0.5 x 106 cell/ml.

Methods: Dual-labeled immunofluorescence analysis to assess co-localization of fascin 1 with ezrin both in Sertoli cells was performed, and Co-IP, by using lysates of seminiferous tubules, was performed using actin and ezrin proteins to identify specific protein-protein interaction with fascin 1.

Results: Firstly, we showed that ezrin and fascin 1, which were components of the ectoplasmic specialization were co-localized in the Sertoli cells and also they were interacted each other. Secondly, we indicated that they were dislocated in the PFOS-treated Sertoli cells in vitro. Because of PFOS (20M), the actin-based cytoskeleton was no longer capable of supporting the distribution and/or localization of actin-regulatory proteins at the cell-cell interface necessary to maintain localization of actin-regulatory at the BTB.

Conclusion: In summary, these findings suggest that ezrin and fascin 1 can work together to preserve BTB integrity by regulating F-actin organization in the PFOS-mediated Sertoli cell disruption.

Keywords: Blood-testis barrier, ectoplasmic specialization, F-actin, ezrin, fascin 1,

ÖZ

Amaç: Bu çalışmada, Ezrin ve Fascin 1'in in vivo olarak baskılanması sonucundaki bulgularla birlikte, Kan testis bariyeri (KTB) yapısının bozulması ve primer Sertoli hücre kültüründe, Perflorooktansülfonat (PFOS) muamelesinden sonra F-aktin demetlenmesinin gösterilmesi amaçlanmaktadır.

Çalışma Tasarımı: Primer Sertoli hücre izolasyonu, kontrol ve PFOS ile muamele edilen (20μM) gruplarla yapıldı. Sertoli hücre konsantrasyonu her iki deney için 0.5 x 106 hücre/ml olacak şekilde hazırlandı.

Metod: Hem Fascin 1'in Sertoli hücrelerinde Ezrin ile birlikte lokalizasyonunu değerlendirmek üzere çift etiketli immünofloresan analizi yapıldı, hem de seminifer tübül lizatlarından aktin ve Ezrin proteinlerinin Fascin 1 proteini ile spesifik protein-protein etkileşimini tanımlamak için Co-IP deneyi uygulandı.

Bulgular: İlk olarak, ektoplazma özelleşmesi bileşenleri olan Ezrin ve Fascin 1 in Sertoli hücrelerinde ko-lokalize olduğu ve birbirleri ile etkileşime girdiği gösterilmiştir. İkinci olarak ise PFOS ile muamele edilen Sertoli hücrelerinde Ezrin ve Fascin 1 dislokasyonu gösterilmiştir. 20μM PFOS uygulandığında aktin tabanlı hücre iskeleti KTB'deki hüce-hücre bağlantı bölgelerindeki aktin düzenleyici proteinlerin yayılmasını ve/veya lokalizasyonunu destekleyememiştir.

Tartışma: Sonuç olarak bu bulgular, PFOS aracılı Sertoli hücre bozulmasında, Ezrin ve Fascin 1'in KTB bütünlüğünü korumak için F-aktin organizasyonunu düzenleyerek birlikte çalışabileceğini desteklemektedir.

Anahtar Kelimeler: Kan-Testis Bariyeri, Ektoplazmik Özelleşme, F-actin, ezrin, fascin

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INTRODUCTION

Studies in the last decade using the blood-testis barrier (BTB) in rats have demonstrated the presence of several signaling pathways [1]. Morphological details of germ cell transport occurs through actin-based cytoskeleton formation during spermatogenesis in rodents [2]. BTB remodeling is important to understand germ cell transport through the actin-based re-bundling mechanism [3]. Ezrin leads actin microfilaments in spermatogenesis to integral membrane protein, as well as peripheral protein in mammalian cells, to organize apical membrane domain, which create a scaffold for signaling molecules to regulate cell migration, proliferation, adhesion, and polarity [4, 5]. A knockdown of ezrin in vivo by RNAi was found to impede spermatid transport, causing defects in spermiation in which spermatids were embedded deep inside the epithelium, and associated with a loss of spermatid polarity [6]. Fascin is a 56-kDa polypeptide, possessing the actin binding and bundling activity by cross-linking filamentous actin into tightly packed parallel bundles [7, 8]. A knockdown of fascin 1 in vivo by 60–70% induced defects in spermatid polarity, which was mediated by a mislocalization and/or downregulation of actin-bundling proteins, impeding F-actin organization and disrupting spermatid polarity. Perfluorooctanesulfonate (PFOS), served as a fabric protector and an active component in stain repellents [9] by 3M in 1949, as a fluorosurfactant. Its toxic effects have begun to emerge, initially detected in wildlife and then in humans, and they include defects in development, cancer, endocrine disruption, neonatal mortality [10, 11], and an increased risk of attention-deficit hyperactivity disorder [12]. PFOS was also found to induce Sertoli cell injury by perturbing actin cytoskeleton through changes in the spatial expression of actin regulatory proteins [13] and the organization of F-actin in Sertoli cells [14]. With this study we aim to investigate how PFOS affects ezrin and fascin 1 expression pattern and prevent the organisation of F-actin bundling in BTB.

