Original Article

Aryl Hydrocarbon Receptor Modulation Impacts on the Blood-testis Barrier Integrity via TJP1 Function

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How to Cite This Article Bustani, Gh. S., & Alghetaa, H. (2025). Aryl Hydrocarbon Receptor Modulation Impacts on the Blood-testis Barrier Integrity via TJP1 Function. *Iranian Journal of Veterinary Medicine, 19*(3), 415-426. https://dx.doi.org/10.32598/ijvm.19.3.1005541

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ABSTRACT

Background: Male infertility is a global concern and it tends to increase due to miscellaneous factors, such as environmental toxins and genetic and lifestyle choices. The aryl hydrocarbon receptor (AHR) has recently attracted attention due to its involvement in male infertility mechanisms and impact on sperm production and function. AHR, a versatile receptor expressed in various tissues, including the testes, regulates the genes involved in spermatogenesis. AHR activation is associated with cell cycle regulation and chromatin condensation during spermatogenesis.

Objectives: This study aimed to investigate the influence of AHR activation on blood-testis barrier (BTB) integrity, focusing on the role of tight junction protein-1 (TJP1) and exploring the effects of AHR modulation on spermatogenesis outcomes in adult male rats.

Methods: Forty adult male rats were divided into four groups according to their treatment regimens. Briefly, the control group was kept without any treatment, the resveratrol (RES) group received an intraperitoneal (IP) injection of 100 mg/kg every 72 h and the AHR antagonist (AHR^-) group received an IP injection of CH223191 at a dose of 10 mg/kg every 72 h. Finally, the dimethyl sulfoxide (DMSO) group received DMSO which was used as a solvent for preparing RES and CH223191. The study lasted for 60 d to cover the entire spermatogenesis cycle. At the study's endpoint, sperm chromatin maturity and condensation were evaluated in addition to *Tjp1* gene expression in testicular tissue, supported by BTB integrity assessment.

Results: The abnormal sperm chromatin maturity (ASCM) and condensation were significantly ($P<0.05$) higher in AHR^- compared to all other groups. *Tjp1* gene expression was significantly upregulated in the RES group compared to the control and AHR^- group, which tightened the BTB and maintained the testicular homeostasis for normal spermatogenesis.

Conclusion: RES treatment positively influenced sperm chromatin maturity, *Tjp1* expression and BTB integrity, suggesting its potential as a protective agent against male reproductive health. Conversely, AHR antagonism leads to compromised sperm chromatin integrity and BTB function, highlighting the critical role of AHR in spermatogenesis and BTB maintenance. These results underscore the importance of AHR modulation in male fertility and provide insights into potential therapeutic interventions.

Keywords: Aryl hydrocarbon receptor (AHR), CH223191, Resveratrol (RES), Spermatogenesis, Tight junction protein 1 (TJP1)

Article info:
Received: 30 May 2024
Accepted: 27 Jul 2024
Available Online: 01 Jul 2025

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Introduction

One of the significant concerns worldwide is infertility, which arises from environmental toxins, genetic predispositions, and lifestyle choices. In recent years, research has increasingly focused on the aryl hydrocarbon receptor (AHR) to elucidate its role in the mechanisms of male infertility and to shed light on the intricate processes involved in sperm production, maturation, and function (Bala et al., 2021; Skakkebæk et al., 2022). AHR is a versatile and dynamic cytosolic receptor found in various tissues throughout developmental and adulthood stages (Gonzalez & Fernandez-Salguero, 1998; Neamah et al., 2020). Many researchers have found that AHR activation is associated with the regulatory genes involved in spermatogenesis. AHR expression in the seminiferous tubule was explored in rats, revealing a specific localization to the primary pachytene spermatocytes during stages VII–XI and round spermatids during the 2nd through 14th stages of the spermatogenic cycle. However, human testes express AHR in all spermatogenic cycle stages (Hansen et al., 2014a; Pohjanvirta, 2012; Schultz et al., 2003).