MATERIAL AND METHODS

Animals

Sprague-Dawley (outbreed) rats were euthanized by CO_2 asphyxiation using slow (20–30%/min) displacement of chamber air with compressed carbon dioxide, in a euthanasia chamber (Braintree Scientific, Braintree, MA). Experimental protocol was approved by animal care and usage committe of Istanbul University and was in accordance with the institiuational Animal Care and Use Committee (IACUC) guide.

Primary Sertoli cell cultures

Sertoli cells were isolated from 20-day-old rat testes and cultured for experiments reported herein, as detailed elsewhere [6]. Cells were cultured in serum-free Ham's F-12 Nutrient Mixture-Dulbecco's modified Eagle's medium (F-12- DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with bovine insulin, human transferrin, EGF, bacitracin, and gentamicin in a humidified CO_2 incubator with 95% air-5% CO_2 (vol/vol) in a humidified atmosphere at 35°C. After isolation, Sertoli cells were plated on Matrigel (BD Biosciences, Billerica, MA)-coated coverslips, 12-well culture dishes, 0.5, and 1.2 x 106 cells/cm2. Cells at these densities were used for the following corresponding experiments: 1) dual-labeled immunofluorescence analysis, including F-actin staining; 2) lysate preparation for immunoblotting and Co-IP.

Toxicants

PFOS (perfluorooctanesulfonic acid, potassium salt, Mr 538.22, Sigma-Aldrich) was dissolved in dimethylsulfoxide (DMSO) as a 100- to 200-mM stock so that the concentration of DMSO in Sertoli cell cultures was approximately 0.02% (vol/vol). PFOS administration used between 3, 6, 24 and 48 hours in primary Sertoli cell culture.

Dual-labeled immunofluorescence analysis

Immunofluorescence analysis using cultured Sertoli cells was performed as described [15]. Primary antibodies for Ezrin (Abcam cat no: ab4069, 1/200 dilution), fascin 1 (Abcam cat no: ab126722, 1/100) , F-actin (Phalloidin, sigma, p5282, 1/150 dilution) used for this study. Secondary antibodies were goat antimouse or goat antirabbit IgG conjugated to either Alexa Fluor 488 or 555 (Invitrogen) and diluted 1:200 in PBS containing 1% BSA (vol/vol). Cells were mounted in ProLong antifade reagent containing

4′,6-diamidino-2-phenylindole (DAPI; Invitrogen), and images were acquired with MicroSuite FIVE software (version 1.224; Olympus Soft Imaging Solutions Corp) using an Olympus DP71 12.5 MP (megapixel) digital camera in an Olympus BX61 motorized fluorescence microscope (Olympus America, Inc). All samples within experimental set vs. controls were processed and analyzed in a single experimental session to avoid inter-experimental variations. A representative set of data was shown herein, but each experiment was repeated at least three times using different preparations of Sertoli cells with similar findings.

Immunoblotting and Co-IP.

Lysates were obtained from Sertoli cells and seminiferious tubules. Tubules isolated from adult rat testes were used within 2 hours after their isolation, which were devoid of Leydig cell contamination. Actin (sc-1616) antibody used for immunoblotting for Co-IP with 1/300 dilution. Co-IP was performed using lysates (around 600 µg protein) from testes or tubules. Chemiluminescence was performed and the immunoblots were analyzed as described [16]. Immunoblotting data were acquired in a Fujifilm LAS-4000 Mini Imaging System and analyzed in MultiGauge software (version 3.1; Fujifilm), which was then quantified by using the Scion Image software package (version 4.0.3.2, Scion; http://scion-image.software.informer.com/) for analysis, as described [17].

Statistical analysis

Significance was determined by one-way ANOVA, followed by Dunnett's procedure using the SigmaStat software package (version 3.5; Systat Software Inc). *; p >0.05.