The effects of AHR on the cell cycle may vary based on the presence or absence of exogenous ligands and specific cellular context. Moreover, the AHR action depends on the absence of an exogenous ligand, which may promote S-phase progression. When AHR binds to its ligand, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), it will lead to inhibit DNA replication in various cell lines (Bock, 2018; Huss et al., 2019; Ribeiro et al., 2018). Furthermore, animals treated with TCDD showed fewer cells in the S phase and arrested cells in the G1 phase (Marlowe et al., 2004). Previous studies show that AHR is crucial in regulating the cell cycle. Furthermore, AHR activity is influenced by the presence or absence of its ligands and the metabolic activity of P4501A1, which regulates cell cycle progression, particularly during the G1-to-S phase transition. Modulating AHR activity and its downstream effects on cell cycle regulators contribute to fine-tuning cell cycle progression.

AHR plays a crucial role in regulating chromatin condensation during prophase, during the first stage of mitosis, and involves the compaction of DNA into a more organized and compact structure, enabling proper chromosome alignment and segregation in subsequent stages (Abdulla et al., 2021; Alghetaa et al., 2023; Zhang et al., 2021). Thus, AHR activation promotes chromatin condensation by enhancing the expression of histone deacetylase (HDACs) enzymes. These enzymes remove acetyl groups from histones, leading to the formation of tight nucleosomes and inactive DNA (Garrison et al., 2000; Tsai et al., 2020; Wang et al., 2017). A substantial body of research suggests that the absence of AHR ligand binding or activation may contribute to inflammation (Meng et al., 2018), apoptosis (Hansen et al., 2014a) and oxidative stress in sperm, leading to DNA damage (Mostafa et al., 2017). Examining AHR expression in the rat seminiferous tubule revealed its affinity for primary pachytene spermatocytes during stages VII–XI and round spermatids through the II–XIV phase of the spermatogenic cycle (Barhoover et al., 2010).

Resveratrol (RES) (3, 5, 4′-trihydroxystilbene) is a non-flavonoid polyphenol organic compound found naturally in various foods, such as grapes, peanuts, and blueberries (Amin et al., 2023; Jawad & Sahib, 2022). RES is widely known for its beneficial biological effects that target multiple molecular pathways, including oxidative stress and the AHR signalling pathway (Alghetaa et al., 2023; Khayoon & Al-Rekabi, 2020). RES is a natural antagonist to AHR but has partial agonistic effects on AHR when used at relatively high doses (Singh et al., 2007; Chitrala et al., 2018). Additionally, RES inhibits the activation of CYP1A1 and CYP1B1 via the AHR/AHR nuclear translocator (ARNT) complex, which has also been correlated with a reduction in reactive oxygen species (ROS) production (Alghetaa et al., 2023; Dawood et al., 2023; Nsaif & Al-Mualm, 2021). The protective impact of RES on ROS production is crucial, given that peroxidation of polyunsaturated fatty acids can lead to a loss of membrane fluidity and decreased activity of membrane enzymes and ion channels, potentially compromising sperm motility (Bustani & Baiee, 2021; Chikhaoui et al., 2023).

Other studies have elucidated the mechanism of RES in AHR, highlighting its ability to restore the reduction in extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) phosphorylation (Abdulla & Al-Okaily, 2022; Alamo et al., 2019; Liu et al., 2020). RES protects cells from DNA damage and apoptosis by modulating anti- and pro-apoptotic mediators and increasing antioxidant status. Studies have shown that RES can inhibit the enzymatic activity of various cytochrome P450s and block their transcription through antagonism of AHR, suggesting that RES could decrease the exposure of cells to carcinogens (Alharris et al., 2017; Mongioì et al., 2021). The tight junction protein-1 (TJP1) plays a crucial role in forming tight cell junctions. It acts as a scaffold protein, cross-linking and anchoring tight junction strand proteins which are fibril-

like structures within the lipid bilayer (Yan et al., 2024; Alghetaa et al., 2017). In spermatogenesis, TJP1 protein is expressed in Sertoli cells and plays a crucial role by providing physical and nutritional support to male germ cells. It is also instrumental in maintaining the integrity of Sertoli cell tight junctions, a major component of the blood-testis barrier (BTB) formation and the progression of spermatogenesis (Ghafouri-Fard et al., 2021).

This study aims to explore the impact of activation or inhibition of AHR signaling on sperm genetic integrity, such as chromatin maturation and condensation, and the effect of AHR signaling dysregulation on blood-testes barrier integrity, focusing on the role of *Tjp1* gene expression.

Materials and Methods

Experimental animals

Forty adult male rats weighed 225-275 grams were utilized in this study. The experimental procedures started following a two-week acclimatization period. The animals were randomly divided into different cages, each containing six animals, at the Animal House of the College of Veterinary Medicine, University of Baghdad. All the animals had free access to food and water throughout the experimental period.

Rats were randomly assigned to four equal groups, including the control group receiving no treatment, the dimethyl sulfoxide (DMSO) group was given an intraperitoneal (IP) injection of DMSO (50% solution), RES group received IP injection of RES at a dose of 100 mg/kg every 72 h (Bordbar et al., 2022) and lastly, AHR^{-} group was IP injection of CH223191, an AHR antagonist, at a dose of 10 mg/kg every 72 h (Cao et al., 2022). All experiments were conducted for 60 days to cover the entire spermatogenesis cycle in rats (Clouthier et al., 1996; Sadighara et al., 2024). At the end of the experiments, five animals from each group were sacrificed to collect the testes and tail of the epididymis for further assessment. The other five animals were used to assess testes barrier integrity.

Preparation of drug solutions

RES was purchased from Hebei Guanlang Biotechnologies and its solution was prepared to achieve a protocol dosage of 100 mg/kg body weight (BW). Briefly, 400 mg RES was dissolved in 2 mL DMSO before being further diluted in another 2 mL distilled water. The resulting solution was accurately mixed for homogeneity using a vortex mixer; then each rat received 1 µL/g BW of this solution (Bordbar et al., 2022). Similarly, the CH223191 solution was prepared by dissolving 40 mg of CH223191 (Hebei Guanlang Biotechnologies) in 2 mL DMSO. Then, once the homogenous solutions are obtained, another 2 mL of distilled water was added. The solution was thoroughly mixed to achieve homogeneity using a vortex mixer, and each rat received 1 µL/g BW of the resulting solution (Alharris et al., 2017; Cao et al., 2022). Two milliliters of DMSO (Hebei Guanlang Biotechnologies) were mixed with 2 mL of distilled water to be used in the DMSO group, where each rat received 1 µL/g BW.

Sample collection

At the end of the experiment, on the 60th day, the animals were anesthetized using ketamine (90 mg/kg BW) and xylazine (40 mg/kg BW). After anesthesia, bilateral testes, epididymis, and blood samples were collected for downstream analysis.

The left tail of the epididymis was rinsed and incubated in 2 mL of normal saline at 37 ˚C. The spermatozoa were cut using microscissors for further evaluation (Ngaha Njila et al., 2019; Al-Mousaw et al., 2022).

Fixation process for cytogenetic examination

The fixation process involved placing a 10 µL aliquot of the sperm sample on a clean microscope slide and smearing, allowing it to air dry briefly. It subsequently immerses the slide in a 3:1 solution of methanol and glacial acetic acid for 5 minutes. Following fixation, the slide was left to air dry completely. This meticulous procedure ensures the preservation of sperm morphology on slides (Tejada et al., 1984).

Abnormal sperm chromatin maturity test (ASCM)

Sperm smears were delicately soaked with 0.1 N hydrochloric acid (HCl) at 5 ˚C for 5 minutes, and then rinsed with distilled water. The smears were stained with 5% toluidine blue (TB) dye in 50% citrate-phosphate buffer (pH=3.5). The evaluation process involved the enumeration of 200 spermatozoa on each slide, using a light microscope with a magnification of 1000×. Sperms with unstained or lightly blue nuclei were categorized as normal sperm (TB−), while those exhibiting dark blue nuclei were considered abnormal (TB+).

This meticulous methodology ensures a comprehensive assessment of sperm chromatin maturity (Abbasi et al., 2011; Pourmasumi et al., 2019) (Figure 1).