RESULTS

Ezrin and fascin 1 expression patterns in Sertoli cells together with their co-immunuprecipitation levels.

We first examined fascin 1 and ezrin expression levels in Sertoli cells (Figure 1A). We showed that ezrin and fascin 1 expressions were co-localized in Sertoli cell cytoplasm with high expression levels. We next observed that ezrin and fascin 1 were co-immunoprecipitated together with actin protein (Figure 1B). We found that anti-fascin-1 IgG was labeled with ezrin expression pattern in seminiferous tubule (ST) lysates.

PFOS perturbs F-actin organization at the BTB, impeding the localization and/or distribution of Ezrin and F-actin at the Sertoli cell-cell interface

PFOS induced changes in the localization of ezrin and distribution of F-actin in the Sertoli cell epithelium by dual-labeled immunofluorescence (Figure 2). It perturbed the organization of F-actin in Sertoli cells. PFOS-treated (6h) Sertoli cells, actin filaments in cell cytosol were truncated and defragmented and actin filaments no longer assumed the orderly bundled configuration, which was not present in the control Sertoli cells (Figure 2.).

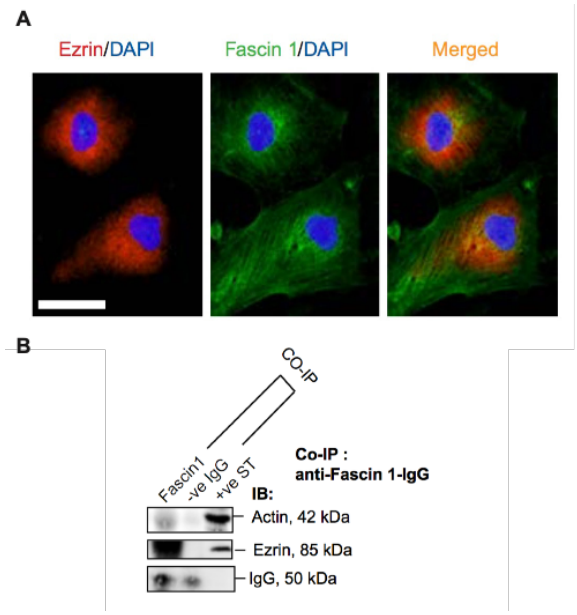

Figure 1. (A) Dual-labeled immunofluorescence analysis to assess colocalization of fascin 1 (green fluorescence) with ezrin (red fluorescence) in Sertoli cells. Sertoli cells nuclei were visualized by DAPI. Scale bar, 15 mm (applies to all micrographs.) (B) Co-IP using lysates of seminiferous tubules was performed using actin and ezrin proteins to identify specific protein-protein interaction with fascin 1. IgG, heavy (50 kDa) chains, served as the protein loading control.

PFOS perturbs F-actin organization at the BTB, impeding the localization and/or distribution of fascin 1 and F-actin at the Sertoli cell-cell interface

PFOS induced changes in the localization of fascin 1 and F-actin in the Sertoli cell epithelium, which were demonstrated by dual-labeled immunofluorescence staining (Figure 3). After PFOS treatment, the expression levels of fascin 1 together with F-actin were affected and these

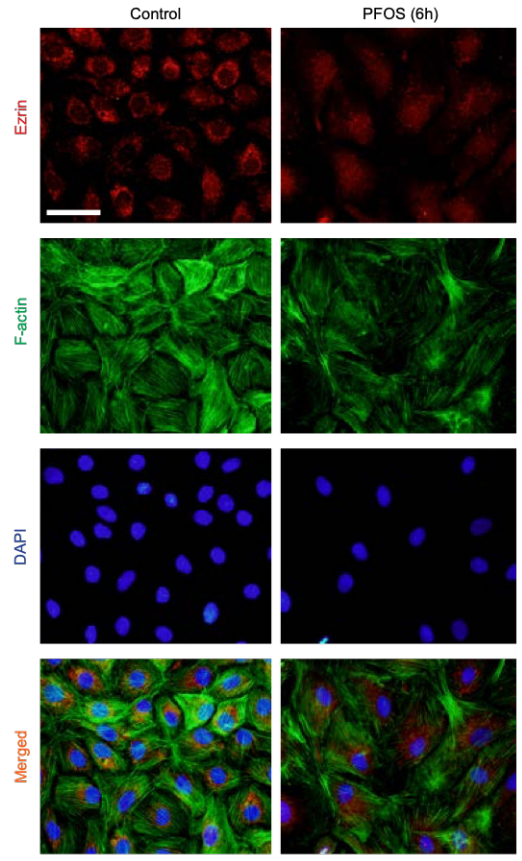

Figure 2. Dual-labeled immunofluorescence analysis to assess colocalization of ezrin (red fluorescence) with F-actin (green fluorescence) in PFOS-treated Sertoli cells. Sertoli cell nuclei were visualized by DAPI. Scale bar, 15 mm (applies to all micrographs).