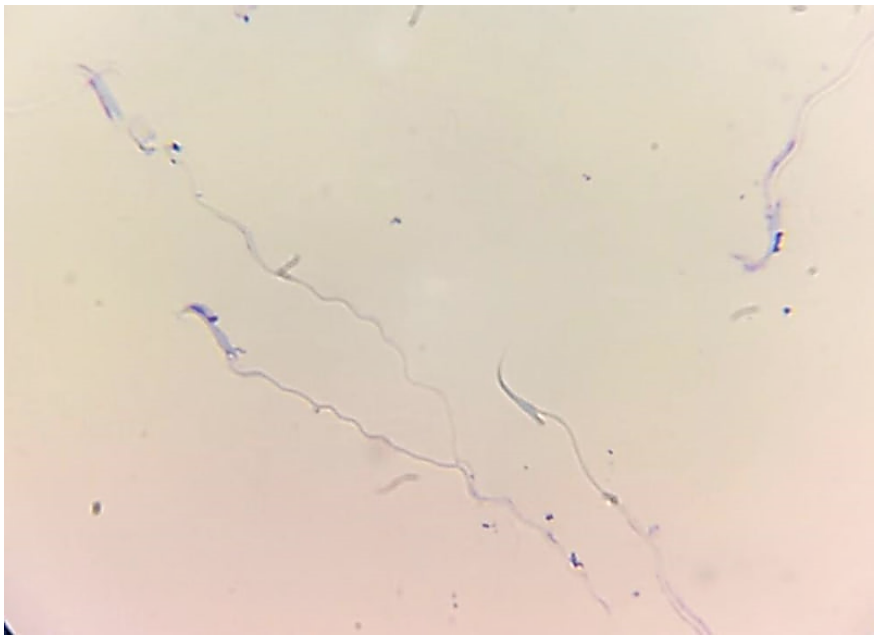

Figure 1. Assessment of sperm chromatin maturity using TB staining

Comparative analysis of TB- and TB+Sperm (×400)

Abnormal sperm chromatin condensation (ASCC) test

Sperm nuclear chromatin condensation was evaluated using aniline blue (AB) staining (Wong et al., 2008). Briefly, after fixation, the slides were immersed in 5% AB solution in 4% acetic acid (pH 3.5) for 5 minutes. Subsequently, the slides were rinsed in distilled water, stained with 0.5% eosin for 1 minute, and air dried. The stained slides were examined at 1000× magnification using a light microscope. Immature sperm, characterized by nuclear histone proteins, appeared dark blue, while mature sperm stained red-pink with protamine. A mini-

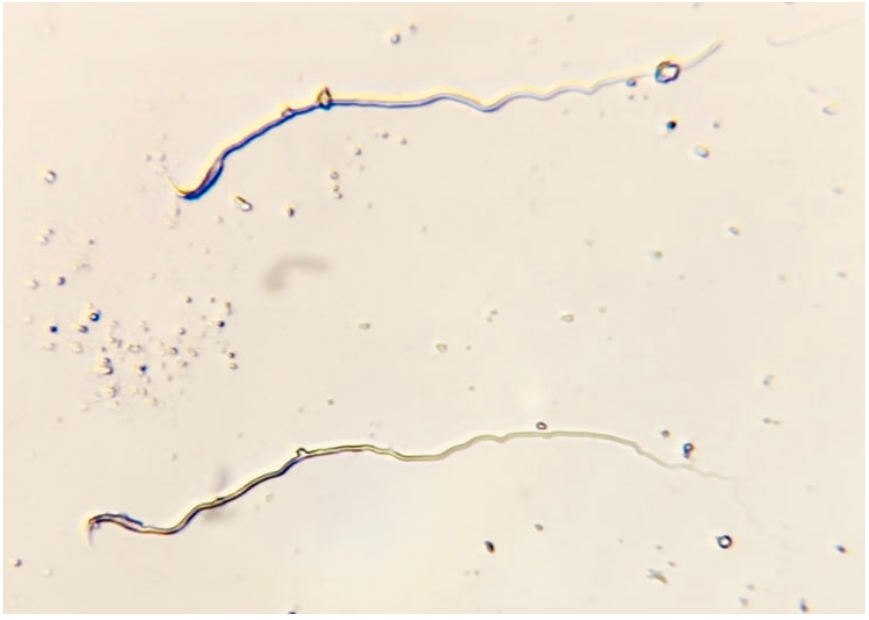

Figure 2. Assessment of sperm nuclear chromatin condensation using AB staining

Comparative analysis of immature and mature sperm (×400)

Table 1. *TJP1* and housekeeping gene *Gapdh* primers used in this study

No.	ID Gene	Reverse	Forward	NCBI Reference Sequence
1	*Tjp1*	5′-TCA CAG TGT GGC AAG CGT AG-3′	5′-TTC CAC AAG GAG CCA TTC CT-3′	NM_001106266.1
2	*Gapdh*	5′-ATG AAG GGG TCG TTG ATG GC-3′	5′-AGA GAC AGC CGC ATC TTC TT-3′	NM_017008.4

mum of 100 spermatozoa per slide was counted to analyze the percentage of red-pink spermatozoa (Figure 2).

Quantitive real-time polymerase chain reaction (qRt-PCR) assessment of *TJP1* gene expression

Gene expression analysis was conducted by extracting ribonucleic acid (RNA) from testicular tissue stored at -80 ˚C using triazole reagent. The extracted RNA was then reverse-transcribed into complementary DNA (cDNA) using a one-step real-time polymerase chain reaction (RT-PCR) premix kit.

Custom-designed primers obtained from GenBank were used to amplify target genes. qPCR was performed using the SYBR green master mix under specific thermocycler conditions (Tables 1 and 2). Real-time monitoring of the amplification process was achieved using a 7500 real-time PCR system, which measured the fluorescence signal emitted by SYBR Green dye throughout each amplification cycle.

BTB test

To assess the testes-barrier integrity, at the end of the 60-day experimental period, five animals from each group were intravenously injected with 0.2 mL of 1% Evans blue in saline via the tail vein. Evans blue, upon intravascular injection, potently binds to albumin, facilitating the study of vascular permeability and albumin leakage in various organs, including the testes (Alves-Lopes et al., 2018; Domínguez-Salazar et al., 2020).

Following a 30-minute interval, the animals were euthanized, and the testes were extracted and incubated in formamide solution for 24 h at 37 ˚C in a water bath. Estimation of extravasated Evans blue levels was performed using a spectrophotometer absorbance at 620 nm and the results were plotted on the slope equation after measuring standards prepared for this purpose (Alves-Lopes et al., 2018).

Statistical analysis

GraphPad Prism software, version 9.1 for windows was employed to analyze the collected data, and one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test and corrected by Holm-Sidak correction for multiple test comparison. Statistical significance was set at $P<0.05$.

Table 2. Thermocycler running conditions

Variables	Phase	Condition (°C)	Time	Cycle
Holding	Holding stage 1	37	15 min	1
	Holding stage 2	95	10 min	1
PCR cycle	Denaturation	95	15 sec	40
	Annealing	60	1 min	
	Extension	72	30 sec	
Melt curve stage	1st	95	15 sec	1
	2nd	60	1 min	1
	3rd	95	30 sec	1

PCR: Polymerase chain reaction.

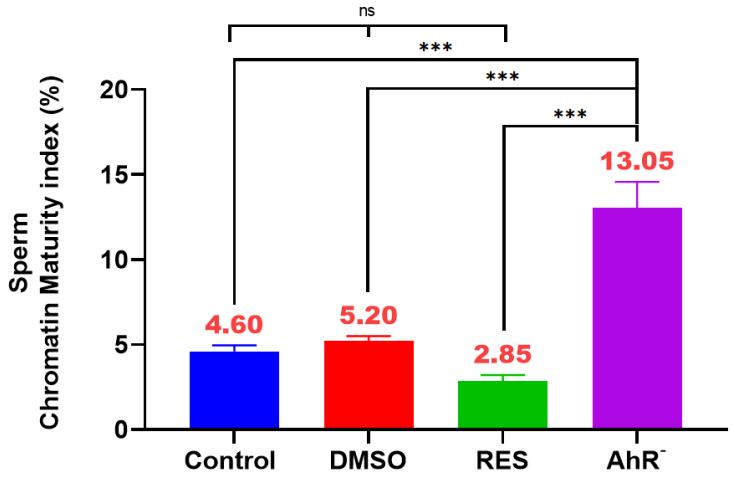

Figure 3. Sperm chromatin maturity

Ns: Non-significant difference between groups.

***P<0.001.

Note: Values are expressed as Mean±SEM; DMSO: Administered DMSO intraperitoneally twice a week for 60 d; RES: Receiving 100 mg/kg RES intraperitoneally twice weekly for 60 d; AhR: Provided with CH223191, intraperitoneally at 10 mg/kg twice a week for 60 d.

Results

ASCM

Analyzed data of spermatozoa in this study revealed that the percentage of mature chromatin in spermatozoa of the AHR$^-$ group has been significantly (P<0.05) increased in comparison with all other study groups. However, administration of RES to the RES group drove the percentage of mature chromatin to the lowest level but was insignificant compared to the control and DMSO groups (Figure 3).