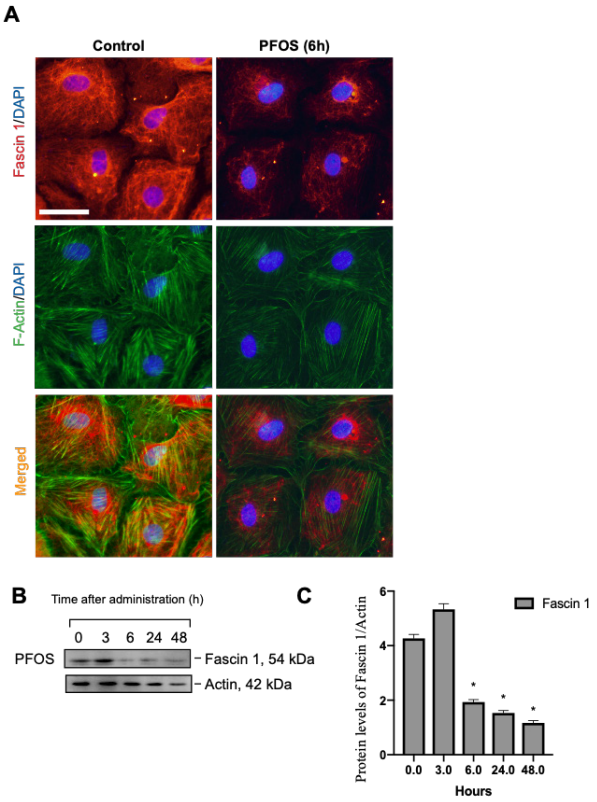

Figure 3. (A) Dual-labeled immunofluorescence analysis to assess colocalization of fascin 1 (red fluorescence) with F-actin (green fluorescence) in PFOS-treated Sertoli cells. Sertoli cell nuclei were visualized by DAPI. Scale bar, 15 mm (applies to all micrographs). (B) Fascin 1 expression levels after PFOS administration (0, 3, 6, 24, 48 hours) with actin as an internal control. (C) Protein levels of Fascin 1 immunoblotting data was analyzed by comparing 5 different hours with at least 3 Fascin 1 bands, which were normalized by actin protein levels.

proteins were mislocalized and their expression levels were decreased (Figure 3A). After PFOS administration in 3, 6, 24 and 48 hours we showed that Fascin 1 expression levels were decreased after 6 hours of PFOS treatment (Figure 3B).

DISCUSSION

Earlier toxicity studies on PFOS have focused mostly on its disruptive effects on thyroid function in humans [18, 19]. In our study, PFOS perturbs the BTB via its effects on F-actin organization related with ezrin and fascin 1 co-operation at the same time. In a recent study that investigated the disruptive effects of PFOS on ezrin and fascin 1 in Sertoli cells, PFOS was found to reduce cell viability by inducing Sertoli cell reactive oxygen species production dose dependently [20]. However, F-actin disruption at the BTB was not studied together with ezrin and fascin expression patterns [21]. Herein we report that PFOS is a potential disruptor for the BTB organization, which was related to ezrin and fascin 1 expression patterns after 6 hour administration. This disrupting effect appears to be mediated initially by changes in the expression and localization of actin bundling proteins ezrin and fascin 1 in Sertoli cells. We have already known that actin bundling proteins leads to regulate germ cell migration during spermatogenesis [22]. These changes, in turn, perturb F-actin organization at the BTB, rendering actin filament bundles, the hallmark ultrastructure of the BTB in mammalian testes [23], which are unable to assume their bundled configuration to support BTB integrity. Instead, actin filaments in PFOS-treated Sertoli cells were truncated and defragmented together with the decreation of ezrin and fascin 1 expression levels. In summary, the regulation of the mechanism of actin bundling in PFOS-treated Sertoli cells can be controlled by ezrin and fascin 1 at the same time by using

the same signalling pathway in BTB. Thus, the responsibility of actin regulating proteins have to be proven by investigating their co-operations in different suggesting toxic models [24, 25] for BTB.

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Conflict of Interest: The author has no conflicts of interest relevant for this article.

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