ASCC

The ASCC is a crucial quality test associated with sperm fertility. AB dye is a traditional method of estimating ASCC. In this study, the collected data revealed that abolishing the AHR pathway using CH223191 led to a significant (P<0.05) increase in the percentage of ASCC in the AHR$^-$ group compared to other groups. In contrast, RES treatment resulted in the lowest value of ASCC in RES among all the study groups (Figure 4).

TJP1 gene expression in testes

Taking testicular tissue for studying this gene expression revealed that inhibiting the AHR pathway led to a significant (P<0.05) reduction in the messenger RNA (mRNA) expression of this protein in the AHR$^-$ group (Figure 5). In contrast, activation of the AHR by using RES has significantly (P<0.05) boosted the gene expression in the RES group compared to the control group as well as the AHR$^-$ group (Figure 5).

Blood-testis barrier

Assessing the BTB using the Evans blue test (Figure 6), distinct variations were observed among the experimental groups. Blocking AHR signals in the AHR$^-$ group has significantly (P<0.05) elevated the extravasated Evans blue dye to the testicular tissue in comparison with control and RES groups (Figure 6). Remarkably, DMSO administration led to a significant (P<0.05) increase in the amount of leaked Evans blue dye in the DMSO group compared to that in the control and RES groups (Figure 6).

Discussion

This study examined the effect of dysregulated AHR signaling on sperm integrity through its genetic components using specific stains, namely TB and AB (Figures 3 and 4). Sperm chromatin maturity reflects the level of compaction and condensation of genetic material within sperm nuclei. In sperm nuclei with loosely packed chromatin or damaged DNA, the phosphate residues of the DNA are more exposed and accessible to TB dye

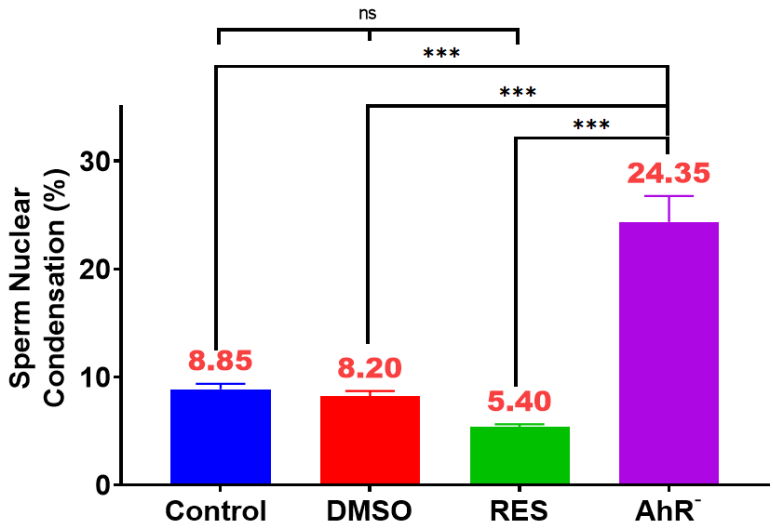

Figure 4. Sperm nuclear chromatin condensation

Ns: Non-significant difference between groups.

***P<0.001.

Note: Values are expressed as Mean±SEM; DMSO: Administered DMSO intraperitoneally twice weekly for 60 d; RES: Receiving 100 mg/kg RES intraperitoneally twice weekly for 60 s; AhR¯: Provided with CH223191, intraperitoneally at 10 mg/kg twice weekly for 60 days.

and metachromatic alterations occur. Chromatin condensation occurs during the final stage of spermatogenesis. Histones pack DNA in the cell nucleus using protamines, resulting in tighter DNA packaging and increased stability of the sperm nucleus. Moreover, chromatin condensation abnormalities can damage DNA (Pourmasumi et al., 2019; Sharma & Agarwal, 2018; Zini et al., 2014). The TB test results presented in Figure 3 provide insights into sperm chromatin maturity, in which the RES group showed an improved sperm chromatin maturity percentage compared to the control and DMSO groups, but with insignificant differences. In contrast, the AHR¯ group exhibited significantly higher values than all other groups, suggesting significantly compromised sperm chromatin maturity, which impairs sperm fertility capacity (Kim et al., 2013).

The results of the current study are those of previous studies that examined the role of AHR in sperm chromatin maturity (Karman et al., 2012). A previous study showed that the sperm of AHR-null mice undergo apoptosis, and the cells also showed an early onset of chromatin condensation, nuclear fragmentation, and DNA laddering together with very low levels of DNA synthesis (Karman et al., 2012; Alharris, et al,. 2022). These results collectively underscore the intricate role of AHR in governing sperm DNA fragmentation, sperm nuclear chromatin condensation, and chromatin maturity (Bustani et al., 2024). Furthermore, previous studies illustrated that the expression of genes involved in sperm chromatin condensation was adversely affected by the loss of AHR, as shown in AHR−/− mice, via different target genes, such as *Prm1*, *Prm2* and *Hsp70a*, which play critical roles in maturity of germ cell and replace histones throughout the late phase of spermatogenesis and are responsible for sperm head condensation (Hansen et al., 2014b).

In addition, we examined the effects of dysregulated AHR signaling on the soundness of the seminiferous tubule barrier through its tight junction protein gene expression, particularly *TJP1* (Figure 5), supported by the ability of this barrier to avoid extravasation of Evans blue dye from circulatory blood into testicular tissue (Figure 6). TJP1 is integral to forming and maintaining tight junctions in the seminiferous epithelium.

A previous study reported that germ cell impairment occurs when TJP1 is disrupted, indicating a potential impact on BTB integrity, which in turn influences the overall composition of the testicular tissue (Noguchi et al., 2021). In this study, RES treatment led to a significant increase in *TJP1* expression (Figure 5), which empowered the BTB in the same treated group (Figure 6).

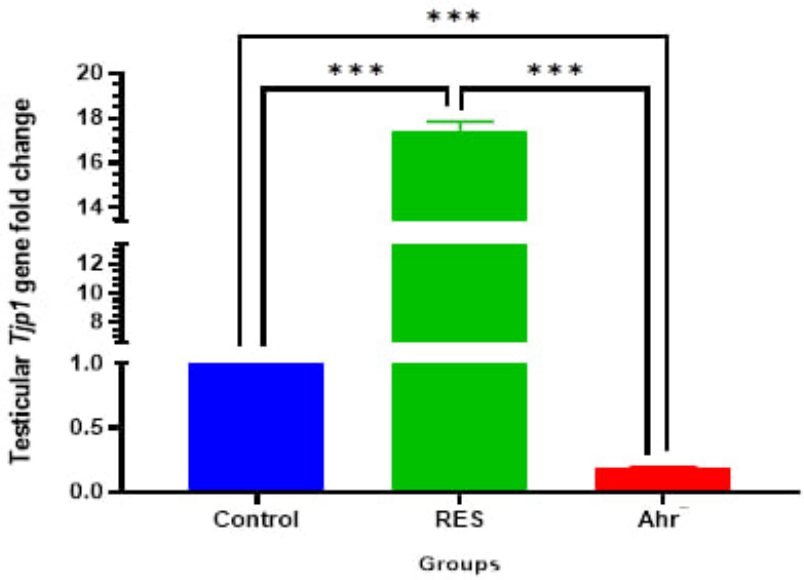

Figure 5. Fold change analysis of *Tjp1* gene expression in response to RES and AHR antagonist treatments

***P<0.001.

Note: Values are expressed as Mean±SEM; RES: Receiving 100 mg/kg RES intraperitoneally twice weekly for 60 d; AhR⁻: Provided with CH223191, intraperitoneally at 10 mg/kg twice weekly for 60 d.

These results are consistent with a previous study that found that RES sustains intestinal barrier integrity via upregulation of *TJP1* expression, improves antioxidant capacity, and alleviates inflammation (Yang et al., 2021). Conversely, the AHR⁻ group significantly reduces *TJP1* expression, indicating a disruption in the barrier function of the testis. These results emphasize the potential regulatory role of RES in enhancing the expression of genes associated with sperm development and integrity, consistent with previous studies that suggest that the sustained normalcy of the BTB hinges primarily on the expression levels of *TJP1*.

In the absence of these proteins, there is a perpetual augmentation of the body's autoimmune response to spermatogenesis, inevitably leading to a diminished efficiency of this crucial reproductive process (Ghafouri-Fard et al., 2021; Koşal et al., 2024; Yan et al., 2024). The results depicted in Figure 6 highlight the BTB and provide crucial insights into its role and potential implications. The BTB plays a pivotal role in the intricate process of spermatogenesis, serving as a robust physical impediment that separates blood vessels from the seminiferous tubules. Formed by tight junctions among Sertoli cells, the BTB meticulously governs the developmental milieu for germ cells, offering protection against toxins and upholding a distinctive chemical composition within the seminiferous tubules lumen. Essential for spermatogenesis, any disruption of the BTB poses a potential threat to fertility (Yan et al., 2024). The BTB's physical structure demarcates the seminiferous epithelium into basal and abluminal compartments, distinguishing it as one of the most impermeable tissue barriers. Beyond its anatomical function, the BTB also serves as an immunological barrier, intricately shaping the physiological environment for spermatogenesis by sequestering germ cells and lymphatic systems while concurrently implementing local immune suppression (Noguchi et al., 2021; Barfourooshi et al., 2023; Alghetaa, et al., 2021).

The result of the RES group showed a significant increase in *TJP1* gene expression (Figure 5) along with a reduction of leaky Evans blue dye (Figure 6) in the testicular tissue compared to the other study groups, which aligns with a previous study that demonstrated the BTB's pivotal role in safeguarding germ cells and shaping the intricate milieu necessary for successful sperm development (Yan et al., 2024). Conversely, the AHR⁻ group displayed a significant reduction in BTB integrity, indicating a negative effect of decreased *TJP1* expression by the antagonist on the BTB.

Conclusion

In conclusion, our study highlights the pivotal role of AhR activation in male reproductive health, par-

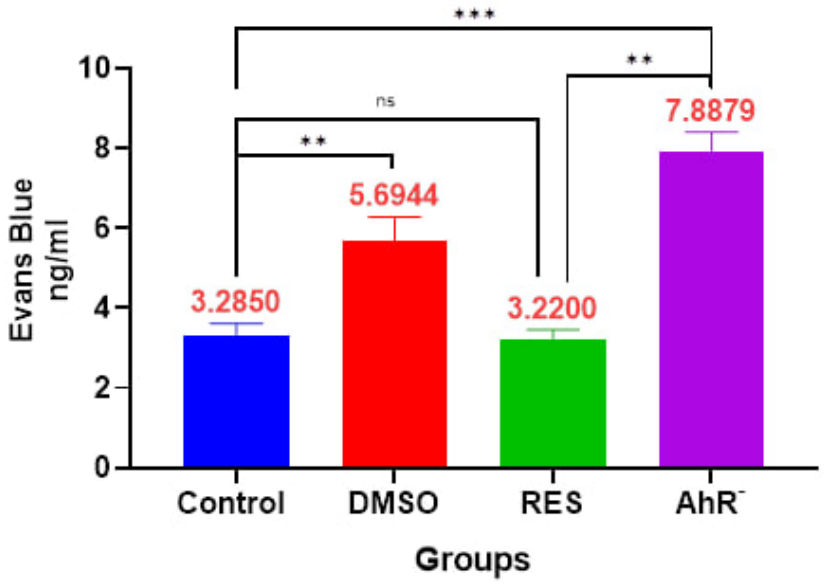

Figure 6. BTB

*P<0.05, ***P<0.001.

Note: Values are expressed as Mean±SEM; DMSO: Administered DMSO intraperitoneally twice weekly for 60 d; RES: Receiving 100 mg/kg RES intraperitoneally twice weekly for 60 d; AhR⁻: Provided with CH223191, intraperitoneally at 10 mg/kg twice weekly for 60 days; Ns: Denotes non-significant differences between groups.

ticularly sperm chromatin maturity and BTB integrity. RES treatment showed promising results in improving sperm chromatin maturity, *TJP1* expression and BTB integrity, suggesting its potential as a therapeutic agent in male infertility. Conversely, AHR antagonism leads to compromised sperm chromatin integrity and BTB function, emphasizing the importance of AHR modulation in spermatogenesis. These results provide valuable insights into potential therapeutic interventions for male reproductive issues.

Ethical Considerations

Compliance with ethical guidelines

This study was conducted in a compliance with the Institutional Animal Care and Use Committee regulations set by the College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq (Code: AUP# N.P.G685).

Funding

This research did not receive any grant from funding agencies in the public, commercial, or non-profit sectors.

Authors' contributions

Experiments, data collection, and writing the original draft: Ghadeer Sabah Bustani; Conceptualization, investigation, review and editing: Hasan Alghetaa; Final approval: All authors.

Conflict of interest

The authors declared no conflict of interest.

Acknowledgments

Authors would like to thank administration of University of Baghdad, Baghdad, Iraq, to facilitate the research conductance.

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